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Rectification of muscle and nerve deficits in paralyzed ryanodine receptor type 1 mutant embryos

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ABSTRACT

Locomotion and respiration require motor axon connectivity and activation of the neuromuscular junction (NMJ). Through a forward genetic screen for muscle weakness, we recently reported an allele of ryanodine receptor type 1 ($Ryr1^{AG}$). Here we reveal a role for functional RyR1 during acetylcholine receptor (AChR) cluster formation and embryonic synaptic transmission. $Ryr1^{AG}$ homozygous embryos are non-motile. Motor axons extend past AChR clusters and enlarged AChR clusters are found under fasciculated nerves. Using physiological and pharmacological methods, we show that contractility can be resumed through the masking of a potassium leak, and evoked vesicular release can be resumed *via* bypassing the defect in RyR1 induced calcium release. Moreover, we show the involvement of ryanodine receptors in presynaptic release at the NMJ. This data provides evidence of a role for RyR1 on both the pre- and postsynaptic sides of the NMJ.

1. Introduction

The mechanisms that establish and maintain axon outgrowth and synaptic function during embryonic development are crucial for proper locomotion (Busetto et al., 2003; Hanson and Landmesser, 2004, 2006; Krull, 2010; Sanes and Lichtman, 1999, 2001) A key component of the locomotor circuit is the neuromuscular junction (NMJ), which is formed and refined by signaling between the motoneuron and the muscle (Sanes and Lichtman, 2001). Therefore, defects in pre- and postsynaptic signaling at the NMJ may have detrimental effects on neonatal survival.

Presynaptic vesicular release of acetylcholine (ACh) by motor nerve terminals leads to focal depolarization of the post-synaptic apparatus. A large action potential sweeps through the muscle fiber to induce global depolarization and muscle contraction. Contractions in muscle transpire through the opening of a muscle-specific voltage gated channel, dihydropyridine receptor (L-type calcium channel DHPR; Cacna1s, CaV1.1, (Franzini-Armstrong and Jorgensen, 1994)), which stimulates ryanodine receptor type 1 (RyR1) in the sarcoplasmic reticulum to flood the myoplasm with calcium. The increased calcium initiates contraction in the skeletal muscle (Numa et al., 1990). These events constitute excitation–contraction coupling (E–C coupling).

Transgenic studies have shown that skeletal muscle DHPR acts independently of E–C coupling to establish muscle prepatterning of AChR clusters, AChR cluster size, the frequency of ACh dependent depolarization, and expression of MuSK (Chen et al., 2011). Moreover, RyR1 is required for establishing muscle prepatterning of AChR clusters and proper axon outgrowth (Hanson and Niswander, 2014). However, the requirement for functional RyR1-DHPR complex in determining AChR cluster size, the frequency of ACh-dependent depolarization, and expression of MuSK is less clear.

The family of ryanodine receptors is required for proper Ca^{2+} release from the sarcoplasmic reticulum in skeletal muscle (RyR1 and RyR3) and heart (RyR2) (Coussin et al., 2000; Giannini et al., 1995; Nakai et al., 1990; Otsu et al., 1990; Takeshima et al., 1989; Zorzato et al., 1990). Mice that lack either *Ryr1* or *Ryr2* die at birth (Takeshima et al., 1994, 1998), whereas mice that lack *Ryr3* are viable (Takeshima et al., 1996), but have deficiencies in synaptic plasticity and locomotion (Futatsugi et al., 1999). Embryonic rat spinal cord and cultured rat motoneurons express RyR1, RyR2, and RyR3 (Dayanithi et al., 2006) and this family of intracellular receptors is required for Ca^{2+} release from the endoplasmic reticulum of neurons (Fill and Copello, 2002). Therefore, RyRs regulate muscle Ca^{2+} release and may be involved in release of Ca^{2+} in the presynaptic terminal that results in release of transmitter.

Here we explore the function of RyR1 as it pertains to mechanisms of motoneuron axon extension, AChR cluster distribution, and presynaptic transmitter release. Mouse embryos that are homozygous for a point mutation in *Ryr1* ($Ryr1^{AG}$) have defects in

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axon extension, abnormally narrow distribution of AChR clusters and increased AChR cluster size. The *Ryr1*$^{AG/AG}$ embryonic muscle has a greater change in internal potassium levels and muscle paralysis but, by inhibiting the potassium leak, the muscle can be stimulated to undergo contractions. In addition, we use this mouse model to uncover a pre-synaptic role of ryanodine receptors at the mouse neuromuscular junction. Overall, these data illustrate that RyRs are involved in vesicular release at the NMJ, RyR1 is required for proper release of transmitter at the neuromuscular junction, and postsynaptic RyR1 is required for the size and distribution of AChR clusters.

2. Material and methods

2.1 Forward genetic screen and mouse strains

ENU mutagenesis was performed as described (Kasarskis et al., 1998) on males of C57BL/6J background and then outcrossed onto 129S1/Svlmj background to score at embryonic day 18.5 for recessive mutations that affect embryonic locomotion. The forward genetic screen and identification of the *Ryr1*^{AG} mutation is described in Hanson et al. (2015). This allele is named *RyR1*^{m1Nisw} but here we will refer to it as *Ryr1*^{AG}. *Ryr1*$^{-/-}$ is the *dyspedic* null mutation (*Ryr1*^{tm1TAlle} allele generated by Richard Allen (Buck et al., 1997)). To examine axon guidance errors in limb muscles, *Ryr1*$^{AG/+}$ mice were crossed with B6.Cg-Tg(Hlxb9-GFP)1Tmj/J (Jackson laboratory, Maine) stated as *Hlxb9-GFP* then outcrossed 6 generations in 129S1/Svlmj background. For the locomotion screen, E18.5 embryos were dissected from timed pregnant dams and placed in room temperature oxygenated mouse Tyrode's solution. To induce limb movement, forelimb and hindlimb footpads were pinched with tweezers to induce paw retraction and cross-extensor reflexes. Touching the forceps to the dorsal rib cage scored s-shaped movements in axial muscles. Touching the forceps to the nose scored neck extension reflex. Touch assay was performed on each embryo in the litter and the genotype of all embryos was determined.

2.2 Calcium transient measurements

Primary myoblasts isolated from E18.5 embryonic muscle were differentiated and then loaded with the fluorescent Ca^{2+} indicator Fluo-3 (Life Technologies, Grand Island, NY) injected by whole-cell configuration at a final concentration of 200 μM. Following loading, Fluo-3 dye was allowed to diffuse within the cell interior for 5 min. The total change in fluorescence ($\Delta F/F$) was determined from the change in peak fluorescence from initial baseline during stimulation, where F was the fluorescence immediately before the test pulse minus the measured average background (non–Fluo-3) fluorescence before dye injection. The average values of fluorescence change (ΔF) for each test potential (V) were fitted according to: $\square\Delta F)=(\Delta F)_{max}/\{1+\exp\ [(V_F-V)/k_F]\}$, where $(\Delta F)_{max}=1093$ au, $V_F=-1.4$ mV and $k_F=5.5$ mV. Fluorescence emission was measured by fluorometer (Biomedical Instrumentation Group, University of Pennsylvania, Philadelphia, PA). Kurt Beam and Roger Bannister provided *Ryr1*-YFP cDNA and performed these experiments.

2.3 [K^+]i/PBFI measurements

For [K+]i measurements, E18.5 diaphragm muscle was mechanically scraped of membrane bordering the muscle and affixed to glass bottom culture dishes (MatTeK) with Vetbond (3 M, St. Paul, MN). The ratiometric cell-permeant potassium indicator PBFI–AM (5 μM; Life Technologies, Grand Island, NY) together with 0.2% Pluronic F-127 for enhanced dye loading was applied to the diaphragm muscle for 30 min in Ringers Solution (3 mM K^+). After loading, muscles were washed for 30 min to remove excess dye and to allow de-esterification of the AM dye. Data was collected from greater than 10 fibers per muscle with 4 muscles per condition. Muscles were analyzed at 33 °C using the 340/380 filters on Zeiss Axio Observer Z1 with Photometrics CoolSNAP HQ2 camera for wide-field imaging. The filtered 340 and 380 intensities were analysized per second and the ratiometric intensity for the fibers were plotted. To calibrate intracellular potassium concentrations, ratiometric PBFI intensities were fitted to the intensities at different K^+ concentrations with the addition of 10 μM gramicidin in extracellular solutions and using the intensity of ~10 fibers of interest per muscle in >3 muscles from each condition. Calibration solutions were prepared with appropriate volumes of a high [K^+] solution with potassium gluconate.

2.4 ATP content

E18.5 limb skeletal muscles were assayed immediately after homogenization using buffer described in Hanson et al. (2015) to determine ATP content with a luciferin–luciferase based bioluminescence assay. The methodology of the ATP determination kit is provided in the experimental protocol by Molecular Probes (A-22066, Eugene, OR, USA). In order to determine ATP content, freshly extracted homogenate was added to a cuvette containing reaction buffer, D-luciferin, luciferase and DTT and placed in a Sirius luminometer v.2.2 (Berthold Detection Systems). Known concentrations of ATP standards were used to establish a standard curve.

2.5 Electrode recordings

Sharp electrode recordings were performed as described (Hanson et al., 2014; Plomp et al., 1992). E18.5 diaphragms were dissected and kept at room temperature in Normal Ringers solution. Diaphragm muscle fibers were impaled with a glass capillary microelectrode (30 MΩ resistance) made using a P-97 microelectrode puller (Sutter Instruments, Novata, CA) and filled with 3 M KCl (Brown et al., 2008). Evoked responses were elicited by a 0.2-ms maximal stimulus applied to the phrenic nerve using a suction electrode and pulse generator (STG 1002, ALA Scientific Instruments, Farmingdale, NY). Evoked and miniature endplate potentials (MEPP) were recorded using an Axoclamp-2A amplifier (Molecular Devices, Sunnyvale, CA) and DigiData 1322A (Molecular Devices). Muscle fiber membrane potentials were adjusted to $E_m=-70$ mV under current clamp. Data was extracted and analyzed with Axoscope 10 software (Molecular Devices).

For EMG recordings and analysis of muscle contractions, explanted diaphragm muscle (Hanson and Niswander, 2014) was warmed to 30 °C and continually supplied with oxygenated Tyrode's solution (3.0 mM KCl). EMG muscle recordings were made using fine-tip suction electrodes pulled from polyethylene tubing (PE-190; Clay Adams, NJ) and recorded *via* amplifiers (AI 401 amplifier connected with Digidata 1322a, Molecular Devices) directly on the computer with Axoscope 10 (Molecular Devices). Significance of data was evaluated by Student's *t*-test, which determined the *P* value (Excel 2008). A *P* value below 0.05 was considered significant. E18.5 diaphragm preparations were treated with pharmacological reagents by addition of drugs to the bath at the indicated concentrations. For contractile studies, a stimulation electrode was placed onto the diaphragm. Evoked response was elicited by a 0.2-ms maximal stimulus compared to locomotor normal littermates within bath. A contractile movement was scored as a 0 if no contraction transpired after stimulation and 1 if contraction was recorded either through EMG recording or a visual