Enhanced Yellow Fluorescent Protein Causes Contractile Dysfunction in Skeletal Muscle with Electrical and Optogenetic Induced Stimulation

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INTRODUCTION: Optogenetic stimulation (OS) of skeletal muscle is an emerging tool used to induce muscle contraction in a noninvasive manner[1]. We have previously established a mouse model to initiate direct OS of muscle in vivo[1]. In this model, skeletal muscle-specific expression of channelrhodopsin-2 (ChR2), a blue light sensitive cation channel with enhanced yellow fluorescent fusion protein (EYFP, as Cre reporter), allows for optical control of muscle activity. In this study we have generated a new transgenic mouse that also expresses ChR2 in skeletal muscle but without the EYFP reporter. The goal of this study was to compare and validate these two muscle-specific optogenetic strains by measuring muscle contractility with electrical and OS and to evaluate skeletal muscle structure using microscopy.

METHODS: The Institutional Animal Care and Use Committee at the University of Michigan approved all animal procedures. Mouse models: All experiments were performed on 11–14-week-old mice. Experimental mice expressed ChR2 with EYFP (ChR2-EYFP) or without EYFP (ChR2-only) in skeletal muscle (using Acta1-Cre mediated recombination; used for all functional assays and imaging; and CK-Cre, used for imaging only). ChR2-only mice were generated following removal of the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) and EYFP fusion gene. Wildtype (WT) littermates (Cre-negative) served as controls. Contractile properties: We compared contractility of gastrocnemius muscles in situ with nerve stimulation among the three groups: WT, ChR2-EYFP, and ChR2-only mice (n=3, 3, and 5 respectively, all male). Isometric contractile forces were measured in response to increasing stimulation frequencies from 40 to 220 Hz and maximum isometric tetanic force was normalized by the physiological cross-sectional area (PCSA) of individual gastrocnemius muscles[2]. Similarly, isometric contractility of isolated extensor digitorum longus (EDL) muscles was measured in vitro for the three groups: WT (n=2, male only), ChR2-EYFP (n=2, male only), and ChR2-only (n=2, 1 male and 1 female). Max. isometric tetanic force was normalized by the PCSA of individual EDLs[3]. OS was performed on EDL muscles using a blue (455 nm) light emitting diode (LED; Thorlabs) with an irradiance ~140 mW/cm^2. We measured peak twitch force with incremental light pulse duration ranging from 1 to 100 ms. The ability of muscle to undergo tetanus was tested by varying LED pulse duration and frequency over 300 ms duration. Microscopy: EDL muscle ultrastructure of WT, ChR2-EYFP and ChR2-only mice was imaged with confocal and transmission electron microscope (TEM) (n=1/group). Statistics: Grouped data were compared using one- or two-way ANOVA in experiments with sample sizes≥3 per group with Tukey's multiple comparison tests on GraphPad. Akaike information criterion (AIC) was used to identify the best fit model to describe the relationship between optogenetic-induced twitch force and light pulse durations.

RESULTS: Although body weights were similar in WT, ChR2-EYFP, and ChR2-only mice, gastrocnemius muscle masses from ChR2-EYFP mice were significantly lower compared with WT and ChR2-only mice ($p<0.01$) (Fig 1 A,B). Isometric tetanic forces of ChR2-EYFP mice were significantly lower than WT and ChR2-only mice at all test frequencies except 40 Hz ($p<0.05$) (Fig 1C). We observed a significant reduction in max. specific tetanic force with nerve stimulation for gastrocnemius muscle of ChR2-EYFP mice compared with WT ($p<0.01$), but not ChR2-only mice (Fig 1D). A similar trend of force reduction was observed for EDL muscles (Fig 2A,B). AIC identified one phase decay as the best fit model describing force outcome as function of pulse duration ($R^2>0.7$). Optogenetic twitch force for EDL muscles increased with pulse duration (1-100 ms) and plateaued at longer pulses (Fig 2C). Twitch force of EDL muscles of ChR2-only mice was higher at shorter pulses than that of ChR2-EYFP mice (Fig 2C,D), and peak force plateaued early compared with ChR2-EYFP mice. Similarly, ChR2-only muscles produced substantially higher forces compared with ChR2-EYFP muscles during partially fused tetanus at 50 and 60 Hz with 10 ms pulse (Fig 2E). Fluorescence imaging consistently showed overexpression of EYFP throughout muscles of ChR2-EYFP (Fig 3A). We identified the presence of vesicles along sarcolemma in TEM images of ChR2-EYFP skeletal muscle (CK-Cre, Fig 3B), which were not present in ChR2-only muscle (data not shown).

DISCUSSION: This study determined the contractile properties of skeletal muscle expressing ChR2 with and without EYFP reporter. Cre reporter strains are useful for lineage tracing, recombination validation, and monitoring expression in living tissues and cultured cells. However, we observed significant dysfunction of EYFP-expressing skeletal muscle. ChR2-EYFP muscle had significantly reduced contractile force with both light and electrical stimulation. Previous studies have shown that ChR2 is colocalizing with sarcolemma, T-tubules, and dihydropyridine (DHP) calcium channel[4]. It is possible that the structural changes caused by ChR2/EYFP overexpression are hindering the functionality of voltage-gated DHP channels, which will negatively impact the Ca^{2+} transient, thereby reducing contractile forces. Thus, while reporters offer many advantages including visualization and tracking cellular processes, the deleterious effects on function should be considered when using these biomarkers for muscle. Further studies are required to determine the underlying mechanisms directing this behavior.

SIGNIFICANCE/CLINICAL RELEVANCE: Use of reporter strains is common in preclinical biomedical research. Reporters are often considered to be inert but their potential effect should be tested in relevant tissues to avoid confounding results that may translate to clinical research.

REFERENCES: 1. Ganji et al, Connective Tiss Res (2021); 2. Larkin et al, Am. J. Physiology (2011); 3. Brooks & Faulkner, J Physiology (1988); 4. Magown et al, Nat Comm (2015).

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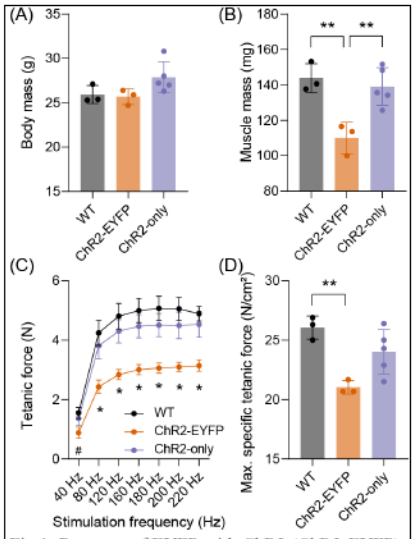

Fig 1: Presence of EYFP with ChR2 (ChR2-EYFP) significantly decreases in situ gastrocnemius muscle functionality compared with WT and EYFP absent (ChR2-only) mice. (A) body mass, (B) muscle mass, (C) absolute force–frequency relationship, and (D) Max. specific tetanic force in gastrocnemius muscle. **, $p<0.01$; * and #, $p<0.05$.

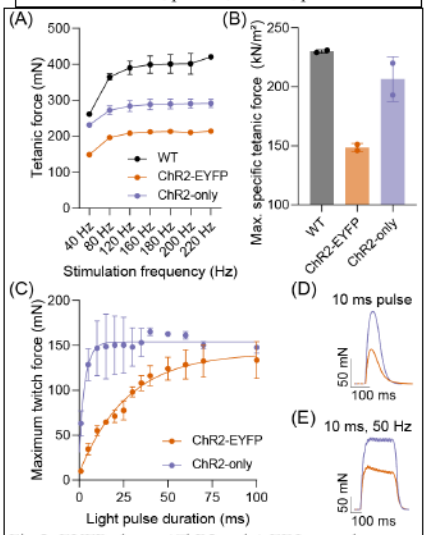

Fig 2: EYFP-absent (ChR2-only) EDL muscle generates higher force in both electrical and opto assays than ChR2-EYFP. (A) absolute force–frequency relationship, (B) Max. specific tetanic force, (C) twitch force with varying light pulse duration in EDL muscle, (D) single opto twitches at 10 ms pulse, and (E) sustained opto contraction at 10 ms, 50 Hz.

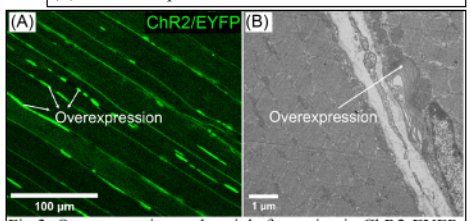

Fig 3: Overexpression and vesicle formation in ChR2-EYFP muscle. Images from (A) confocal microscopy and (B) TEM.