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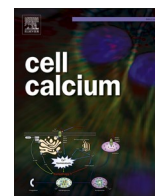
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Handling a mature calcium signature through optogenetics improves the differentiation of primary murine myotubes

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ABSTRACT

Calcium takes part in numerous cellular processes such as proliferation, migration, differentiation, or cell death and plays a particular role in myogenesis of skeletal muscle. Indeed, intracellular calcium signaling participates, in a non-negligible manner, to the “on” signal of muscle differentiation from undifferentiated cells to differentiated myotubes. Therefore, this differentiation can be modulated by controlling calcium activity with electrical or optogenetic stimulation approaches. In this study, we used the optogenetic tool channelrhodopsin 2 (ChR2) to control calcium activity and to modulate skeletal muscle differentiation. Using primary cultures of mouse myotubes, we showed that ChR2 stimulation was well-adapted to control intracellular calcium activity at the single cell or whole culture scale. To modulate the calcium-dependent myotube differentiation, we used an optical stimulation protocol based on GCaMP6s-decoded spontaneous calcium activity patterns of differentiated myotubes. The optical training of myotubes increased the fusion index and their contractile ability. This study demonstrates that handling a mature calcium signature with such optogenetic tool improves the differentiation of primary murine myotubes.

Abbreviations

2-APB	2-Aminoethoxydiphenyl borate
AAV	Adeno-associated virus
AP	Action potential
BKCa	Large conductance calcium-activated potassium channel
ChR2	channelrhodopsin 2
DAPI	4',6-diamidino-2-phenylindole
CPA	Cyclopiazonic acid
DHPR	Dihydropyridine receptors
DMSO	Dimethyl Sulfoxide
FRAP	Fluorescence recovery after photobleaching
GEC1	Genetically encoded calcium indicator
GFP	Green fluorescent protein
GLUT4	Glucose transporter 4
LED	Light emitting diode
NCX	Na-Ca exchanger
RT	Recovery time
RyR	Ryanodine receptors

SERCA	Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase
TG	Thapsigargin
TT	T tubule
TTP	Time to peak
TTX	Tetrodotoxin

1. Introduction

Calcium is an ubiquitous ion and a second messenger involved in the regulation of a large variety of cellular processes such as exocytosis, contraction, metabolism, transcription, proliferation and fertilization [1]. In skeletal muscle, calcium is an essential player in muscle differentiation and function. During myogenesis, it participates in cell migration [2,3], myoblast fusion [4–6], and differentiation [7–12]. In vitro experimentations on muscle cells demonstrated the presence of spontaneous membrane potential [13] and calcium oscillations [14–19] which were correlated to myogenesis processes [11,20].

Numerous studies have shown that electrical stimulation of muscle cells in culture increased the expression of contractile proteins as well as

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the ability of myotubes to contract [21–23]. A wide variety of electrical stimulation protocols mimicking physical exercise have been experimented to study their physiological consequences on muscle cells differentiation and maturation [for review see 23]. However, electrical stimulation can produce toxic effect on cells, resulting of electrode corrosion, production of gasses and reactive oxygen species [25]. In addition, the *in vivo* electrical stimulation approach does not allow to target with precision a single cell type leading to the stimulation of neighbouring tissues [26].

Since the mid-2000s, optogenetic tools are developed to control the membrane potential of excitable cells by using light activated ion channels [27]. For example, the channelrhodopsin 2 (ChR2), discovered from the algae *Chlamydomonas reinhardtii* [28], can generate cationic inward currents responsible for the generation of action potentials in neurons when expressed and stimulated with blue light [29]. This optogenetic tool makes it possible to efficiently control the membrane potential with a high spatio-temporal resolution. Such tools can be expressed specifically in a cell type, which limits any side effects linked to the stimulation of neighbouring cells [26]. In muscle, ChR2 has been shown to effectively control contractile function by reproducing a large part of the force generated by an electrical stimulation [30]. It has also been shown that optical stimulation of ChR2 is an effective method to trigger membrane depolarization and associated calcium transients in C2C12 myotubes [31] making it possible to improve their maturation [32].

Nevertheless, previous experiments aiming at evaluating the effects of optical stimulation on skeletal muscle cells were performed on cells displaying low levels of maturation, i.e., mainly small myotubes from cell lines [30,31].

The present study was designed to control, through ChR2 optical stimulation, intracellular calcium signaling and associated differentiation processes in primary mouse myotubes. Mouse muscle primary cultures display promptly large and elongated contractile myotubes that can result from the fusion of hundreds of myoblasts indicating a higher maturation level compared to studies performed on C2C12 cells [33,34]. We first investigated the efficacy of ChR2 expression and the capacity of such preparation to be optically stimulated at the whole culture scale. Second, faced with the diversity of empirically developed stimulation protocols used to modulate myoblast differentiation and knowing that calcium activity plays a key role in these processes, we decided to design an optical stimulation pattern that reproduces spontaneous calcium activity of mature cells. After an exhaustive analysis of spontaneous calcium dynamics of primary myotubes during differentiation using the genetically encoded calcium indicator GCaMP6s, we chose and applied an optical stimulation protocol during differentiation that mimicks the calcium signature of mature myotubes. The effect of this customized stimulation pattern was then evaluated in term of fusion and contractility.

2. Materials and methods

2.1. Cell culture

The investigation was conducted in agreement with European Community Council directives as well as with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). Primary culture of myotubes was established from satellite cells of 3/6-week-old C57BL/10 mice killed by cervical dislocation. Briefly, muscle tissue was extracted from hindlimbs, was minced in PBS then treated with 1A-type collagenase (1,5 mg/mL, Cat. No C9891, Sigma-Aldrich, Lyon, France) and dispase II (2 mg/mL, Sigma-Aldrich, Lyon, France) at 37 °C for 1 h ± 15 min. The pieces of remaining tissue were triturated with decreasing diameter glass pipettes and several centrifugation and filtration processes were applied to eliminate the last tissue fragments. Myoblast suspension was enriched by a plating step for 45 min at 37 °C to decrease

the fibroblast fraction. 0.4×10^6 cells were directly seeded on glass-bottom culture dishes previously coated with matrigel (1 mg/mL, Corning, Wiesbaden, Germany) in a proliferative medium composed of Dulbecco's modified Eagle's medium (DMEM, Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS, Biowest, Nuaille, France), 10% horse serum (Gibco, ThermoFisher Scientific, Villebon sur Yvette, France) and 1% penicillin/streptomycin (P/S, Sigma-Aldrich, Lyon, France). Two days after, differentiation was started by replacing proliferative medium by a differentiation medium (DMEM, 5% horse serum, 1% penicillin/streptomycin).

2.2. AAV infection

During seeding, isolated cells were directly infected for 48 h with associated adenovirus (AAV). 5×10^9 viral particles/mL of AAV1-ACAGW-ChR2-Venus (pACAGW-ChR2-Venus-AAV [35] was a gift from Karel Svoboda; Addgene viral prep #20,071-AAV1; <http://n2t.net/addgene:20,071>; RRID:Addgene_20,071) or 2.5×10^9 viral particles of AAV1 CAG-GCaMP6s-WPRE-SV40 (pAAV.CAG.GCaMP6s.WPRE.SV40 [36] was a gift from Douglas Kim & GENIE Project, Addgene viral prep #100,844-AAV1, <http://n2t.net/addgene:100,844>; RRID:Addgene_100,844) were added to the proliferative medium.

2.3. Drugs and treatments

In the pharmacological experiments, tetrodotoxin (TTX, Sigma-Aldrich, Lyon, France), a blocker of voltage-gated Na⁺ channels (Na_v), nifedipine (Sigma-Aldrich, Lyon, France), a blocker of L-type Ca²⁺ channels, paxilline (Sigma-Aldrich, Lyon, France), a blocker of large conductance K⁺ channels, thapsigargin (TG, Sigma-Aldrich, Lyon, France), a blocker of Sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) pumps, 2-Aminoethoxydiphenylborate (2-APB, Sigma-Aldrich, Lyon, France), a non-specific inhibitor of IP3Rs, TRP channels and store-operated Ca²⁺ entry, ORM-10,103 (Sigma-Aldrich, Lyon, France), a blocker of Na-Ca exchangers (NCX) and the vehicle dimethyl sulfoxide (DMSO, Sigma-Aldrich, Lyon, France), were added to the medium to modulate spontaneous calcium oscillations or light-induced calcium transients.

2.4. Spontaneous calcium fluorescence recording

For the characterization of spontaneous calcium activity of C57BL/10 myotubes in differentiation, GCaMP6s fluorescence was recorded with a fast-spinning disk confocal microscope composed of an Olympus IX81 microscope (IX81-ZDC, Olympus, Tokyo, Japan) enclosed in a thermostatic chamber (Solent Scientist) coupled to an Andor Revolution Imaging System (Andor Technology, Oxford Instruments, Belfast, UK). This system was equipped with an EMCCD/iXion + -DU-897 camera coupled to a sutter filter wheel and a Yokogawa CSU-X1 (Yokogawa, Tokyo, Japan). IQ3 acquisition software (Andor Technology, Oxford Instruments, Belfast, UK) was used to acquire images of 512 × 512 pixels. Fluorescence recordings were performed at the physiological temperature 37 °C and perfused with 5% CO₂ in atmosphere. GCaMP6s was illuminated at 488 nm with an argon laser and fluorescence emission was collected with a GFP filter set (dichroic mirror: 488 nm, emission filter: 525 nm). Several acquisition protocols were used to record GCaMP6s fluorescence. Fast acquisitions were performed with a x40 objective at an acquisition speed of 40 frames/s for 2 min. Another acquisition protocol was realized with a x40 objective at 1 frame/s for 15 min for calcium oscillation characterization and slow acquisitions were also assessed with a x10 objective at 5 frames/min for long acquisition experiment of 8 to 12 h. From fluorescence image series, fluorescence measurements were extracted under ImageJ software (NIH, Bethesda, MD, USA). Fluorescence changes were expressed as the fluorescence normalized to basal values ($\Delta F/F_0$). Calcium response parameters as amplitude peak ($\Delta F/F_0$, U.A.), area (U.A), time to peak

(TTP, s), recovery time (RT, s), event frequency (events/min), percent of time spent in activity (%) and inactivity time between each event (s) were analysis with a macro-software developed under the image-processing IDL 6.1 language (Research Systems Inc., Boulder, Colorado).

2.5. Confocal microscopy of Chr2-Venus fluorescence

After cell fixation with 4% paraformaldehyde (4% PFA) for 10 min, Chr2-Venus myotubes were stained with 4',6-diamidino-2-phenylindole (DAPI, 1/200, Sigma-Aldrich, Lyon, France) for 5 min to identify nuclei and mounted in Mowiol (Sigma-Aldrich, Lyon, France). Confocal images and 3D-sections of endogenous fluorescence of Venus protein were then obtained at D3 and D7 by confocal scanning microscopy using a FV-1000 system mounted on an Olympus IX81 inverted microscope (Olympus, Tokyo, Japan). Cells were excited with a 488 nm laser at x40 objective and fluorescence images were acquired with Fluoview software (Olympus, Tokyo, Japan). Images were then analysed with Bitplane Imaris 9 software (Oxford instruments Company, Abingdon, UK).

2.6. Optical stimulation training

To perform optical stimulation training, we used a homemade irradiation system composed of 8 LEDs (470 nm, 3 W, 700 mA, Ledxon, Geisenhausen, Germany) arranged in derivation on a steel plate supplied with a generator connected to a Powerlab A/D converter (PowerLab 4/26, AD Instrument, Australia) and controlled by a computer using Chart 5 software (AD Instrument, Australia). Customized optical stimulation training was conducted on myotubes for 48 h between the D3 and D5 with a specific irradiation protocol composed of 470 nm light stimulation trains of 6 Hz (pulses of 10 ms) at an intensity of 3.2 mW/mm². These stimulations were imposed for 23 s every 40 s.

2.7. Light-evoked calcium fluorescence recording

For the identification of Chr2-positive myotubes, cells were rapidly excited with a 488 nm laser to reveal Venus fluorescence recorded through a GFP filter set. Fast spinning disk confocal images were acquired using the Olympus IX81 microscope coupled to an Andor Revolution Imaging System (Andor Technology, Oxford Instruments, Belfast, UK). IQ3 acquisition software used to acquire images of 512 × 512 pixels. For Ca²⁺ imaging, cells loaded with Rhod-2 AM (5 μM, Invitrogen, ThermoFisher Scientific, Villebon sur Yvette, France) were illuminated at 561 nm and fluorescence emission was collected with a red filter set (dichroic mirror: 561 nm, emission filter: 607 nm). Optical stimulation at 488 nm was performed with the use of the FRAP (Fluorescence Recovery after photobleaching) module of the Andor Revolution Imaging system on a small area of 16 μm² delimited in the center of the myotube. For large-field light stimulation, calcium fluorescence recording was monitored using a Zyla 5.5 camera (Andor Technology, Oxford Instruments, Belfast, UK) by epifluorescence macroscopy with a 1x objective lens and a 6.3x zoom mounted on a MVX10 Olympus microscope (Olympus, Tokyo, Japan). Myotubes expressing Chr2-Venus were identified with a GFP filter set (excitation, bandpass 460–480 nm; dichroic mirror, 485 nm; emission, bandpass 495–540 nm). For Ca²⁺ imaging, cells loaded with 5 μM Rhod-2 AM were illuminated with a mercury lamp X-Cite 120 Q (Olympus, Tokyo, Japan) and calcium fluorescence was collected with a red filter set (excitation filter: bandpass 535–555 nm; dichroic mirror: 565 nm, emission filter: bandpass 570–625 nm). During the recording, myotubes were photo-stimulated with blue light protocols composed of several pulse duration and frequencies. Light stimulation was produced by the homemade stimulator arranged below the culture dish at 470 nm with a light intensity of 3.2 mW/mm². Fluorescence recordings were acquired at 20 frames/s using the CellSens software (Olympus, Tokyo, Japan) and each recording was analysis under ImageJ software (NIH, Bethesda, MD, USA). Fluorescence changes were expressed as the fluorescence

normalized to basal values ($\Delta F/F_0$). Each culture dish was divided into 5–10 regions of interests randomly selected. These experiments were performed at 22 °C and in a standard Tyrode solution. From fluorescence image series, fluorescence measurements were extracted under ImageJ software (NIH, Bethesda, MD, USA) and amplitude peak was analyzed with the macro-software developed under IDL language.

2.8. Fusion index

35 mm culture dishes containing myotubes were fixed with 4% PFA and stained with DAPI (1/200) at D3, D5 and D7 of the differentiation. Chr2-Venus positive myotubes and DAPI-stained nuclei were counted under a confocal microscope with a 20x objective, a 100 W X-Cite lamp and an UV (excitation filter: bandpass 360–370 nm; dichroic mirror: 400 nm; emission filter: 420 nm) and GFP filter sets. The index fusion was calculated by dividing the number of nuclei in Chr2-venus positive myotubes by the number of Chr2-venus positive myotubes.

2.9. Contractility

The contractility of myotubes was monitored using the same MVX10 Olympus macroscopic system described in 2.7. Myotubes expressing Chr2-Venus were identified with a GFP filter set and were photo-stimulated with blue LED flashes produced by the homemade stimulator (470 nm, 1 Hz, 20 ms) arranged below the culture dish. Myotubes were illuminated from above with a weak white light which was insufficient to induce stimulation of the channelrhodopsin. Image series of contractility were achieved with a bright field filter at 20 frames/s using the CellSens software (Olympus, Tokyo, Japan). Each culture dish was divided into 5–10 regions of interests randomly selected. Flash-saturated frames were deleted, and the difference extraction image was obtained after the standard deviation of the movie [32]. The difference extraction image was overlapped on the GFP image of Chr2-Venus positive myotubes to detect light-induced contracted myotubes. The numbers of fluorescent myotubes and contractile fluorescent myotubes were then counted. All the image analysis was performed using ImageJ software (NIH, Bethesda, MD, USA).

2.10. Statistical analysis

All statistical analysis were described in the figures' legends and were performed with Prism 8 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Control of calcium activity in primary cultures with Chr2-optical stimulation

Optogenetic approach already led us to a fine-tuning control of membrane potential and calcium activity in C2C12 muscle cells by inducing inward cationic currents [31,32]. To determine if Chr2 expression could be an adapted tool to handle calcium activity in entire myotubes primary cultures, we infected primary cultures through a viral transduction with the AAV1-ACAGW-Chr2-Venus. In our conditions, Chr2-Venus positive myotubes were easily discriminated from the non-fluorescent ones and represent a large portion (70–75%) of total multinucleated myotubes. A strong expression of Chr2 was observed from the third day of differentiation (D3), with a substantial fraction located at the plasma membrane and particularly on the substrate side of the myotube (Fig. 1A). It can be noticed that, at D3, these cultures already display small myotubes with more than a tenth of nuclei. At D7, the expression of Chr2-Venus is largely located at the plasma membrane (Fig. S1). We then investigated the ability of Chr2 expressing myotubes to respond to light-induced excitation by monitoring the calcium activity of these cells with the red calcium probe Rhod-2 (Fig. 1B). The light stimulation was performed using the FRAP unit of our confocal

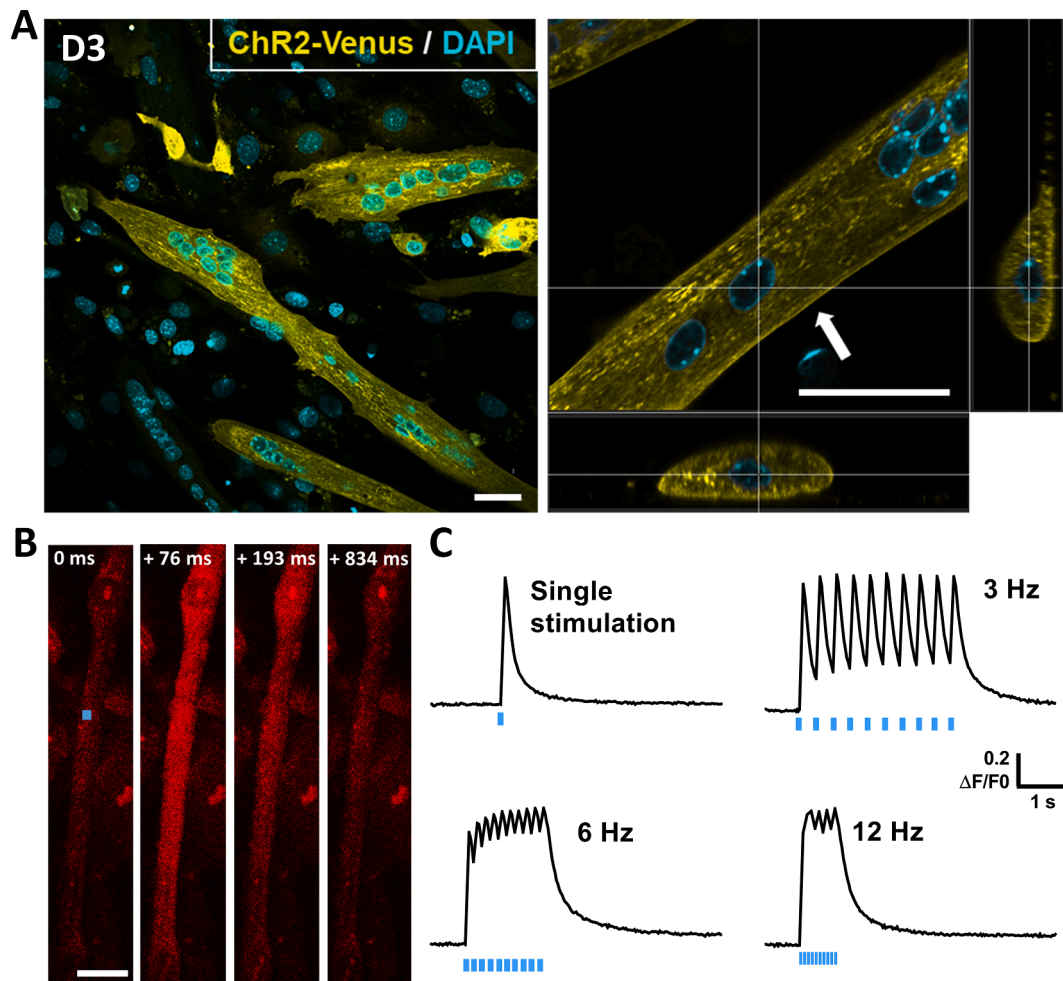


Fig. 1. Membrane localization and functionality of Chr2 at the single cell scale. (A) Expression of Chr2-Venus in C57BL/10 myotubes at D3. The right panels show the images in 3D view. Chr2 is detected by the fluorescence of the Venus protein (yellow) and the nuclei are labeled with DAPI (blue). The white arrow shows the membrane localization of Chr2-Venus. Scale bar: 30 μm . (B) Fluorescence images of a C57BL/10 myotube loaded with the Rhod-2 calcium probe (5 μM) before light stimulation ($t = 0$ ms) and after light stimulation (+76 ms, +193 ms and +834 ms) at 488 nm using a FRAP module. The stimulation zone is delimited in the center of the myotube and represents a surface of 16 μm^2 (blue square). Scale bar: 20 μm . (C) Examples showing the fluorescence measurements ($\Delta F/F_0$) of the Rhod-2 probe during a single stimulation or after 10 repeated light stimulations (488 nm, blue rectangle) at several frequencies (3, 6 et 12 Hz) in the same myotube expressing Chr2-Venus.

microscope to stimulate a single cell. An optical stimulation at 488 nm of a small area of 16 μm^2 delimited in the center of a myotube was able to induce a calcium transient (Fig. 1C) displaying a fast elevation of intracellular calcium followed by a progressive decrease (Fig. 1C: single stimulation). To evaluate the ability of Chr2-positive primary myotubes to respond to a complex optical stimulation challenge, we imposed escalating frequencies of optical stimulation up to 12 Hz. This induced repetitive calcium transients synchronized with the optical stimulation. A sustained calcium elevation was observed during the application of repetitive stimulations and decrease to basal level after turning off the light (Fig. 1C: 3, 6 and 12 Hz and video 1 as supplementary data).

At the entire culture scale, we developed a self-made stimulation system, composed of blue LEDs controlled by a computer, and that can be placed under culture dishes inside cell culture incubator (Fig. S2). This stimulation system can also be coupled with a microscope to directly visualize the effects of optical stimulation on cell culture. Microscope images allow to identify Chr2-expressing primary myotubes on large optical fields of 8.2 mm^2 (Fig. 2A). Chr2-Venus infected primary cultures were loaded with Rhod-2-AM calcium fluorescent probe and series of fast images (54 images/s) were recorded during underneath blue lightening for stimulation (see video 2 in

supplementary data). Fig. 2B shows a computed image displaying the standard deviation (SD) of Rhod-2 fluorescence in each cell during the fast Rhod-2 calcium imaging under optical stimulation revealing that Chr2-GFP expressing myotubes displayed large intracellular calcium variations in response to optical stimulation. Escalating stimulation time or frequency led also to calcium transients synchronized with the stimulation events (Fig. 2C). These results are consistent with the fact that optical stimulation allows to excite the majority of primary myotubes from a whole cell culture with a high temporal resolution.

To identify the molecular players involved in the formation of these optical-induced calcium transients and to compare them with those involved in the excitation-contraction coupling, a pharmacological approach was carried out to target channels and receptors involved in calcium homeostasis. The inhibition of L-type calcium channels with nifedipine, large conductance potassium channels with paxillin or Na_v channels with TTX significantly reduced the amplitude of calcium increases (Fig. 2D and S3), showing that calcium response to stimulation follows excitation-contraction pathways known for differentiating myotubes. Blockade of sarcoplasmic reticulum (SR) pump also reduced calcium response to optical stimulation, indicating that calcium increase may be due to release from the SR. Inhibition of NCX exchanger seemed

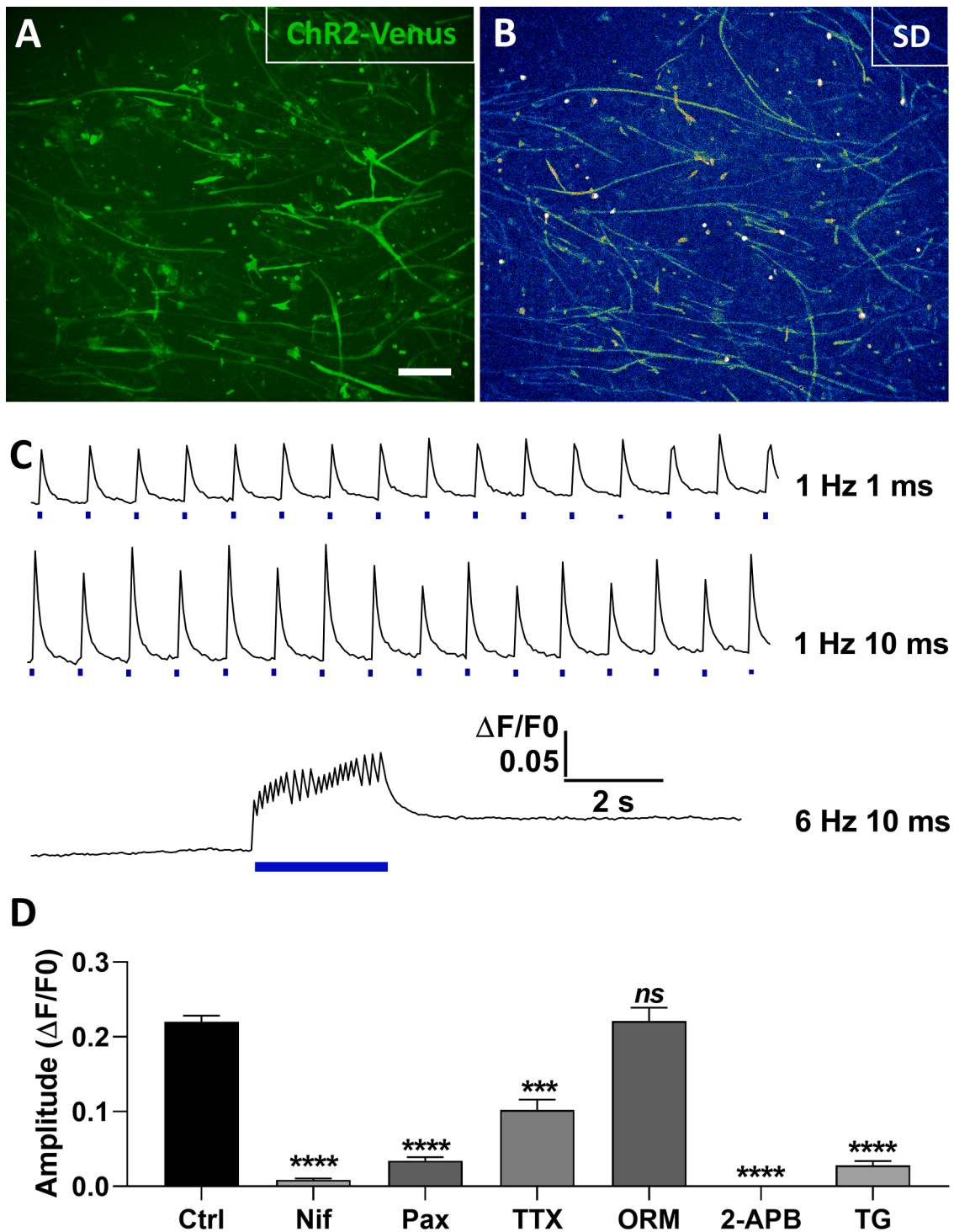


Fig. 2. Optically-induced calcium transients in the scale of a whole cell culture of C57BL/10 myotubes. **A:** Fluorescence images in a large optical field of 8.2 mm² of Chr2-Venus protein. **B:** computed image displaying standard deviation of Rhod-2 fluorescence in each pixel, recorded during optical stimulation in a culture of C57BL/10 myotubes at D5. Scale bar: 300 μ m. **C:** Examples showing Rhod-2 fluorescence measurements during the application of several light stimulation protocols (1 Hz, 1 ms; 1 Hz, 10 ms and 6 Hz, 10 ms) at 470 nm (3.2 mW/mm²; blue rectangle) on a culture of C57BL/10 myotubes at D7. **(D)** Pharmacology of the calcium response induced by light stimulation on C57BL/10 myotubes expressing Chr2. The amplitude peak ($\Delta F/F_0$) of the optical-induced calcium transients (470 nm; 3.2 mW/mm²) is determined in untreated myotubes (Ctrl) then treated with nifedipine (10 μ M; $n = 10$), paxillin (10 μ M; $n = 25$), TTX (2 μ M; $n = 19$), ORM-10,103 (3 μ M, $n = 19$), 2-APB (50 μ M; $n = 11$) and thapsigargin (1 μ M; $n = 19$) for 10 min. Each histogram bar represents the mean value \pm SEM. Statistical analysis are carried out by a Kruskal-Wallis test followed by a Dunn test (***: $p < 0.001$ and ****: $p < 0, 0001$).

not to affect this optically induced calcium increase. These data show that optical-induced calcium transients depend on the known excitation-contraction coupling actors. Furthermore, the stability of optical-induced calcium activities over time encouraged us to consider stimulation protocols of long durations during cell cultures.

3.2. Evolution of calcium signature of primary myotubes during differentiation

Since no exploration of the differentiation of primary cultures was performed through optogenetics, our aim was to apply optical stimulations via ChR2 to handle calcium activities in the way to modulate the differentiation processes of mouse muscle primary cultures. To date, wide variety of electrical stimulation protocols in terms of frequency, voltage and intensity has been tested on the differentiation and the maturation of muscle cells [for review see 23]. It is well known that calcium activity plays a key role in these processes, we then hypothesized that the application, on young myotubes, of stimulations that mimics the spontaneous calcium activity observed in mature myotubes, this would be able to improve muscle differentiation. To design an optical stimulation pattern that reproduces spontaneous calcium activity of mature cells, we therefore explored the evolution of calcium signature of primary myotubes during differentiation.

Spontaneous calcium signature was then exhaustively explored by using an optogenetic approach, in physiological conditions (37 °C with 5% CO₂), through the expression of the genetically encoded calcium indicator GCaMP6s. Fluorescence images displayed a good cytoplasmic expression, and not nucleic, of the GCaMP6s protein in the majority of myotubes (Fig. 3A). When intracellular calcium increased, GCaMP6s emitted a strong green fluorescence with a homogenous repartition in the cytoplasm easily detectable at several magnifications (Fig. 3A, B and video 3 as supplementary data). GCaMP6s expression was detected as soon as the third day of differentiation (Fig. 3B). Recording of spontaneous global calcium oscillations revealed multiple event profiles of varying durations (Fig. 3C). To study and evaluate the evolution of the spontaneous global calcium activity, 1474 oscillations were recorded in cultures from D3, D5 and D7 and all plotted in a distribution histogram of durations (Fig. 3D). This distribution was decomposed, with the help of Gauss functions, into 3 classes: class of short duration (0 to 12 s), intermediate duration (12 to 40 s) and long duration (up to 40 s) events. Most of calcium events belong to the short duration class which make up 61% of total calcium events. The intermediate duration class represented 29% of total calcium events. Finally, the last class composed of long duration calcium events represented 10% of total calcium events. The average duration of these spontaneous calcium oscillations was 23 ± 1 s ($n = 1429$). During myotube differentiation, the duration of spontaneous calcium oscillations evolved, and the proportion of short duration calcium oscillations decreased to make way for longer calcium oscillation durations (Fig. 3E). These data show that spontaneous calcium oscillations pattern evolved from short to longer events during myotube differentiation.

To decipher spontaneous calcium signature during myotube differentiation, we analyzed several parameters to compare their evolution within the different classes. The amplitude of calcium oscillations increased in more differentiated myotubes for the intermediate and long duration oscillations, but no difference was observed in the short duration class (Fig. 4A). Amplitude was also found higher in longer duration oscillations compared to short duration class. The area of spontaneous calcium oscillation was found higher in long duration class and increased in more differentiated myotubes (Fig. 4B). We then decided to analyze the time to peak (TTP) and the recovery time (RT) kinetic parameters which give information on the time necessary to reach the maximal calcium concentration and the time necessary to decrease intracellular calcium concentration to basal value respectively. TTP was found higher in long duration class but decreased during the differentiation only in long duration oscillations (Fig. 4C). Concerning

the RT, this parameter increased when the duration of the oscillations was higher, and in more differentiated myotubes (Fig. 4D). We then determined other parameters such as calcium oscillation frequency, the time percent in activity as well as the inactivity duration between each oscillation. The calcium event frequency was around 1.04 ± 0.08 Hz ($n = 95$, Fig. 4E), the percentage of time spent in activity was around $44.1 \pm 3.6\%$ ($n = 73$, Fig. 4F) and the inactivity time was around 27 ± 1.5 s ($n = 91$, Fig. 4G) in myotubes in differentiation. However, no evolution of these parameters was observed during the differentiation (Fig. 4E-G). All these data (summarized in Table 1) reveal first that, globally, calcium oscillations increased in amplitude with differentiation and second, that intermediate and longer events, becoming a major part of the signature in mature cells, displayed higher values in both calcium increase (TTP) and recovery (RT).

The design of a stimulation protocol needed to determine a total duration of stimulation as well as the pulse frequency. For that, we decided, in the same perspective, to analyze spontaneous calcium oscillations in differentiated myotubes during several hours (between 8 and 12 h, Fig. 5A). We noticed that 69% of myotubes (11 myotubes among 16) presented a continuous and stable calcium activity (Fig. 5B) whereas 31% of them seemed to take a several hours rest between intense activities (Fig. 5C). Additionally, by using fast acquisition recording, we detected, an intrinsic oscillatory frequency during the plateau of long duration calcium events (Fig. 5D) with an average frequency around 4.2 ± 0.7 Hz ($n = 10$) and a maximum value of around 6 Hz in differentiated myotubes. Therefore, these results demonstrate changes in calcium homeostasis during myotube differentiation with a generally higher intracellular calcium exposure in differentiated myotubes, due to higher and longer elevations of calcium. These spontaneous calcium oscillations occur during several hours and oscillate at a high frequency.

Spontaneous calcium oscillations already observed in skeletal muscle culture of myotubes have been demonstrated to take their origin from ionic channel activities located at the sarcolemma [14,17,18,13,37]. To determine the origin of spontaneous calcium activity in our model of GCaMP6s expressing primary myotubes, we investigated, through pharmacological approach, the blockade effect of channels and receptors involved in this activity. We observed that the inhibition of voltage-gated membrane channels such as voltage gated sodium channels, L-type calcium channels and BK channels with TTX, nifedipine or paxilline respectively, led to a total inhibition of spontaneous calcium oscillations (Fig. S4A-C). Nevertheless, the treatment with Ni²⁺, an inhibitor of T-type calcium channels, did not affect this calcium activity (Fig. S4D). We then investigated the role of SERCA pump and NCX exchanger. The inhibition of SERCA pump with CPA provoked a large elevation of intracellular calcium with the arrest of spontaneous calcium oscillations (Fig. S4E). However, NCX exchanger inhibition with ORM-10,103 did not affect spontaneous calcium activity (Fig. S4F). The use of 2-APB, a non-specific inhibitor of IP3Rs, TRP channels and store-operated Ca²⁺ entry strongly inhibited spontaneous activity (Fig. S4G). The DMSO vehicle did not impact calcium activity (Fig. S4H). This pharmacological investigation demonstrated the involvement of voltage-gated ion channels in the formation of spontaneous calcium activity with the specific recruitment of intracellular calcium stores and a major role of SERCA pumps to restore basal concentration of calcium.

3.3. Modulation of muscle differentiation through optical stimulation

Based on all information obtained on spontaneous calcium signature in primary myotubes in differentiation, we designed an optical stimulation protocol mimicking the spontaneous calcium signature observed in differentiated myotubes (Fig. 6A). This protocol was composed of trains of stimulation of blue light flashes (470 nm) of 10 ms with a light power of 3.2 mW/mm^2 applied at a 6 Hz frequency corresponding to the intrinsic frequency of spontaneous calcium oscillations in mature myotubes (Fig. 5D). The stimulation trains were applied for 23 s, the average

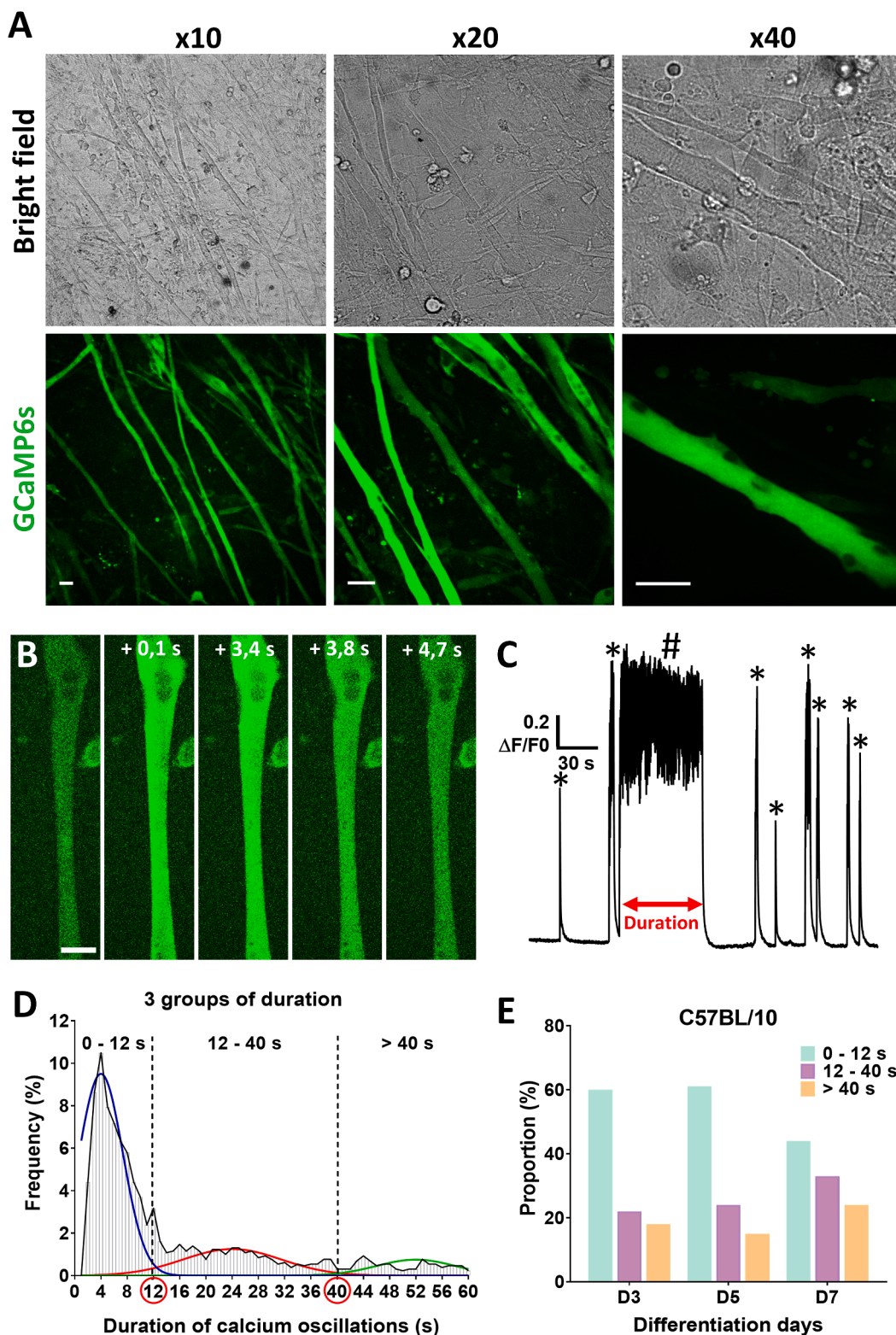


Fig. 3. Determination of 3 groups of spontaneous calcium oscillation duration in C57BL/10 myotubes expressing GCaMP6s. (A) Bright field and GCaMP6s fluorescence (green) images at different magnifications in a culture of C57BL/10 myotubes at D7. Scale bar: 40 μm . (B) Series of GCaMP6s fluorescence images of a myotube at D3 at several time intervals during a spontaneous calcium oscillation. Scale bar: 20 μm . (C) Representative curve showing the variation of spontaneous calcium activity (normalized fluorescence, A.U.) over time (s) in a C57BL/10 myotube at D3. In this example, myotube shows oscillations in a range of few seconds (*) to several tens of seconds (#). (D) Distribution histogram (%) of the duration (s) of all spontaneous calcium oscillations at D3, D5 and D7. 3 groups of duration were delimited by using Gaussian function. The first group represents calcium oscillations of short duration (from 0 to 12 s, blue line). The second group represents calcium oscillations of intermediate duration (from 12 to 40 s, red line). The last group represents calcium oscillations of long duration (more than 40 s, green line). (E) Organization of the different groups of oscillation duration during differentiation of C57BL/10 myotubes. The distribution (%) of short-duration oscillations is visible in green, intermediate-duration oscillations in purple and long-duration oscillations in orange. (D,E) Data were obtained on a set of 3 independent cultures.

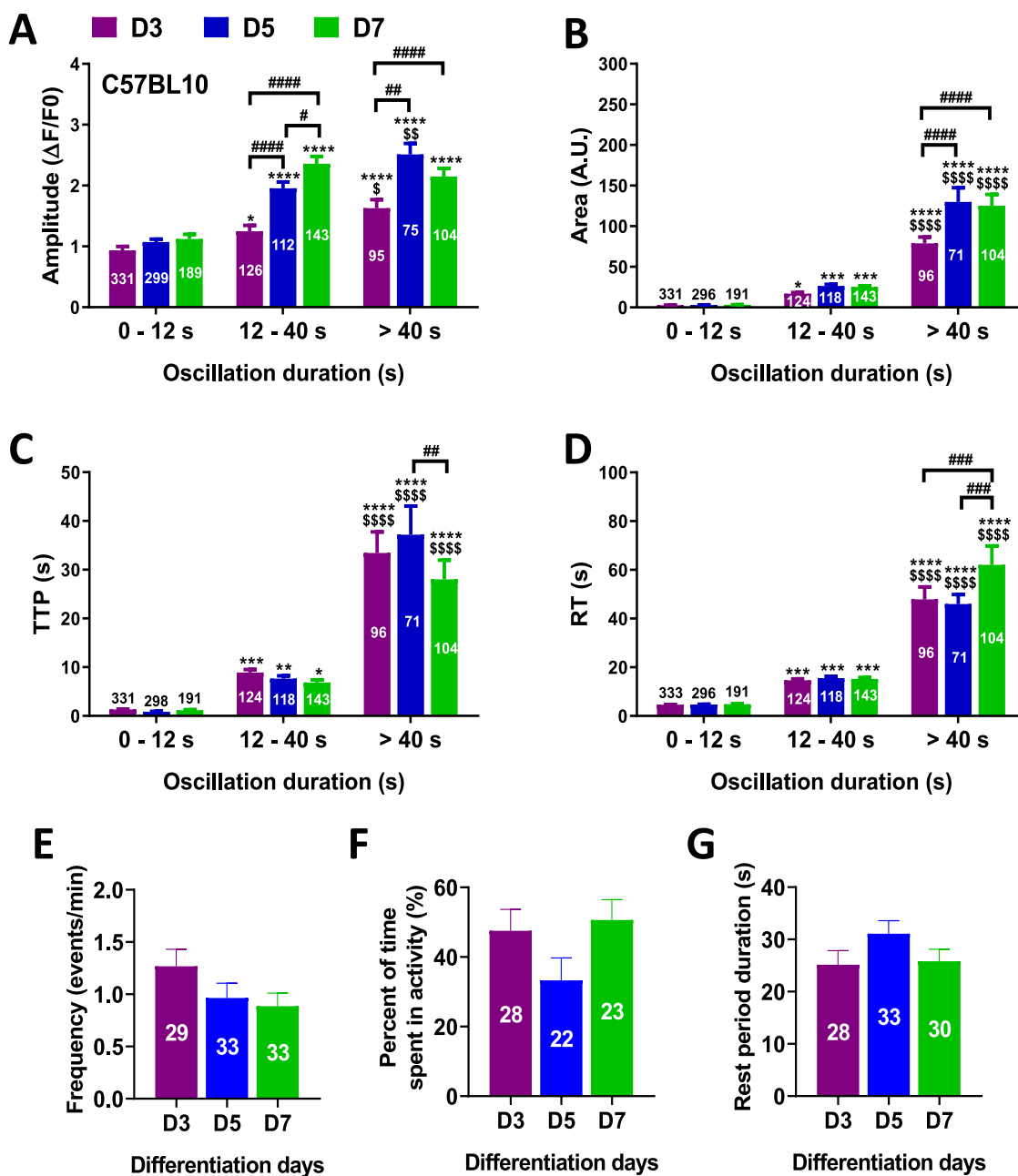


Fig. 4. Characterization of spontaneous calcium signature in differentiating C57BL/10 myotubes. Evolution of calcium parameters such as (A) the amplitude ($\Delta F/F_0$), (B) the area (A.U.), (C) the time to peak (TTP,s), (D) the recovery time (RT,s) in spontaneous calcium oscillations as well as (E) the frequency (events/min), (F) the percentage of time spent in activity (%) and (G) the rest period duration (s). (A-D) A comparison of the different parameters of calcium activity was carried out between the short (0–12 s), intermediate (12 to 40 s) and long duration (> 40 s) classes. The days of differentiation are represented by a color (D3: red, D5: blue and D7: green). Each histogram bar represents the mean value \pm SEM. The number of samples n is detailed in each histogram bar and represents the number of calcium variations analyzed (A-D) or the number of myotubes analyzed (E-G). * indicates a significant difference with the duration class 0 - <12 s of the same day of differentiation (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$). \$ indicates a significant difference with the duration class 12 - 40 s of the same day of differentiation (\$: $p < 0.05$; \$\$: $p < 0.01$; \$\$\$: $p < 0.0001$). # indicates a significant difference between differentiation days of the same duration class (#: $p < 0.05$; ##: $p < 0.01$; ###: $p < 0.001$; ####: $p < 0.0001$). For figures A-D, the statistical approach is carried out by a two-way ANOVA test coupled with a multiple Tukey test. For figures E-G, the statistical approach is performed by a one-way ANOVA test coupled with a multiple Dunn test.

duration of the spontaneous calcium oscillations and the rest period duration between each stimulation train was 40 s, a value close to the inactivity duration observed between each oscillation (Fig. 4G). Stable profiles of spontaneous calcium activity for several hours (Fig. 5B) led us to apply this protocol over a long period of 48 h, between day 3 and day 5 of differentiation of Chr2 expressing myotubes (Fig. 6B). To evaluate

the effect of optical stimulation on entire myotubes cultures during differentiation, two major parameters have been analyzed: the ability of myoblasts to fuse, with the calculation of fusion index parameter and their capacity to contract, in determining the percentage of contractile myotubes. Results show that the application of this customized protocol significantly increased the fusion index by 45% of Chr2-Venus

Table 1

Comparisons and evolutions of spontaneous calcium oscillation parameters in differentiating C57BL/10 myotubes.

Differentiation days	Duration classes	Amplitude ($\Delta F/F_0$)	Area (A.U.)	TTP (s)	RT (s)	Freq. (events/min)
D3	0 - 12 s	0.93 \pm 0.06	2.7 \pm 0.2	1.3 \pm 0.1	4.6 \pm 0.1	1.27 \pm 0.16
	12 - 40 s	1.25 \pm 0.10	16.8 \pm 1.5	8.9 \pm 0.6	14.6 \pm 0.6	
	> 40 s	1.63 \pm 0.14	78.9 \pm 7.7	33.5 \pm 4.3	47.9 \pm 5.1	
D5	0 - 12 s	1.07 \pm 0.05	2.9 \pm 0.2	0.9 \pm 0.1	4.7 \pm 0.1	0.96 \pm 0.14
	12 - 40 s	2.00 \pm 0.10 (####)	26.5 \pm 1.9	7.7 \pm 0.6	15.6 \pm 0.7	
	> 40 s	2.51 \pm 0.18 (\$; ####)	129.9 \pm 17.5	37.2 \pm 5.9	46.0 \pm 3.9	
D7	0 - 12 s	1.12 \pm 0.08	3.2 \pm 0.3	1.2 \pm 0.1	4.8 \pm 0.2	0.89 \pm 0.13
	12 - 40 s	2.36 \pm 0.12 (##)	25.1 \pm 1.6 (####)	6.9 \pm 0.5	15.2 \pm 0.6	
	> 40 s	2.15 \pm 0.13 (####)	125.2 \pm 13.8 (####)	28.0 \pm 4.0 (\$\$)	62.1 \pm 7.7 (\$\$; ###)	

Spontaneous calcium oscillations were obtained by recording GCaMP6s fluorescence in differentiating C57BL/10 myotubes under spinning disk confocal system at 37 °C with 5% of CO₂ in the atmosphere.

\$: significant difference between D5 and D7; #: significant difference between D3/D5 or D3/D7 (Two-way ANOVA test coupled with multiple Tukey test).

A.U., arbitrary units; TTP, Time To Peak; RT, Recovery Time; Freq, Frequency.

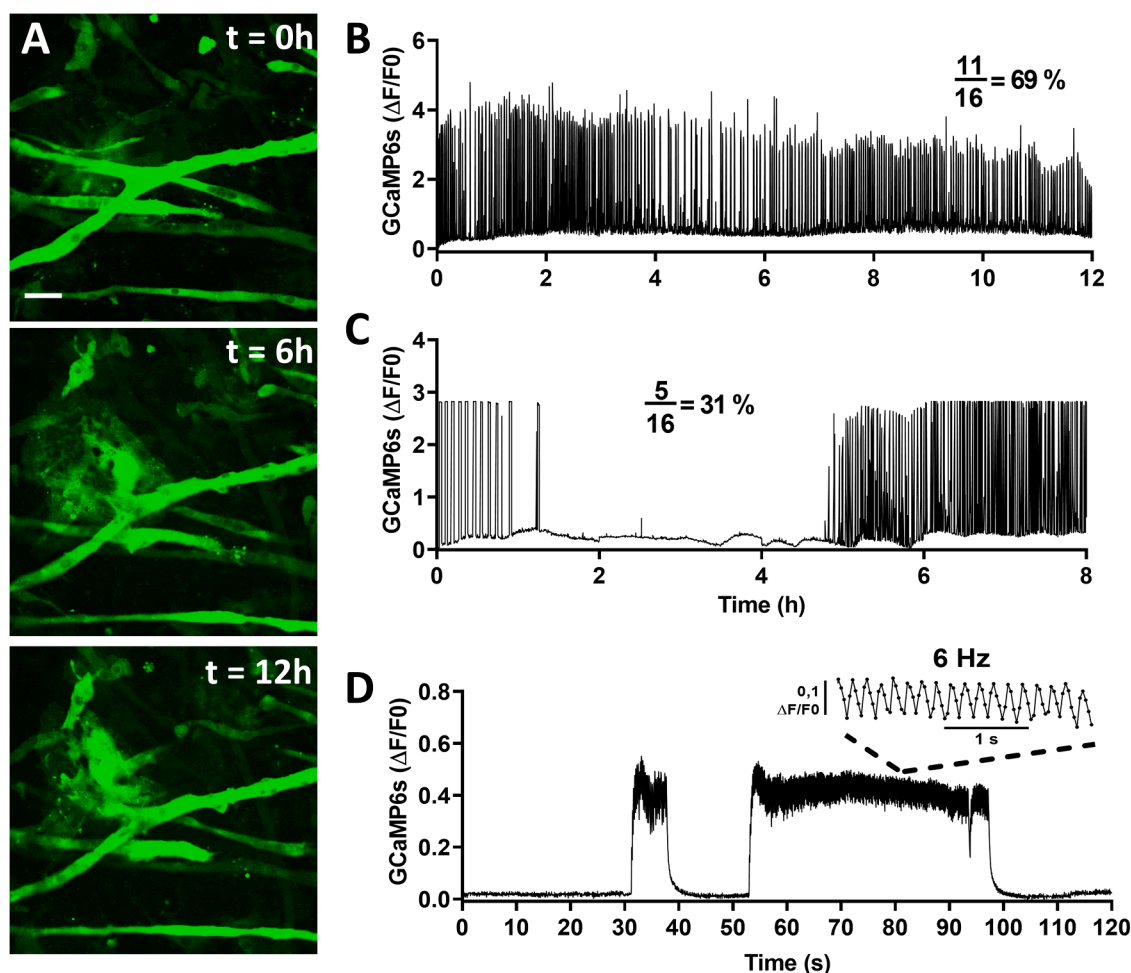


Fig. 5. Profiles of spontaneous calcium activity during a long period of time and characterization of the intrinsic oscillatory frequency. (A) Series of GCaMP6s fluorescence images at several time intervals: $t = 0$ h (top panel), $t = 6$ h (middle panel) and $t = 12$ h (bottom panel). Scale bar: 50 μ m (B, C) Examples of spontaneous variations of calcium ($\Delta F/F_0$) exhibiting a (B) continuous and (C) discontinuous profile during a long period of recording (between 8 and 12 h) of GCaMP6s fluorescence in differentiated C57BL/10 myotubes at D7. 11 out of 16 myotubes (69%) exhibit a continuous oscillatory profile while 5 out of 16 myotubes (31%) exhibit a discontinuous oscillatory profile. (D) Example of intrinsic oscillatory frequency around 6 Hz in a spontaneous calcium variation of a differentiated C57BL/10 myotube ($n = 10$). Magnification is performed in an oscillatory temporal window. Each point represents a fluorescence image. Fluorescence acquisition frequency: 40 Hz.

expressing stimulated myotubes at D7 compared to unstimulated myotubes (Fig. 6C, D). Moreover, the analysis of contractile ability of myotubes expressing ChR2-Venus showed a significant relationship between the optical stimulation of myotubes and the increase in their capacity to contract at D5 (+ 10%) and D7 (+ 32%) when they were stimulated in

culture (Fig. 6E, F). These results display the modulatory effect of optical stimulation on the differentiation processes such as fusion and their capacity to become contractile. They also allowed to demonstrate that the application of an optical stimulation pattern that reproduces spontaneous calcium activity of mature cells improves muscle

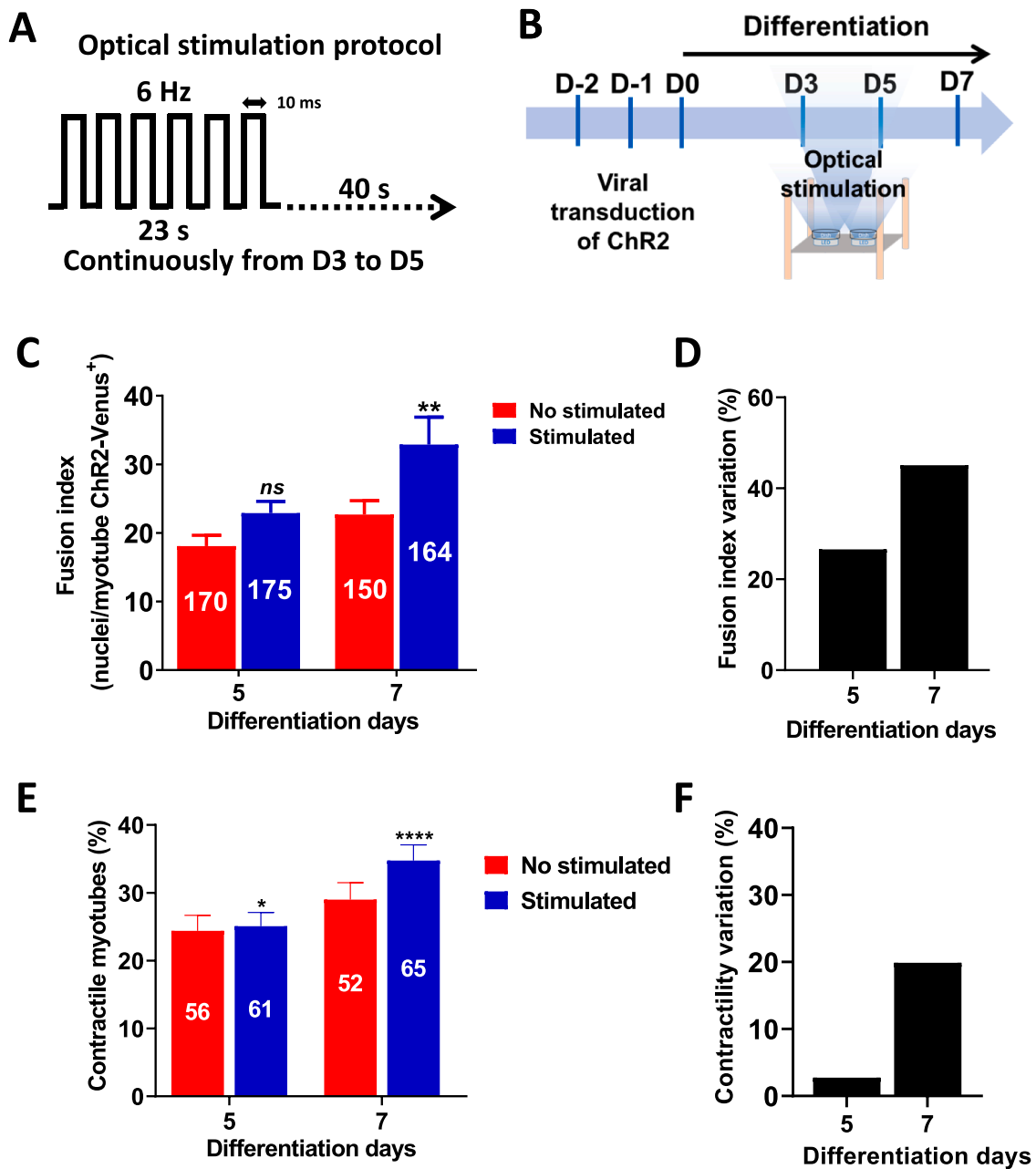


Fig. 6. Modulation of differentiation processes by optical stimulation in culture. A) Scheme of the optical stimulation protocol developed from the spontaneous calcium signature. The customized protocol was applied for 2 days from D3 to D5. B) Chronology of the application of optical stimulation during the culture of C57BL/10 myotubes expressing ChR2-Venus. C) Fusion index for unstimulated (red) and stimulated myotubes with the optical stimulation protocol (blue, $N = 3$). Data represent the mean value \pm SEM. Statistical analysis is performed by a two-way ANOVA test followed by a Sidak test (*: $p < 0.05$; **: $p < 0.01$). C) Variation in fusion index representing the percentage difference in the number of nuclei per myotube in the optically stimulated cultures compared to those not stimulated. D) Fraction (%) of contractile myotubes expressing ChR2-Venus unstimulated (D5, $n = 56$; D7, $n = 52$) or stimulated (D5, $n = 61$; D7, $n = 65$, $N = 3$) with the customized optical protocol. The contractile responses of the myotubes were induced and recorded using repeated blue light stimulations composed of pulses of 20 ms at 1 Hz. The statistical approach is performed by a Fisher exact test (* $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$). F) Change in contractility representing the percentage difference in the proportion of the number of contractile myotubes between optically stimulated cultures compared to unstimulated ones.

differentiation.

4. Discussion

In this study, we evaluated the effect of ChR2 stimulation on differentiation of mouse muscle primary cultures. Stimulation of ChR2 allowed to finely control calcium activity in such cultures. The application of an optical-stimulation protocol that mimics calcium signature of mature myotubes, determined with GCaMP6s, allowed to improve

maturation of primary myotubes. Light-induced calcium variations occurred through the excitation-contraction coupling mechanism. Hence, expression of ChR2 allowed, by handling mature calcium signature, to enhance primary myotube differentiation

In this primary culture model, we first performed an approach of localized stimulation of ChR2 with an area 10 times smaller than the area of a neuromuscular junction [38]. We have shown that stimulation of ChR2 induced calcium transients that were synchronized with the frequency of the optical stimulation. These results confirmed our

previous study on C2C12 cells [3,31] evidencing the potential of such optogenetic tool to control cell excitability and calcium activity in muscle cells with a fine spatiotemporal resolution. The optical stimulation approach by our self-made optical stimulation system showed also effective control of calcium activity and evidenced the efficacy of stimulation at the whole culture scale [32]. We characterized the different calcium players involved in the response to calcium stimulation and a strong involvement of classical actors of the excitation-contraction coupling mechanism was found [39]. The weak effect of voltage gated sodium channels inhibition on calcium transients showed a weak involvement of these channels in responses to optical stimulation, which confirms the first observations made on C2C12 myotubes [31,32]. We think that the ChR2-induced membrane depolarization is sufficient to reach the activation threshold of DHPs, bypassing the action of Nav. Also, the lack of effect of NCX exchanger inhibition showed the predominant role of the SERCA pump for calcium decreases during spontaneous oscillations.

4.1. Characterization of spontaneous calcium activity

The optogenetic tool GCaMP6s was used to characterize spontaneous calcium activity during differentiation of primary myotubes because it displays several advantages as compared to conventional calcium probes. Indeed, the GCaMP6s calcium indicator generates a bright fluorescence signal with a good signal/noise ratio, resistance to photobleaching and resistance to temperature allowing its use at 37 °C for long periods [36]. GCaMP6s fluorescence measurements showed that myotubes exhibit spontaneous calcium oscillations which progressively changes during differentiation with higher amplitude and longer duration. These results are in agreement with previous observations [40,14,17]. Nevertheless, these results are in contradiction with those obtained on the *Xenopus* myotubes model showing that the evolution of long duration Ca^{2+} transients (LTDs) towards short duration Ca^{2+} transients (STDs) would report the onset of myofibrillogenesis [41]. Moreover, it is established that the differentiated myotubes of rodents are able to generate faster and longer calcium responses [40] demonstrating a greater solicitation of calcium during differentiation. The pharmacological approach of these spontaneous calcium oscillations revealed a strong involvement of voltage-dependent channels such as voltage-dependent sodium channels and voltage-dependent L-type calcium channels in the formation of these oscillations. These calcium oscillations could be induced by the presence of spontaneous oscillations of the membrane potential [18,13]. Analysis of the calcium signature by GCaMP6s at a high acquisition rate revealed the presence of an intrinsic oscillatory frequency of approximately 6 Hz within long duration calcium events of differentiated myotubes. This high frequency is compatible with membrane potential variations driven by voltage gated ionic channels and triggering calcium oscillation. Interestingly, several studies have connected intracellular calcium oscillations to gene expression. Indeed, depending on the oscillatory frequency, calcium variations can be responsible for the specific activation of signaling cascades such as calcineurin/NFAT at low frequency and NF- κ B at high frequency, promoting selective gene expression [42,43]. The oscillatory frequency could be responsible for the specific activation of such mechanisms involved in myotubes differentiation. In addition, GCaMP6s fluorescence recordings over a long period of several hours revealed two oscillatory profiles of the primary myotubes: a main profile with continuous oscillations over time and a discontinuous profile showing rest periods between oscillations. This resting time could condition a breaking signal of the activation of calcium-dependent signaling pathways such as the calcineurin/NFAT pathway [44,45].

4.2. Modulation of myogenesis by optogenetics

The characterization of spontaneous calcium dynamics from differentiated myotubes made it possible to design an optical stimulation protocol applied to undifferentiated myotubes to modulate their differentiation state. The optical stimulation of myotubes increased the fusion index and their contractile ability. These data are in accordance with those obtained with electrical stimulation [20–22] which showed an increase of the expression of contractile machinery proteins and sarcomere assembly. These data also confirmed that optogenetics was a well suitable method to modulate myogenic processes as described in the C2C12 cell line where they used a low-frequency optical stimulation protocol [32]. It would be interesting to investigate if application of low-frequency protocols [21,32,24] would be efficient to modulate primary culture differentiation. As described, primary myotubes display spontaneous calcium oscillations and it would be interesting to altern ChR2 stimulation with the stimulation of an inhibitory optogenetic tool such as halorodopsin pump to keep a total control of membrane electrical activity. Indeed, we cannot exclude that spontaneous calcium activity adds up optically induced calcium variations modulating differentiation processes. Lots of electrical stimulation protocols were adapted of chronic exercise to understand the effect of physical training on cell and tissue transformations [24,46,47]. Even if frequency patterns of stimulation need to be adapted to fully differentiated muscle fibers, stimulation of ChR2 could reproduce these chronic exercises to better understand mechanisms implicated in beneficial health-related effects of exercise.

5. Conclusion

Our present work highlights the interest of using optogenetics to modulate myogenic processes through the control of calcium activity. This method has less invasive and more selective characteristics than the electrical stimulation approach and this makes it a good exploratory tool for cell and tissue functionality. We demonstrated that the application of an optical-stimulation protocol that mimics calcium signature of mature myotubes allowed to improve maturation of primary myotubes, revealing that myogenesis needs a specific temporal stimulation-pattern to occur.

CRediT authorship contribution statement

Charles-Albert Chapotte-Baldacci: Investigation, Formal analysis, Writing – original draft. **Christian Cognard:** Methodology. **Patrick Bois:** Funding acquisition. **Aurélien Chatelier:** Conceptualization, Methodology, Validation, Writing – review & editing, Supervision. **Stéphane Sebille:** Conceptualization, Methodology, Software, Validation, Writing – review & editing, Supervision.

Conflict of interest

No conflict of interest is declared by the authors.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.ceca.2022.102546](https://doi.org/10.1016/j.ceca.2022.102546).

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