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Disrupted T-tubular network accounts for asynchronous calcium release in MTM1 deficient skeletal muscle

by

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**Running title:** Simulation of Ca release in MTM1 muscle

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32 **Key points summary**

- 33 • Myotubular myopathy is a fatal disease due to genetic deficiency in the  
34 phosphoinositide phosphatase MTM1.
- 35 • Although causes are known and corresponding gene-therapy strategies are developed,  
36 there is no mechanistic understanding of the disease-associated muscle function  
37 failure.
- 38 • Resolving this issue is of primary interest both for fundamental knowledge of how  
39 MTM1 is critical for healthy muscle function but also for establishing the related  
40 cellular mechanisms most primarily or stringently affected by the disease, and thus of  
41 potential interest as therapy targets.
- 42 • The mathematical modelling approach used in the present work proves that the  
43 disease-associated alteration of the plasma membrane invagination network is  
44 sufficient to explain dysfunctions of excitation-contraction coupling, providing the  
45 first integrated quantitative framework explaining the associated contraction failure.

46

47 **Abstract**

48 In mammalian skeletal muscle propagation of surface membrane depolarization into the interior  
49 of the muscle fibre along the transverse (T-) tubular network is essential for the synchronized  
50 release of calcium from the sarcoplasmic reticulum (SR) via Ryanodine receptors (RyR) in  
51 response to the conformational change in the voltage-sensor dihydropyridine receptors.  
52 Deficiency in 3-phosphoinositide phosphatase myotubularin (MTM1) has been reported to  
53 result in the disruption of the T-tubules and the appearance of delayed SR calcium release. Here  
54 confocal calcium transients recorded in muscle fibres of MTM1-deficient mice were compared  
55 to results from a model where propagation of the depolarization along the T-tubules was  
56 modelled mathematically with disruptions in the network assumed to modify the access and  
57 transmembrane resistances as well as the capacitance. If, in simulations, T-tubules were  
58 assumed to be partially or completely inaccessible to the depolarization and RyR at these points  
59 to be prime for calcium-induced calcium release, all features of measured SR calcium release  
60 could be reproduced. We conclude that the inappropriate propagation of the depolarization into  
61 the fibre interior is the initial critical cause of severely impaired SR calcium release in MTM1  
62 deficiency, while  $\text{Ca}^{2+}$ -triggered opening of RyRs provides an alleviating support to the  
63 diseased process.

64

## 65 **Introduction**

66 In skeletal muscle the release of calcium ions from the sarcoplasmic reticulum (SR), the  
67 intracellular calcium store, is the key step controlling contraction (Schneider, 1994). The  
68 opening of the calcium release channels, the ryanodine receptors (RyR; Fleischer et al., 1985),  
69 in the SR membrane is therefore a strictly controlled process which is achieved by the molecular  
70 interaction between RyRs and the voltage sensors of the transverse-(T-) tubular membrane, the  
71 dihydropyridine receptors (DHPR; Ríos and Pizarro, 1991). Under physiological conditions  
72 RyRs remain closed until a depolarization of the surface and T-tubular membranes, normally  
73 in the form of an action potential, initiates a conformational change in the DHPR which in turn  
74 leads to the opening of RyRs and the subsequent fast release of calcium from the terminal  
75 cisternae of the SR (see for review Ríos et al., 1992; Baylor and Hollingworth, 2011;  
76 Hernandez-Ochoa and Schneider, 2018) and thus to contraction; a process referred to as  
77 excitation-contraction coupling (ECC). Relaxation is achieved by returning the released  
78 calcium back into the SR via the SR calcium pump (Melzer et al., 1986).

79 In mammals three different isoforms of RyR – type 1, 2, and 3 (RyR1, RyR2, and RyR3) – are  
80 expressed in a tissue specific manner (Marks et al., 1989). For striated muscles, RyR2 is found  
81 in the heart, while RyR1 and 3, with RyR1 being the dominant isoform (in most cases with  
82 more than 95%) are present in skeletal muscle (Sorrentino and Volpe, 1993). Importantly,  
83 isolated and reconstituted RyRs, all isoforms, display a clear sensitivity to calcium ions,  
84 namely, a small increase of intracellular calcium concentration ( $[Ca^{2+}]_i$ ) at their intracellular  
85 surface opens (calcium-induced calcium release; CICR), while a greater increase in  $[Ca^{2+}]_i$   
86 inactivates the channel (Endo, 2009). In heart muscle this calcium sensitivity plays a vital role  
87 in ECC as RyR2 is always activated by CICR (Gyorke & Gyorke, 1998). In contrast, in the  
88 skeletal muscles of mammalian species calcium ions, *i.e.* CICR, do not seem to have any role  
89 in RyR opening *in situ*, under physiological conditions.

90 A number of mutations in the *ryr1* gene have been described to date, many associated with  
91 altered handling of  $[Ca^{2+}]_i$  resulting from leaky (see *e.g.* Lawal et al., 2018) or less/non-  
92 conducting (Lawal et al., 2018) release channels. Nevertheless, CICR in adult skeletal muscle  
93 fibres even carrying these *ryr1* mutations has not been documented. On the other hand,  
94 situations where the DHPR and RyR interaction has not developed yet or has artificially been  
95 altered gave evidence that calcium ions can initiate the release of calcium from the SR. For the  
96 former, CICR was demonstrated in T-tubule devoid regions of skeletal myotubes in culture

97 (Yang et al, 2001) or in Cav1.1<sup>ΔE29</sup> (Sultana et al., 2016). For the latter, the presence of calcium  
98 sparks in enzymatically isolated adult skeletal muscle fibres subjected to osmotic challenge or  
99 to membrane permeabilization by Saponin provide the clear evidence (see *e.g.* Wang et al.,  
100 2005, and Pierantozzi et al., 2019, respectively).

#### 101 *Myotubularin, MTM1 deficiency, calcium sparks*

102 In 2009, skeletal muscle ECC was for the first time documented to be a major target of  
103 myotubular myopathy, the X-linked form of centronuclear myopathy due do deficiency in the  
104 phosphoinositide phosphatase MTM1 (Al Qusairi et al., 2009). Although mild and intermediate  
105 forms of the disease do exist, most of the patients experience the severe form which is  
106 associated with stringent muscle weakness, hypotonia, and respiratory distress from birth on,  
107 leading to death within the first months of life (see for review Annoussamy et al., 2019). The  
108 *Mtm1*-KO mouse model recapitulates the main features of the human disease (Buj-Bello et al.,  
109 2002) and has allowed a detailed characterization of the pathological mechanisms responsible  
110 for muscle weakness. This revealed an unanticipated accumulation of molecular and functional  
111 defects of the ECC machinery including T-tubule network disruption, triad disorganization,  
112 reduced protein amount of DHPR  $\alpha 1$  subunit and of RyR1, reduced voltage-activated DHPR  
113  $Ca^{2+}$  current, reduced, and spatially erratic at the subcellular level, voltage-activated RYR1-  
114 mediated SR  $Ca^{2+}$  release, conspicuous presence of spontaneous SR  $Ca^{2+}$  release activity at rest  
115 under the form of  $Ca^{2+}$  sparks (Al Qusairi et al., 2009; Kutchukian et al., 2016, 2019). The  
116 pathogenic mechanisms leading to these defects remain unclear (for a recent review, see  
117 Gómez-Oca et al., 2021). Interactions of MTM1 with other proteins, specifically BIN1 and  
118 DNM2, are likely implicated, as they are believed involved in processes of membrane curving,  
119 membrane tubulation and tubule fission, and could thus play a role in proper t-tubule  
120 maintenance in the differentiated fibres. In addition, accumulation of MTM1 phosphoinositide  
121 substrates may also directly affect RyR1-mediated SR  $Ca^{2+}$  release (Rodriguez et al., 2014).

122 The above observations lead us to propose that adult skeletal muscle fibres from MTM1  
123 deficient mice display CICR during normal excitation (Kutchukian et al., 2019). While the data  
124 were conclusive, there was no evidence that CICR could indeed generate the spatio-temporal  
125 profiles of  $[Ca^{2+}]_i$  seen in MTM1 deficient muscle fibres during voltage-clamp activation. Here  
126 we provide a detailed simulation of the activation and inactivation of RyRs under conditions  
127 where DHPR-and calcium-activated channels can co-exist but are spatially segregated, with the  
128 latter confined to areas where the T-tubular network is disrupted. By comparing measured and

129 simulated line-scan images of  $[Ca^{2+}]_i$  we demonstrate that this co-existence of differentially  
130 gated RyRs can explain all observed features of the spatially and temporarily inhomogeneous  
131 calcium transients seen in skeletal muscles of MTM1 knock-out mice.

132

## 133 **Methods**

### 134 Ethical Approval

135 All animal experiments were performed according to the ethics rules of the French Department  
136 of Veterinary Services and of the French Ministry for Higher Education, Research and  
137 Innovation, and followed the guidelines specified by the revised European Directive  
138 2010/63/EU and the local animal ethics committee of the University Claude Bernard Lyon 1  
139 (#DR2015-62).

### 140 Experimental procedures

141 The confocal Ca<sup>2+</sup> measurements described in the present manuscript are from a series of  
142 experiments reported by Kutchukian et al. (2016, 2019). In brief, single muscle fibres were  
143 isolated from the *flexor digitorum brevis* and interosseus muscles of 4-5-week old WT and  
144 *Mtm1*-KO male mice in the 129PAS background, using collagenase (Sigma, type 1) treatment  
145 for 60 min at 37 °C, followed by gentle mechanical trituration of the muscles. Single isolated  
146 muscle fibres were voltage-clamped with the silicone voltage-clamp technique (Jacquemond,  
147 1997) using a micropipette filled with a solution containing (in mM) 120 K-glutamate, 5 Na<sub>2</sub>-  
148 ATP, 5 Na<sub>2</sub>-phosphocreatine, 5.5 MgCl<sub>2</sub>, 12 EGTA, 4.8 CaCl<sub>2</sub>, 0.1 rhod-2, 5 glucose, 5 HEPES,  
149 while the extracellular solution contained (in mM) 140 TEA-methane-sulfonate, 2.5 CaCl<sub>2</sub>, 2  
150 MgCl<sub>2</sub>, 1 4-aminopyridine, 10 HEPES and 0.002 tetrodotoxin. Once the voltage-clamp pipette  
151 tip was inserted in a muscle fibre, a 30 min period was allowed before starting the  
152 measurements, to allow for equilibration of the fibre cytosol with the pipette solution.  
153 Membrane depolarizing steps were delivered from a holding value of -80 mV. Fluorescence of  
154 the Ca<sup>2+</sup>-sensitive dye rhod-2 in the fibre's cytosol was detected using the confocal line-scan  
155 configuration (1.15 ms per line) of a Zeiss LSM 5 Exciter microscope (Zeiss, Oberkochen,  
156 Germany) equipped with a 63× oil immersion objective (numerical aperture 1.4), with the line  
157 parallel to the longitudinal axis of the fibre. Voltage pulses-activated rhod-2 fluorescence  
158 changes were expressed as F/F<sub>0</sub> with F<sub>0</sub> the pre-pulse baseline fluorescence as described by  
159 Kutchukian et al. (2019). All experiments were performed at room temperature (20–22 °C).

160 Imaging of the t-tubule network was achieved with the membrane staining dye di-8-anepps as  
161 described by Kutchukian et al. (2016): isolated fibres were incubated for 60 minutes in the  
162 presence of 10 μM of the dye.

### 163 Mathematical models



164 *Intramembrane charge movement*

165 The steady-state membrane potential ( $V_m$ ) dependence of intramembrane charge movement,  
166  $Q_a(V_m)$ , that can directly gate the opening of the adjacent RyR (also referred to as charge1; see  
167 *e.g.* Brum & Ríos, 1987) was assumed to follow a Boltzmann-distribution described with the  
168 appropriate steepness factor ( $k_a$ ) and mid-point voltage ( $V_{50,a}$ )

169 
$$Q_a(V_m) = \frac{1}{1+e^{-(V_m-V_{50,a})/k_a}} \cdot \quad \text{Eqn. 1a.}$$

170 Distribution between charge in the inactivated (also referred to as charge2; Brum & Ríos, 1987)  
171 and non-inactivated (charge1) positions based on the holding potential ( $V_h$ ) was also assumed  
172 to follow a two-state Boltzmann distribution

173 
$$Q_i(V_h) = \frac{1}{1+e^{(V_h-V_{50,i})/k_i}}, \quad \text{Eqn. 1b.}$$

174 with a steepness factor and a mid-point voltage of  $k_i$  and  $V_{50,i}$ , respectively. These, and all  
175 subsequent parameters used in the model calculations are presented in Table 1.

176 Transitions of charge1 between the resting and activating states following a change in trans-  
177 membrane voltage was calculated based on a second-order single-barrier Eyring-rate model  
178 (Brum and Ríos, 1987) with

179 
$$\tau(V_m) = \frac{2 \cdot \tau_{max} \cdot \tanh\left(\frac{V_m - V_{50,a}}{2 \cdot k_a}\right)}{(V_m - V_{50,a})/k_a}, \quad \text{Eqn. 2.}$$

180 where  $\tau(V_m)$  and  $\tau_{max}$  are the time constants of charge transitions at the membrane potentials of  
181  $V_m$  and  $V_{50,a}$ , respectively. Examples of non-linear capacitive currents representing transfers of  
182 intramembrane charge between the resting and activating states with different  $V_m$  and  $V_h$  are  
183 presented in Fig. 1, 11, and 13.

184 *Membrane potential*

185 Spreading of the change in membrane potential into the depth of the fibre was assumed to satisfy  
186 the differential equation (see Adrian et al., 1969)

187 
$$\frac{\partial V_m}{\partial t} + \frac{1}{\tau} V_m = \frac{1}{r} \frac{\partial}{\partial r} \left( r D \frac{\partial V_m}{\partial r} \right), \quad \text{Eqn. 3.}$$

188 where  $r$  is the radial distance from the longitudinal axis of the fibre (*i.e.* space; note that the  
 189 fibre was assumed to be cylindrically symmetrical),  $t$  is the time from the change in voltage,  $D$   
 190 (a diffusion coefficient-like parameter) is equal to the ratio of the specific conductance ( $G_{lum}$ ;  
 191 dimension  $1/\Omega/m$ ) and capacitance ( $C_{tm}$ ; dimension  $F/m^3$ ) of the T-tubular lumen and  
 192 membrane, respectively ( $D = G_{lum}/C_{tm}$ ; dimension  $m^2/s$ ), while  $\tau$  (a time constant-like  
 193 parameter) is the ratio of the specific capacitance and conductance ( $G_{tm}$ ;  $\tau = C_{tm}/G_{tm}$ ; dimension  
 194 s) of the given T-tubule along which the potential spreads inward.

195 Given the boundary conditions,  $V_m(r=a, t=0) = V_h$ , where “a” is the radius of the fibre, the  
 196 solution of Eqn. 3 is

$$197 \quad \frac{V_m(r,t) - V_m(r,t=0)}{V_m(r,t=\infty) - V_m(r,t=0)} = 1 - 2 \sum_{j=1}^{\infty} \frac{1 + \beta_j^2 e^{-(1 + \beta_j^2)t/\tau}}{1 + \beta_j^2} \frac{J_0(r^* \alpha_j)}{\alpha_j J_1(\alpha_j)}, \quad \text{Eqn. 4.}$$

198 with  $J_i$  being the  $i^{\text{th}}$  first order Bessel function,  $\alpha_j$  the  $j^{\text{th}}$  positive root of  $J_0$ ,  $r^* = r/a$ ,  $\beta_j = \rho \alpha_j$ ,  
 199  $\rho = \sqrt{D\tau}/a$ , while  $a$ ,  $D$ , and  $\tau$  have their defined meaning. Note that in the steady-state ( $t =$   
 200  $\infty$ ) the solution of Eqn. 4 is the function of the electrical properties of the given T-tubule ( $\rho$ )  
 201 and the relative distance ( $r^*$ ) from the surface (Fig. 2). Note also that while  $\tau$  is the time constant  
 202 of the T-tubule membrane,  $\rho$  can be considered as its space constant normalized to the fibre  
 203 radius. Figs. 3&4 present the calculated time courses of membrane voltage, amount of charge  
 204 in the activating position, and the corresponding charge movement current for the different  $\rho$   
 205 and  $\tau$  values. It was apparent that if  $\rho$  was considered to be close to normal ( $\rho=5$  for Fig. 3) the  
 206 effect of  $\tau$  was marginal. On the other hand, if  $\rho$  was set to 1 (Fig. 4; considerable T-tubule  
 207 dysfunction) the effect of  $\tau$  became significant.

## 208 *RyR activation*

209 The activation of the RyR calcium release channels was, unless otherwise specified (see below),  
 210 taken to be the consequence of the conformational change in the adjacent voltage sensors  
 211 (DHPRs). Namely, when the voltage sensors (those in the charge1 position) in response to a  
 212 depolarizing pulse move to the activated state the neighboring RyRs open. These channels will  
 213 hereon be termed as charge- (Q-) gated.

214 Release channels were also assumed to have a calcium activating site (Sárközi et al., 2000;  
 215 Ogawa et al., 2021). Upon binding of  $Ca^{2+}$  to this site (Fig. 5A) the channel undergoes a  
 216 conformational change and opens. The process is implemented using a four-state model (Fig.

217 5B) with two closed and two open states from each of which one is  $\text{Ca}^{2+}$  bound and one is not.  
218 These channels will hereon be termed as calcium- ( $\text{Ca}^{2+}$ -) gated.

219 Note that in the framework of the model a RyR calcium release channel is either Q-gated or  
220  $\text{Ca}^{2+}$ -gated, *i.e.*, a Q-gated channel cannot be activated by  $\text{Ca}^{2+}$  only by the voltage sensor.  
221 Moreover, channels that are located in normally functioning T-tubules are always Q-gated. On  
222 the other hand, some RyRs in disrupted T-tubules can be  $\text{Ca}^{2+}$ -gated.

### 223 *RyR inactivation*

224 All channels (both Q- and  $\text{Ca}^{2+}$ -gated) can undergo a calcium-dependent inactivation. The  
225 channels are assumed to have a binding site for  $\text{Ca}^{2+}$  which, if occupied, initiates a  
226 conformational change to a state where the channel cannot be opened (Fig. 5A). The process is  
227 modeled using a four-state scheme similar to that described for the activation site (Fig. 5C).  
228 The entire process of inactivation was assumed to be independent of all other properties of the  
229 channel (whether Q- or  $\text{Ca}^{2+}$ -gated and is in a closed or an activated state). It is further assumed  
230 that the parameters describing the activation and inactivation of RyR by calcium is independent  
231 whether the T-tubular network is disrupted or not.

### 232 *Voltage dependence of RyR opening*

233 Fig. 6A presents the calculated time course of SR calcium release expressed as the relative  
234 proportion of open RyR-s in a fibre with normal T-tubules (*i.e.*  $\rho=5$  and  $\tau=20$  ms; based on  
235 values for  $G_{lum}$ ,  $G_{tm}$ , and  $C_{tm}$  as estimated by Adrian et al., 1969) for depolarizations to the  
236 indicated voltages from a holding potential of -80 mV. For a Q-gated channel to be activated  
237 four adjacent voltage sensors (based on the arrangement of DHPR-s and RyR-s in the junctional  
238 area; see *e.g.* Flucher and Franzini-Armstrong, 1996.) were assumed to be needed in the  
239 activating position. This gave rise to a rightward shift and steeper voltage dependence of SR  
240 calcium release (Fig. 6B; black symbols and trace) as compared to that of intramembrane  
241 charge movement (Fig. 6B; red trace).

242 To further enable the comparison with previously published data, the declining phase of the  
243 time course of channel opening during the depolarizing pulse – representing the calcium  
244 dependent inactivation of RyR – was fitted by a single exponential function. The obtained time  
245 constants ( $\tau_{ina}$ ) were then plotted as a function of the  $[\text{Ca}^{2+}]_{junc}$  ( $[\text{Ca}^{2+}]_{junc}$ )  
246 expressed as relative to the maximal attainable  $[\text{Ca}^{2+}]_{junc}$  ( $[\text{Ca}^{2+}]_{junc,rel}$ ; Fig. 6C). These data  
247 points were then fitted with

248 
$$\tau_{ina}([Ca^{2+}]_{junc,rel}) = \frac{K_{d,rel} + [Ca^{2+}]_{junc,rel}}{[Ca^{2+}]_{junc,rel} \cdot k_i}, \quad \text{Eqn. 5.}$$

249 where  $K_{d,rel}$  is the dissociation constant of the inactivation site normalized to the maximal  
250 attainable  $[Ca^{2+}]_{junc}$  and  $k_i$  is the rate constant of the conformational change of RyR (see *e.g.*  
251 Sárközi et al., 1996). As demonstrated in Fig. 6C the data points were well fitted with Eqn. 5  
252 giving rise to a  $k_i$  of 78.7 1/s in accordance with previously published data from measurement  
253 on isolated fibres (Sárközi et al., 1996).

254

255 **Results**

256 *Disrupted T-tubular network in MTM1 deficient skeletal muscle fibres*

257 Fig. 7 presents transmitted light (panels A&B) and fluorescence images (panels C&D) of  
258 control and MTM1 deficient muscle fibres collected with a confocal microscope to demonstrate  
259 how the absence of this phosphoinositide phosphatase affects the T-tubular network. To this  
260 end, fibres were stained with di-8-Anepps, a hydrophobic voltage-sensitive fluorescent dye that  
261 has been extensively used to stain the surface membrane of excitable cells (*e.g.* Al-Qusairi et  
262 al., 2009; Kutchukian et al., 2016). Fig. 7C demonstrates that under physiological conditions  
263 the staining reveals a regular double-rowed pattern consistent with the organization of the T-  
264 tubular network. On the other hand, in the image of the fibre from the MTM1 knock-out animal  
265 (Fig. 7D) large patches of non-stained areas are present indicative of disrupted T-tubules in  
266 these parts of the muscle fibre. However, the image also demonstrates that there are zones with  
267 normal staining as well. These observations suggest that in MTM1 deficient fibres areas with  
268 both normal and modified ECC should be present.

269 *Implementation of the model for ECC with a modified T-tubular network*

270 To understand the implications of disrupted T-tubules on ECC, a computer routine was written  
271 to simulate the spread of excitation along the T-tubules and the consequent activation and  
272 inactivation of RyRs at a given release site together with the diffusion of calcium ions between  
273 these sites. For simplicity simulation was performed in one dimension – along the scan-line –  
274 only. The events at different release sites were assumed not to influence one another apart from  
275 the  $\text{Ca}^{2+}$  arriving from the adjacent sites and modifying the local  $[\text{Ca}^{2+}]$ . As the scan-line was  
276 positioned parallel with the longitudinal axis of the fibre the relative distance ( $r/a = r^*$ ) was  
277 taken to be identical for all spatial positions.

278 As evidenced from Eqn. 4, the spatial differences in charge movement activation depend only  
279 on how the specific parameters ( $D$  and  $\tau$ ) of the T-tubular membrane vary along the scan-line.  
280 To incorporate this into the model,  $\tau$  and the dimensionless parameter  $\rho$  were allowed to vary  
281 along the scan-line to describe any disruptions in the T-tubule network at the given spatial  
282 position. This is reflected both in the spatial variation in  $V_h$  (see Fig. 8A) and in the time course  
283 of the membrane potential change that drives the conformational change of the voltage sensors  
284 (see Fig. 2).

285 Q-gated channels were assumed to be activated immediately when all four of their voltage  
286 sensors moved to the activating position. They remained open until one of the voltage sensors  
287 returned to its resting state unless they became inactivated due to the calcium-dependent  
288 inactivation process. To incorporate in the model that calcium ions may activate the channels a  
289 dimensionless parameter ( $R_m$ ) was introduced which described the proportion of  $\text{Ca}^{2+}$ -gated  
290 channels at a given spatial position (see Fig. 8A). By definition  $0 \leq R_m(i) \leq 1$  for all “ $i$ ”  
291 (where “ $i$ ” refers to a given spatial coordinate in the image along the scan-line); and  $R_m(i)=0$  if  
292 all channels are Q-gated and  $R_m(i)=1$  if all channels are  $\text{Ca}^{2+}$ -gated at that position (if, *i.e.*, 30%  
293 of the channels are  $\text{Ca}^{2+}$ -gated then  $R_m(i)=0.3$ ; see Fig. 8A).  $\text{Ca}^{2+}$ -gated channels could also  
294 undergo calcium-dependent inactivation, as mentioned above.

295 Once the spatial profiles of T-tubule properties and channel activation were set the simulation  
296 was run to follow the release and diffusion of calcium ions along the scan-line. The program  
297 generated the spatio-temporal profiles in each point of the scan-line of intramembrane charge  
298 movement, RyR activation and inactivation, and the calcium concentration. The latter, as a line-  
299 scan image was then compared to those measured experimentally.

300 As an example, Fig. 8 presents the result of the simulation where the scanning line was assumed  
301 to traverse three different compartments of the fibre. In certain spatial positions – exemplified  
302 by position #1 in Fig. 8A – the T-tubule network was assumed to be intact and RyRs were thus  
303 taken to be all Q-gated (*i.e.*  $\rho=5$  and  $R_m=0$ , and consequently the holding potential,  $V_h$ , was  
304 calculated to be -80 mV; left panel in Fig. 8A). In another spatial position (position #2 in  
305 Fig. 8A) the T-tubule network was assumed to be completely disrupted ( $\rho=0.2$ ; calculated  $V_h =$   
306 -8 mV; see also Fig. 2A) while all RyRs were still taken to be Q-gated. Finally, in a part of the  
307 fibre, with completely disrupted T-tubules, 30% of the calcium release channels were assumed  
308 to be  $\text{Ca}^{2+}$ -gated ( $R_m=0.3$ ). As demonstrated in Fig. 8 this gave rise to spatially inhomogeneous  
309 intramembrane charge movement and calcium release with delayed openings of RyR. Note also  
310 that while calcium release terminated instantly upon fibre repolarization at positions where only  
311 Q-gated channels were present (position #1 in Fig. 8), this was not the case where calcium was  
312 allowed to govern channel opening (position #3).

### 313 *Spatially and temporarily heterogeneous calcium release in MTM1 deficient skeletal muscle*

314 Fig. 9 presents line-scan images of calcium transients measured on enzymatically isolated  
315 skeletal muscle fibres from myotubularin deficient mice in response to depolarizing pulses of  
316 500 ms in duration and various amplitudes. The images were selected to demonstrate critical

317 features of the calcium release process in MTM1 deficient muscle fibres that are not seen in  
318 healthy controls. First, and foremost, as demonstrated in all panels of Fig. 9 (see spatial  
319 positions pointed at by black arrows) there are positions within the fibre that behave essentially  
320 identical to that seen under control conditions. Namely, large calcium release occurs that is  
321 synchronous with the depolarizing pulse and displays a fast rising phase, fast inactivation, and  
322 termination upon returning to the holding potential.

323 On the other hand, four distinct features can be identified in these images that are not present  
324 in control muscle fibres. First, as seen in panels A (position at the red arrow) and Ca, there are  
325 regions of the fibre where, despite of the depolarization, none or very little calcium release  
326 occurs. Secondly, as exemplified in Fig. 9B, propagating calcium waves seem to occur in these  
327 cells. This is substantiated by the linear wave front along the spatial dimension and the delays  
328 in calcium release activation as demonstrated by the time-courses of the calcium transients  
329 depicted at positions marked by the blue and red arrows shown below the line-scan image.  
330 Thirdly, at distinct spatial positions calcium release activation is delayed as compared to the  
331 onset of fibre depolarization (see *e.g.* positions in Fig. 9Ca marked by blue and red arrows and  
332 the corresponding traces below the image) independent of any visible calcium wave. Finally,  
333 by comparing panels Ca and Cb of Fig. 9 – in which consecutive line-scans and corresponding  
334 transients are presented from the same spatial positions of the same fibre with depolarizations  
335 to -10 and 0 mV, respectively – it is evident that this delay in the activation of SR calcium  
336 release is voltage dependent. To further the comparison green arrows are positioned at the same  
337 point in time on the lowermost traces (corresponding to the spatial position marked by red  
338 arrows at the line-scan images). The time point was selected to mark the peak of the transient  
339 in panel Ca of Fig. 9. Note that the peak of the transient in panel Cb occurs earlier than that in  
340 panel Ca, demonstrating that a shorter delay for activation corresponds to a larger  
341 depolarization.

#### 342 *Lack of or delayed activation of SR calcium release*

343 It is evident from the model used, and is demonstrated in Fig. 8A (spatial position #2), that if  
344 the T-tubules are critically disrupted (in that particular example  $\rho=0.2$ ) and there are no  $\text{Ca}^{2+}$ -  
345 gated RyRs in the given region of the fibre calcium release from the SR will not occur. Under  
346 these conditions the resting  $V_h$  is very low (-8 mV in Fig. 8A) giving rise to an essentially  
347 complete inactivation of the voltage sensors and their consequent inability to activate the release  
348 channels. This, taken together with the disrupted T-tubular network (see Fig. 8) would simply

349 explain the observation that some regions are devoid of SR calcium release in the line-scan  
350 images.

351 However, the introduction of  $\text{Ca}^{2+}$ -gated RyRs in such a region would give rise to essentially  
352 instantaneous (the delay would only be the time needed for calcium to diffuse from a  
353 neighboring release site) calcium release. To generate an appreciable delay in activation of SR  
354 calcium release based on calcium-induced calcium release only, one should introduce a large  
355 area with no  $\text{Ca}^{2+}$ -gated RyRs as was done in Fig. 8A (see the delay in the onset of the calcium  
356 wave). This, nevertheless, was not the case in the images presented in Fig. 9C where the delay  
357 was present in spatially neighboring locations (*i.e.* within the resolution of the confocal system).

358 To explain this observation, we explored the possibility according to which altered T-tubular  
359 properties could underlie the delayed onset of SR calcium release. Simulations were run using  
360 different values for  $\rho$  and  $\tau$  ranging from 0.2 to 5 and from 20 to 600 ms, respectively, with only  
361 Q-gated calcium release channels present. The surface membrane was depolarized to -10 mV  
362 (identical in size to the depolarization used in the actual measurement presented in Fig. 10A)  
363 for 100 ms. Fig. 10B thus presents simulated line-scan images where two distinct areas were  
364 considered within the muscle fibre. In the region represented by the black arrow  $\rho$  and  $\tau$  were  
365 set to their normal values (5 and 20 ms, respectively), while in the affected region  $\rho$  was reduced  
366 to 0.8, 0.9, and 1 for panels Ba, Bb, and Bc, respectively, with  $\tau$  being set at 200 ms. Note that  
367 the onset of SR calcium release under these conditions was substantially delayed as compared  
368 to the start of the depolarization. Furthermore, as evidenced from the images the smaller the  $\rho$   
369 the greater was the delay. This dependence of the onset of SR calcium release activation on  $\rho$   
370 is presented in Fig. 10C where the position of the peak of the transients is displayed as a function  
371 of  $\rho$ . If the T-tubular network is functioning close to normal ( $\rho > 2$ ) the activation of the interior  
372 of the fibre is readily achieved, while if the T-tubular access is reduced the activation is  
373 substantially prolonged, and furthermore, this prolongation is extremely sensitive to the space  
374 constant when it is close to the radius of the fibre (*i.e.* when  $\rho$  is equal to or less than 1). The  
375 calculated changes in membrane voltage and intramembrane charge movement are presented  
376 in Fig. 11. It should be noted that if  $\rho$  is reduced below 0.7 no detectable calcium release occurs  
377 (in the absence of Ca-gated channels) due to the insufficient activation of the Q-gated channels  
378 (data not shown).

379 *Voltage dependence of activation delay*



380 To further test the potential of the model, depolarizing pulses of increasing amplitudes were  
381 applied to characterize the voltage dependence of the delay of SR calcium release in areas of  
382 affected T-tubules. To generate the images in panel B of Fig. 12 the previously introduced  
383 method (Fig. 10B) was followed. The fibre was assumed to have an area of normal (represented  
384 by the black arrows) and an additional area with disrupted T-tubules (represented by the blue  
385 arrows). The surface membrane was depolarized to -20, 0, and +20 mV for panels Ba, Bb, and  
386 Bc, respectively, to explore the voltage range used in the actual measurements (line-scans from  
387 such measurements are presented in panels Aa and Ab). To simulate the T-tubule mal-function  
388  $\rho$  and  $\tau$  were set to 0.8 and 200 ms, respectively, values corresponding to those used for Fig. 10.  
389 The corresponding changes in membrane voltage and intramembrane charge movement are  
390 presented in Fig. 13. Note that the delay for calcium release activation, as also evidenced from  
391 the traces below the line-scan images in panel B was clearly voltage-dependent. Furthermore,  
392 in line with the observations from the actual measurements, greater depolarizations brought  
393 about shorter delays. The membrane potential-dependence of release activation – assessed as  
394 the time-to-peak of the transients – is presented in Fig. 12C for the voltage range of -20 to  
395 +20 mV for two values of  $\rho$ . In T-tubules with a low space constant ( $\rho=0.8$ ) the voltage  
396 dependence is steep, while for only a slightly less affected T-tubule ( $\rho=1$ ) this dependence is  
397 shallow.

### 398 *Calcium waves*

399 Calcium waves have previously been described in both cardiac (Wier & Blatter, 1991) and  
400 skeletal muscle (Cully et al., 2014), therefore, we expected that our model would simply  
401 reproduce the presence of propagating wave fronts of calcium release activation (as presented  
402 in Fig. 14A from an actual measurement) once Ca-gated release channels and, consequently,  
403 CICR is present in the model. This assumption was clearly substantiated by the simulation  
404 presented in Fig. 8A. The calcium waves described so far had a velocity around 0.2  $\mu\text{m}/\text{ms}$   
405 (Zhou et al., 2005).

406 Using 0.53  $\mu\text{m}^2/\text{ms}$  for the diffusion coefficient of  $\text{Ca}^{2+}$  in the myoplasm (a value taken from  
407 the literature; see Table 1) our model readily generated a propagating calcium wave as presented  
408 in Fig. 14Bb. For the simulation we followed the idea used for Figs. 10&12, namely, the fibre  
409 was divided into two areas one with normal and another with disrupted T-tubules. In this case,  
410 however, affected T-tubules were assumed to be essentially incapable of conducting the change  
411 in the surface membrane potential into the interior of the fibre (*i.e.*  $\rho$  was set to 0.2) but to have

412 some  $\text{Ca}^{2+}$ -gated channels ( $R_m$  was set to 0.3 for the simulations in Fig. 14). This setting  
413 (Fig. 14Bb) gave rise to a calcium wave with a propagation velocity (calculated from the slope  
414 of the wave front) of  $0.285 \mu\text{m}/\text{ms}$ , essentially in line with previous measurements (see above).  
415 Varying the diffusion coefficient of  $\text{Ca}^{2+}$  in the range of 0.3 (corresponding line-scan is shown  
416 in Fig. 14Ba) to  $0.6 \mu\text{m}^2/\text{ms}$  had little effect on the speed of propagation as presented in  
417 Fig. 14C (black symbols). These findings convinced us that CICR and calcium wave  
418 propagation properly incorporated into our model.

419 However, values calculated from actual measurements (red symbols in Fig. 14C) were scattered  
420 over a much wider range, reaching propagation speeds as great as  $2.4 \mu\text{m}^2/\text{ms}$ ; an order of  
421 magnitude greater than those published earlier. As propagation speed based on CICR critically  
422 depends on the rate and amount of calcium released from the internal calcium stores,  
423 simulations were repeated with changing the assumed free SR  $\text{Ca}^{2+}$  concentration (using  
424  $0.53 \mu\text{m}^2/\text{ms}$  as the diffusion coefficient for calcium in the myoplasm). Although the  
425 propagation speed indeed became faster with increased SR calcium load as presented in  
426 Fig. 14D, doubling the load only increased the speed by 26.3%. These simulations convinced  
427 us that the extremely fast wave fronts could not be explained by assuming CICR to be present  
428 in these fibres. However, it should be emphasized that in the majority of fibres the propagation  
429 velocities were essentially identical to those obtained from our simulations suggesting that  
430 CICR was indeed responsible for the development of propagating wave fronts in those fibres.

#### 431 *Wave front of calcium release activation*

432 The extremely large apparent propagation speed for the activation of SR calcium release  
433 observed in a subpopulation of fibres required alternative ways, other than CICR, to explain.  
434 The observations that different  $\rho$  values – *i.e.* different extents of T-tubular disruption – can  
435 result in different delays for the onset of calcium release activation and that T-tubular defects  
436 seemed to appear in patches (see Fig. 7) suggested a possible solution. In all of the simulations  
437 above the spatial profile of  $\rho$  was assumed to change suddenly (from a control value of 5 to  
438 different but lower values to represent the defect) and then remain constant. What if  $\rho$  was to  
439 change “smoothly”, *i.e.* following a given continuous spatial profile, that represents a T-tubular  
440 defect that is not identical within a region, rather it becomes worth and worth.

441 Simulations presented in Fig. 15 explore this possibility. Panels Fig. 15A&C present two  
442 simple spatial profiles for  $\rho$ , namely,  $\rho$  was assumed to decrease linearly or following a second  
443 order polynomial from a starting of “1” to a final value of “0.8” for panels A and C, respectively,

444 in the affected region. As in all simulations, a portion of the fibre was assumed to have normal  
445 T-tubules ( $\rho=5$ ). Results of the simulations with the linear and quadratic functions for  $\rho$  are  
446 presented in panels Fig. 15B and D, respectively. In both cases an apparent traveling waveform  
447 is observed which has faster apparent speed of traveling (greater slopes) than those presented  
448 in Fig. 14B. This later observation is quantified in Fig. 15E where the apparent speeds  
449 – calculated from the slopes – at positions marked by corresponding letters “a”, “b”, and “c” on  
450 the line-scan image in panels B and D, respectively, are plotted together with the measured  
451 propagation speeds (values taken from Fig. 14C). The simulated values fall perfectly in the  
452 range of extremely large measured speeds suggesting that a progressive decline in T-tubular  
453 function in the affected domains could be the underlying reason for apparent traveling wave  
454 fronts of SR calcium release.

455 Taken together, our simulations with the simple assumptions of altered T-tubular accessibility,  
456 i.e. reduced  $\rho$  and  $\tau$ , and the appearance of  $\text{Ca}^{2+}$ -gated channels in areas where voltage control  
457 was completely lost were able to describe not only qualitatively but also quantitatively the  
458 complex observations that in certain domains release activation is delayed, this delay is strongly  
459 voltage dependent, and traveling wave fronts of release activation can occur with a large range  
460 of apparent traveling speeds.

461

462 **Discussion**

463 *Diseased Ca<sup>2+</sup> release reproduced with numerical simulations*

464 We provide here quantitative mechanistic understanding of critical hallmarks of the defective  
465 SR Ca<sup>2+</sup> release process that is associated with muscle contraction failure in myotubular  
466 myopathy. Results from the modelling demonstrate that various extents of T-tubule disruption  
467 (in concurrence with the experimental observations) combined with the consequent opened  
468 option for RyR1 channels to switch from a charge(Q)-gated to a Ca<sup>2+</sup>-gated operating mode,  
469 reproduces the diseased Ca<sup>2+</sup> transients. There are two important accompanying correlates: -T-  
470 tubule disruption is a key initial determinant of the disease-associated functional deficit, -Ca<sup>2+</sup>-  
471 gated RyR1 channels provide an emergency support alleviating (though not relieving) the  
472 impact of the defective normal Ca<sup>2+</sup> release process. Of importance, the similarity between Ca<sup>2+</sup>  
473 release symptomatology between MTM1-deficient muscle and other centronuclear myopathies  
474 (Kutchukian et al., 2017; Huntoon et al., 2018; Silva-Rojas et al., 2022) promotes the likelihood  
475 that identical or closely related pathophysiological mechanisms are at work in those diseases as  
476 well.

477 SR Ca<sup>2+</sup> release in healthy mammalian muscle fibres exclusively relies on Q-gated RyR1  
478 channels; it exhibits very standardized features in terms of voltage-control of onset and  
479 termination, voltage-dependency of activation, kinetics, spatial homogeneity, which overall  
480 contribute to the stringent safety of the control of muscle contraction. In contrast, MTM1-  
481 deficient muscle fibres exhibit a diversity of SR Ca<sup>2+</sup> release alterations including spatial  
482 heterogeneity, kinetic defects, depressed amplitude, propagating events and voltage-free  
483 spontaneous activity (Al-Qusairi et al., 2009; Kutchukian et al., 2016, 2019). So far, a unifying  
484 modelling framework capable of explaining defects in voltage-activated Ca<sup>2+</sup> release in this  
485 disease model had remained unavailable. The present work relies on a history of previously  
486 worked-out quantitative biophysical knowledge of the properties of the T-tubule network, of  
487 the EC coupling process and of the RyR channels, including Ca<sup>2+</sup>-dependency of their  
488 activation and inactivation, to provide such framework. The model considers healthy SR Ca<sup>2+</sup>  
489 release areas where opening of RyR channels is triggered by the voltage-dependent DHPR Q  
490 displacement in the T-tubule membrane, followed by RyR closure due to Ca<sup>2+</sup>-dependent  
491 inactivation, together with diseased Ca<sup>2+</sup> release areas where Q-dependent RyR gating is  
492 compromised because of limited T-tubule accessibility to voltage changes. This is sufficient to  
493 predict spatially inhomogeneous ICM activation due to locally shunted T-tubule voltage

494 distribution, delayed  $\text{Ca}^{2+}$  transients and the voltage-dependency of the delay. Under these  
495 conditions, implementing the added possibility for a fraction of the RyR channels to undergo  
496  $\text{Ca}^{2+}$ -dependent activation is sufficient to recapitulate the existence of the observed propagating  
497 transients. Importantly, the strength of the model is that the two-above disease-related  
498 assumptions are validated by the images of the T-tubule network in the diseased fibres (see Fig.  
499 7) and by the consensual concept according to which control of RyR activation by the DHPR  
500 precludes the  $\text{Ca}^{2+}$ -activation mode (see Ríos, 2018).

501 It is important to stress that the apparent speeds of propagation for the observed calcium wave  
502 fronts showed a large scatter with values ranging from 0.16 to 2.48  $\mu\text{m}/\text{ms}$  (see Figs. 14 and  
503 15). To explore the possible underlying mechanisms one must first consider the number of  
504 evidence that show that if the T-tubular network is disrupted – *e.g.* by Saponin permeabilization  
505 or by osmotic stress (Bodnar et al., 2014; Wang et al., 2005, respectively) – or not yet developed  
506 – as in cultured myotubes (Shirokova et al., 1999) – the RyRs are prone to be activated by  
507 calcium. This raised the possibility that the wave front is due to a propagating calcium wave.  
508 While the values at the lower end would be consistent with a CICR-based propagation (Figuroa  
509 et al., 2012), those of the higher end are clearly not. A number of possibilities arise to explain  
510 the apparent fast propagation of the calcium wave front. *In situ* the traveling of an action  
511 potential (AP) along the longitudinally positioned T-tubules would be an ideal candidate. These  
512 APs have indeed been demonstrated to exist and to travel with a speed of  $13 \pm 3 \mu\text{m}/\text{ms}$   
513 (Posterino et al., 2000), comparable to the values measured here. However, under our  
514 experimental conditions (the presence of 2  $\mu\text{M}$  tetrodotoxin and tetraethylammonium being the  
515 major cation in the External solution) APs could not develop, thus this possibility must be  
516 excluded. Nevertheless, longitudinally positioned T-tubules provide an alternative solution.  
517 Namely, electrotonic propagation. If these T-tubules are also affected as the transversally  
518 running T-tubules, and there is no reason to believe that they aren't, slowed propagation could  
519 take place in them, too. This framework would, however, require that the transversal T-tubules  
520 of the affected area should be gradually more and more affected otherwise the excitation would  
521 reach a given spatial point faster along the corresponding transversal T-tubule than *via* a distant  
522 transversal T-tubule followed by propagation along a longitudinal T-tubule. This scenario is  
523 not very different from what we have assumed in Fig. 15 (and in the corresponding text), *i.e.*  
524 gradually changing  $\rho$ , the only simplification is that spread along longitudinal T-tubules was  
525 not modeled. Taken together, our model – and the observations – are consistent with the

526 possible presence of CICR-based propagation of calcium waves but inevitably require  
527 additional, most likely gradual loss of T-tubule function in the affected areas.

### 528 *Limitations of and simplifications in the models used*

529 Although the models were effective in reproducing the most important features associated with  
530 MTM1 deficiency, it should be emphasized that a number of simplifications were made when  
531 collating the events of EC-coupling. Furthermore, certain details seen in line-scan images  
532 representing modified SR calcium release were not explored here.

533 First, and foremost, interactions between adjacent T-tubules other than the diffusion of calcium  
534 from a release site to its neighbors were not considered. This approximation neglects the  
535 presence of longitudinal T-tubules (Franzini-Armstrong, 1991) that were shown to conduct  
536 electrical activation between sarcomeres even in the absence of surface membrane  
537 depolarization (Launikonis and Stephenson, 2004), and the percentage of which was shown to  
538 be increased in MTM1-deficient muscle fibres (Al Qusairi et al., 2009). A scenario where  
539 excitation along first a non-affected transversal and then along a longitudinal T-tubule would  
540 reach areas where the corresponding T-tubule is not functioning is, therefore, not unimaginable.  
541 In this framework access distances to otherwise activatable RyRs could be much greater than  
542 the radius of the fibre. This would then lead to activation delays even if T-tubular conductance  
543 ( $G_{lum}$  and  $G_{tm}$ ) and capacitance ( $C_{tm}$ ) are not altered. While this possibility cannot be ruled out,  
544 the observed delays (under certain conditions greater than 100 ms) would, if T-tubules are  
545 functioning normally, require access distances to exceed the fibre radius several fold. This is  
546 unlikely to be the case based on the staining of T-tubules (Fig. 7), which suggested that the size  
547 of disrupted areas was clearly less than that of the fibre diameter. Nevertheless, this alternative  
548 pathway for activation with modified T-tubular parameters, *i.e.* reduced  $\rho$  and/or increased  $\tau$ ,  
549 could explain the extremely long delays seen in a few line-scan images.

550 Intramembrane charge movement was described using a simple two-state model with Eyring-  
551 rate kinetics (Brum and Ríos, 1987). This is clearly an over-simplification of the process. A  
552 number of reports, including those from our laboratory, have confirmed that distinct kinetic  
553 components are present on charge movement currents, referred to as the ‘hump’ component or  
554  $I_{\gamma}$  (see *e.g.* Huang, 1991; Szücs et al., 1991). This component was suggested to be the result of  
555 calcium release from the SR and thus generating a positive feed-back in RyR activation (Pizarro  
556 et al., 1991), resulting in a steeper voltage-dependence of SR calcium release than that expected  
557 from the charge movement alone. Furthermore, both orthograde and retrograde coupling

558 between DHPRs and RyRs, based on their physical interaction, have been described (Dirksen,  
559 2002). These, and a number of other information including, but not restricted to, whether the  
560 four S4 segments in a given DHPR would contribute equally to the activation or the four DHPRs  
561 that have been suggested to interact with a single RyR (Huang et al., 2011) would all participate  
562 in the activation, make the precise simulation of intramembrane charge movement and its role  
563 in the activation process to be problematic. However, we did not aim to describe the exact  
564 voltage-dependence of the processes that were analyzed here, rather, only the tendency how the  
565 size of the depolarization affects them was assessed. Since no in-depth analysis was needed the  
566 model used here was, in our opinion, sufficient to give the necessary insight.

567 The simulation of the release of calcium from the SR also involved a number of simplifications.  
568 RyRs in mammalian muscle were described to be organized in a double-row pattern where only  
569 every second calcium release channel is coupled physically to the DHPRs (Protasi, 2002). In  
570 our simulations all RyRs in a given triad were assumed to behave identically. This is an over-  
571 simplification not only because of the above mentioned morphological arrangement, but also  
572 because the T-tubular membrane might be differentially affected within the region of the triad  
573 giving rise to the possibility of differentially gated RyRs. It is also feasible that the  
574 pathophysiology is not restricted to the T-tubular membrane, but the SR membrane is affected,  
575 too, which again could give rise to differentially gated channels within a given triad.  
576 Furthermore, albeit the regulation of the calcium release channel by calcium is included into  
577 and is an essential part of the model simulations, not only are the gating schemes for both  
578 calcium-dependent activation and inactivation simplified (see *e.g.* Dura et al., 2003; another  
579 model with elaborate description) but the luminal regulation of the channel (Quin et al., 2009)  
580 was not included either. However, the time-course (and voltage-dependence) of the simulated  
581 SR calcium release (Fig. 6) in non-affected areas closely resembled that published earlier  
582 confirming that the model used here reproduces the most important features of the release  
583 process, thus enabling us to conduct not only a qualitative but a quantitative comparison of  
584 measured and simulated values. Nevertheless, it should be noted that when selecting the  
585 parameters describing the activation and inactivation schemes we, while trying to use values as  
586 close to those published for the given processes, kept in mind that the final wave-form and  
587 magnitude of SR calcium release should be as close to those measured earlier as possible.

588 This raises the question of how adequate was the selection of parameters used in the model  
589 simulations. Importantly, the parameters used represent, to the best of our knowledge, the  
590 physiological values of the given processes. Even if these would be adequate to describe the

591 physiological case, they might not be relevant for the areas with affected T-tubules. This is  
592 substantiated by the finding that altered membrane properties critically affect the functioning  
593 of transmembrane proteins, including ion channels (Pouvreau et al., 2004). This, and the  
594 aforementioned complexity of the interaction between the molecules involved, led us to use as  
595 few adjustable parameters as possible. During the simulations only two parameters, the  
596 dimensionless parameter  $\rho$  and the  $\tau$  (space- and time-constant parameters, respectively)  
597 describing the loss of proper conduction of the depolarization along the T-tubules were  
598 systematically altered. They were selected based on two simple reasons. First, their  
599 physiological meaning can easily be understood, namely, how far and how fast can the  
600 depolarization spread into the interior of the fibre, and, secondly, the actual changes in the  
601 electrical parameters of the T-tubules (access resistance, transmembrane resistance,  
602 capacitance) do not need to be directly assessed. It is important to note here that the range tested  
603 for these parameters – from 0.2 to 5 and from 20 to 600 ms for  $\rho$  and  $\tau$ , respectively – albeit  
604 seem arbitrary they actually cover all possibilities that could occur within the fibre. Values less  
605 than 0.2 or greater than 5 for  $\rho$  would not further alter intramembrane charge movement as there  
606 was no activation at all at  $\rho = 0.2$  or homogenous and full activation at  $\rho = 5$ . Similarly, values  
607 greater than 600 ms for  $\tau$  would not alter the outcome as no appreciable activation is seen during  
608 a depolarizing pulse already with  $\tau = 600$  ms. Altogether, the above limitations and  
609 simplifications should not compromise any of the conclusions from the simulations.

#### 610 *Comparison with cardiac cells*

611 To our knowledge, MTM1-deficiency was the first reported model of skeletal muscle disease  
612 associated with stringent disruption of the T-tubule network. In cardiac cells, there has been  
613 several reports of alike alterations, with the T-tubule network yielding reduced density, spatial  
614 disorganization/patchy pattern, associated with heart failure (Louch et al., 2004, 2006; Heinzl  
615 et al., 2008; Lyon et al., 2009; see for review Dibb et al., 2022). In concurrence with our  
616 simulations, detachment of T-tubules from the surface membrane in cardiac cells was shown to  
617 alter action potential propagation in the disconnected portions of the network, but also in the  
618 still connected portions, due to worsened coupling with the surface membrane (Sacconi et al.,  
619 2012). Importantly, electrical properties of cardiac T-tubules were also shown to be heavily  
620 determined by their constriction/dilation status (Uchida and Lopatin, 2018), which is something  
621 that could also play a role in our skeletal muscle disease models. For instance, muscle fibres  
622 from the dynamin2 R465W mouse model of centronuclear myopathy do not exhibit the gross  
623 alterations of the T-tubule network observed in MTM1-deficient fibres. Still, they also suffer



624 from spatial loss of SR  $\text{Ca}^{2+}$  release synchrony, of propagating events and of spontaneous  $\text{Ca}^{2+}$   
625 sparks (Kutchukian et al., 2017). It is thus very possible that changes in the constriction or  
626 geometrical status of individual T-tubules in that model, play a role in the  $\text{Ca}^{2+}$  release  
627 alterations.

628 The comparison with cardiac muscle is also interesting because cardiac EC coupling fully relies  
629 on  $\text{Ca}^{2+}$ -activated RyRs in normal conditions. Still, some qualitative hallmarks of defective  $\text{Ca}^{2+}$   
630 release due to T-tubule disorganization are similar to those in skeletal muscle, as for instance  
631 reduced synchrony due to orphaned RyRs being activated by  $\text{Ca}^{2+}$  released by- and diffusing  
632 from- still intact dyadic regions. Within the early steps T-tubule disorganization, activation of  
633 RyRs by  $\text{Ca}^{2+}$  in muscle fibres necessarily acts as a compensatory gain of function mechanism,  
634 allowing affected regions at immediate proximity of healthy regions to experience close to  
635 normal  $\text{Ca}^{2+}$  release. Of course, in the absence of compensatory reconstruction of the T-tubule  
636 network, progression of the defects irremediably compromises EC coupling function, leading  
637 to the fatal outcome in myotubular myopathy. Strategies aimed at promoting T-tubules  
638 reconstruction have already been considered in the cardiac context (see Dibb et al., 2022) and  
639 would certainly be of therapeutic relevance for myotubular and other forms of centronuclear  
640 myopathy.

641

642 In summary, within the framework of the present knowledge of EC coupling function,  
643 modelling the consequences of altered T-tubular network in muscle fibres is sufficient to  
644 recapitulate the  $\text{Ca}^{2+}$  release defects experimentally detected in MTM1-deficient muscle fibres.  
645 Our proposal is that that T-tubule destruction is a leading determinant of  $\text{Ca}^{2+}$  release  
646 dysfunction and EC coupling failure in myotubular myopathy and, as such, should be  
647 considered a therapeutic target.

648

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816

817 **Additional information section**

818 **Data availability statement**

819 All data supporting the results presented in the manuscript. The simulation program is available,  
820 upon reasonable request, from the last author (LC).

821

822 **Competing interests**

823 The authors declare no conflict of financial and non-financial competing interests on behalf of  
824 all authors.

825

826 **Author contributions**

827 All animal experiments were performed at Institut NeuroMyoGène, University Claude Bernard,  
828 Lyon 1. CK and VJ performed the electrophysiological and confocal imaging experiments. AB-  
829 B generated the MTM1-KO mouse. LC, DB and PS created the model of Ca release. LC wrote  
830 the simulation program. LC, PS and CT performed the simulations. DB, PS, LC and VJ  
831 contributed to data interpretation and discussion and to critical revisions of the manuscript. All  
832 authors approved the final version of the manuscript, agree to be accountable for all aspects of  
833 the work in ensuring that questions related to the accuracy or integrity of any part of the work  
834 are appropriately investigated and resolved. All persons designated as authors qualify for  
835 authorship, and all those who qualify for authorship are listed.

836

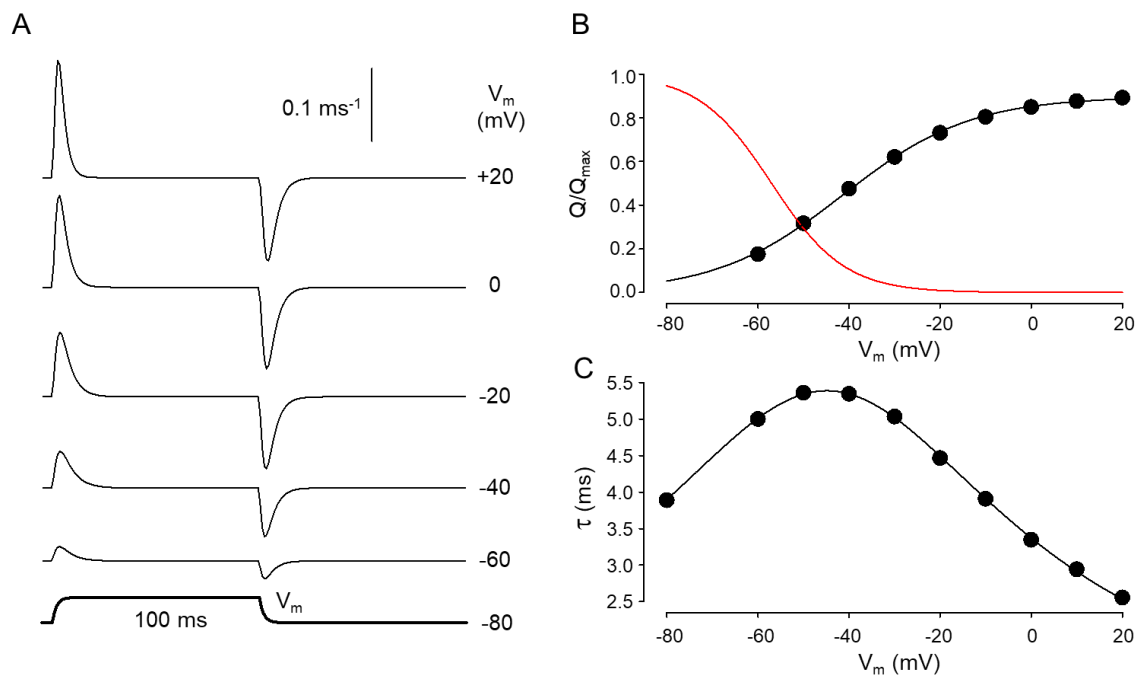
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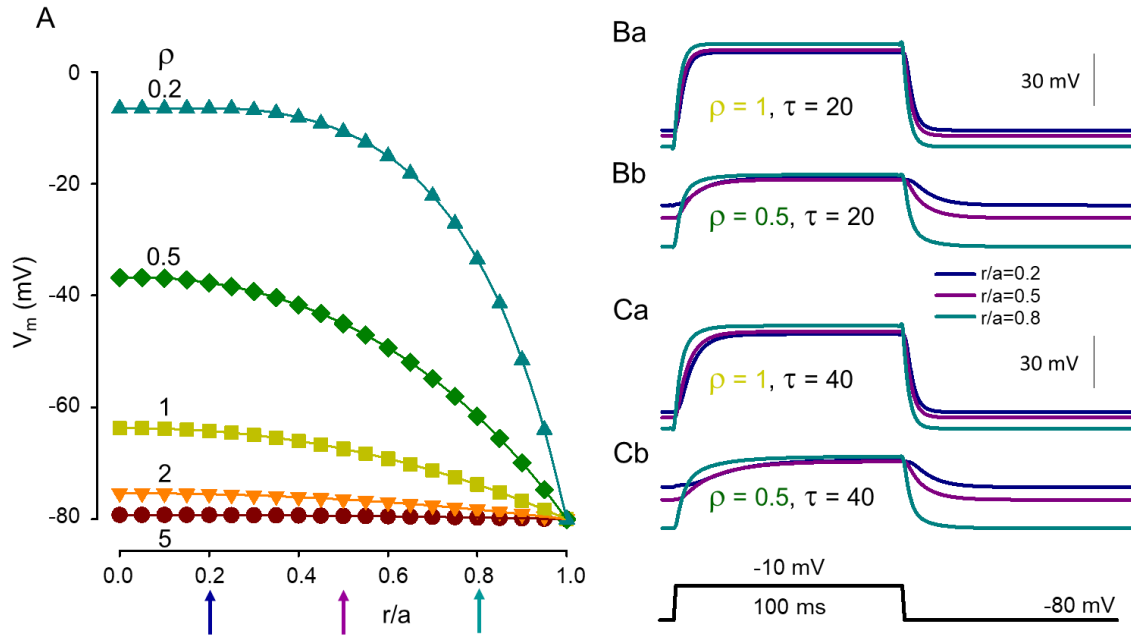
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849

850 **Figure 1.** Voltage dependence of intramembrane charge movement. (A) Simulated non-linear  
 851 capacitive currents ( $I_Q$ ) in response to 100 ms long depolarizing pulses to the indicated  
 852 membrane potentials ( $V_m$ ). Its time course is presented below the traces. The holding potential  
 853 ( $V_h$ ) was set to -80 mV. (B) Voltage dependence of charge activation (circles) together with the  
 854 best fit of Eqn. 1a (black curve) to the data points. The parameters of the fit are  $Q_{\text{max}}=0.90$ ,  
 855  $V_{50,a}=-41.3$  mV, and  $k_a=14.0$  mV. Note that the maximal available charge does not reach unity  
 856 as some DHPRs are in the inactivated state at  $V_h=-80$  mV. The voltage dependence of  
 857 inactivation (Eqn. 1b) is also shown (red curve). (C) Voltage dependence of the time constant  
 858 for charge transfer. The curve represents the best fit of Eqn. 2 to the data points with  $\tau_{\text{max}}=5.4$   
 859 ms,  $V_{50}=-44.9$  mV, and  $k=15.8$  mV. Parameters for the simulation are given in Table 1. For  
 860 further details see text.

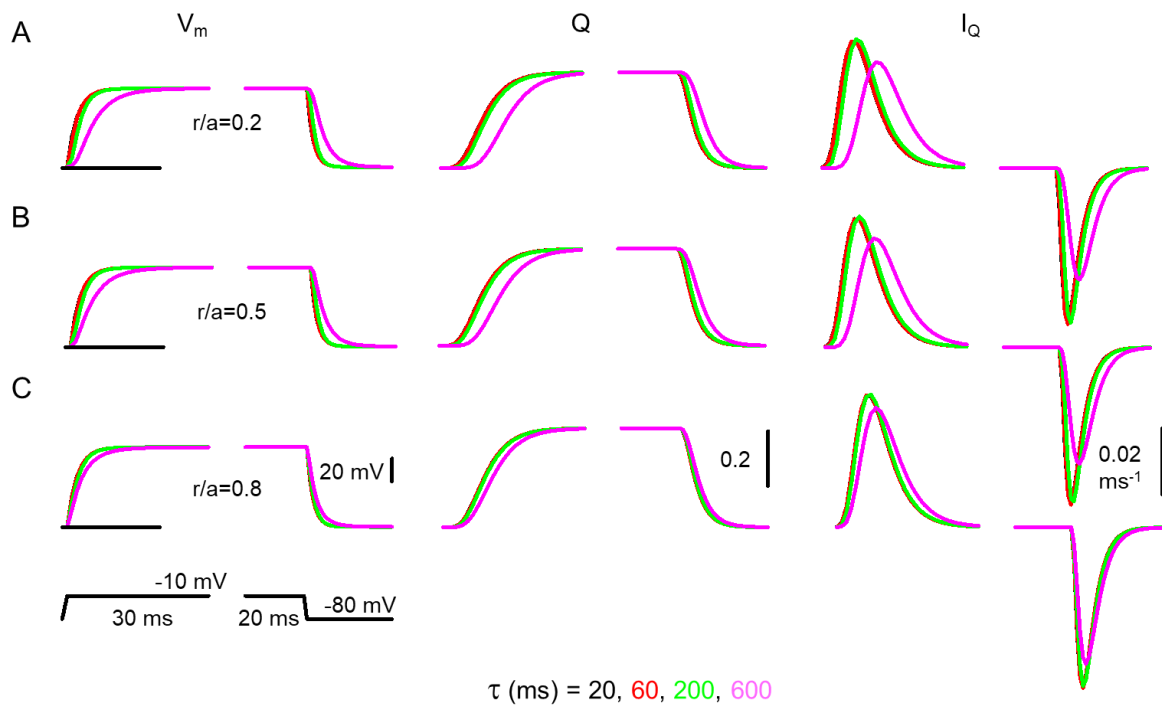
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862

863 **Figure 2.** Simulated profiles of membrane potentials with different T-tubule parameters. (A)  
 864 Resting membrane potential profiles within the voltage-clamped muscle fibre at different values  
 865 for  $\rho$  (*i.e.* steady-state solutions of Eqn. 4 for  $\rho = 5, 2, 1, 0.5, 0.2$ ). Relative spatial positions  
 866 were defined as the distance from the longitudinal axis ( $r$ ) of the fibre divided by the radius ( $a$ )  
 867 of the fibre. Holding potential ( $V_h$ ) was set to  $-80$  mV. (B & C) Changes in the membrane  
 868 potential at ( $r/a = 0.2, 0.5$ , and  $0.8$ ; blue, violet, and light blue traces, respectively) in response  
 869 to a depolarizing step of 100 ms to  $-10$  mV. Panels Ba & Ca and Bb & Cb present the cases of  
 870  $\rho = 1$  and  $0.5$ , respectively. Values for  $V_h$  at a given  $r/a$  were taken from panel A.  $\tau$  was set to  
 871 20 and 40 ms for panels B and C, respectively.

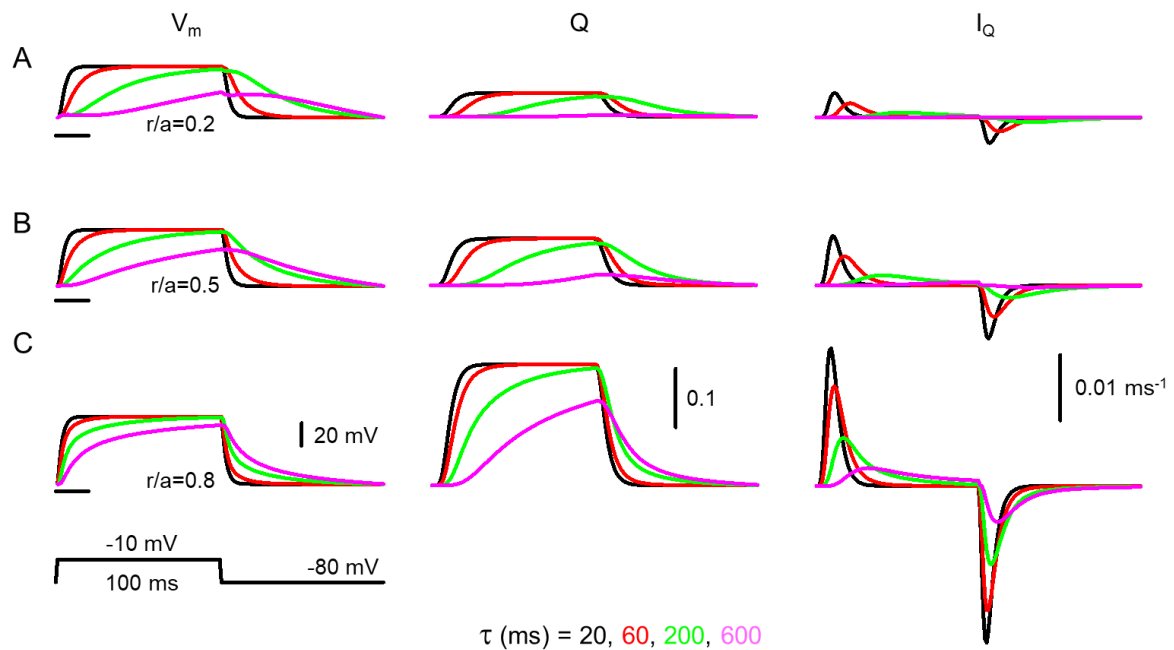
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873

874 **Figure 3.** Simulated time-courses of membrane potential ( $V_m$ ), charge in the activating position  
 875 ( $Q$ ), and charge movement current ( $I_Q$ ). (A-C) Simulations were carried out at different relative  
 876 spatial positions ( $r/a$ ) within the fibre (0.2, 0.5, and 0.8 for panels A, B, and C, respectively)  
 877 with  $\rho$  set to 5 and  $\tau$  set to 20, 60, 200, and 600 ms (black, red, green, and magenta traces,  
 878 respectively). The fibre was depolarized to -10 mV for 100 ms from a holding potential of -80  
 879 mV (indicated by the horizontal black lines at the  $V_m$  traces). Note that an extreme value of 600  
 880 ms (compare with the control value of 20 ms; see parameters in Table 1) for  $\tau$  is needed to  
 881 obtain appreciable difference in the time-course of  $V_m$  and  $I_Q$  at normal  $\rho$  (*i.e.* at  $\rho = 5$ ).

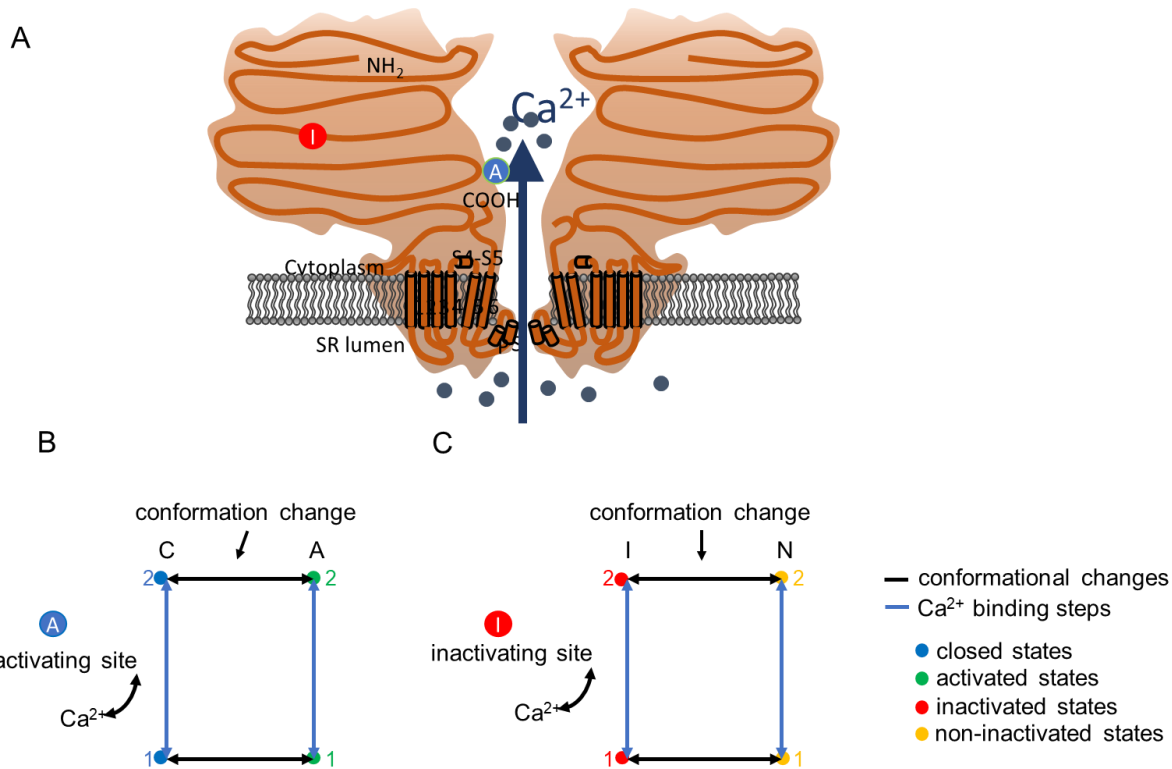
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883

884 **Figure 4.** Simulated time-courses of membrane potential ( $V_m$ ), charge in the activating position  
 885 ( $Q$ ), and charge movement current ( $I_Q$ ). (A-C) Simulations were carried out at different relative  
 886 spatial positions ( $r/a$ ) within the fibre (0.2, 0.5, and 0.8 for panels A, B, and C, respectively)  
 887 with  $\rho$  set to 1 and  $\tau$  set to 20, 60, 200, and 600 ms (black, red, green, and magenta traces,  
 888 respectively). The fibre was depolarized to -10 mV for 100 ms from a holding potential of -80  
 889 mV (indicated by the horizontal black lines at the  $V_m$  traces). Note that already a small change  
 890 in  $\tau$  to 60 ms (compare with the control value of 20 ms; see parameters in Table 1) results in an  
 891 appreciable difference in the time-course of  $V_m$ ,  $Q$ , and  $I_Q$  at reduced  $\rho$  (*i.e.* at  $\rho = 1$ ). Note also  
 892 that at large  $\tau$  the amount of charge moved by the depolarizing pulse is considerably reduced.

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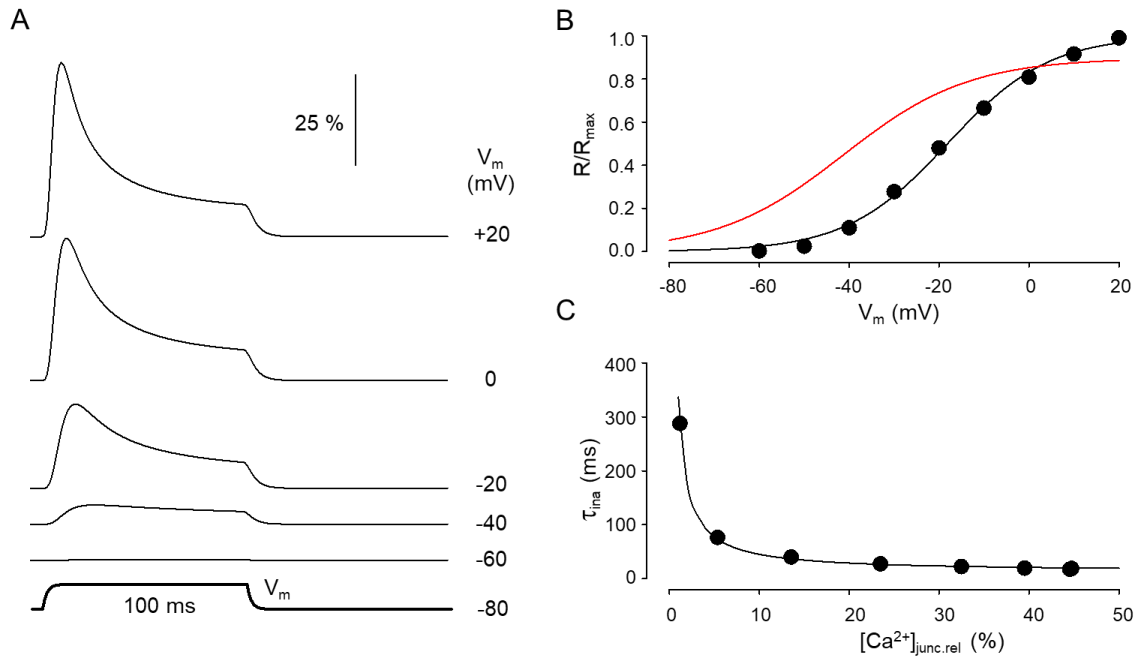


894

Channel open if : A & N

895 **Figure 5.** Model of RyR Ca<sup>2+</sup> release channel and its gating. (A) Inactivating-sites (I): N-  
 896 terminal; around amino acids 1873–1903. Activating-site (A): C-terminal; amino acids 4007–  
 897 5037. B&C. Gating schemes of RyR for activation and inactivation with calcium. (B) Four-  
 898 state model of channel activation. (C) Four-state model of channel inactivation. Note: the  
 899 channel is open if A(ctivated) and N(ot-inactivated).

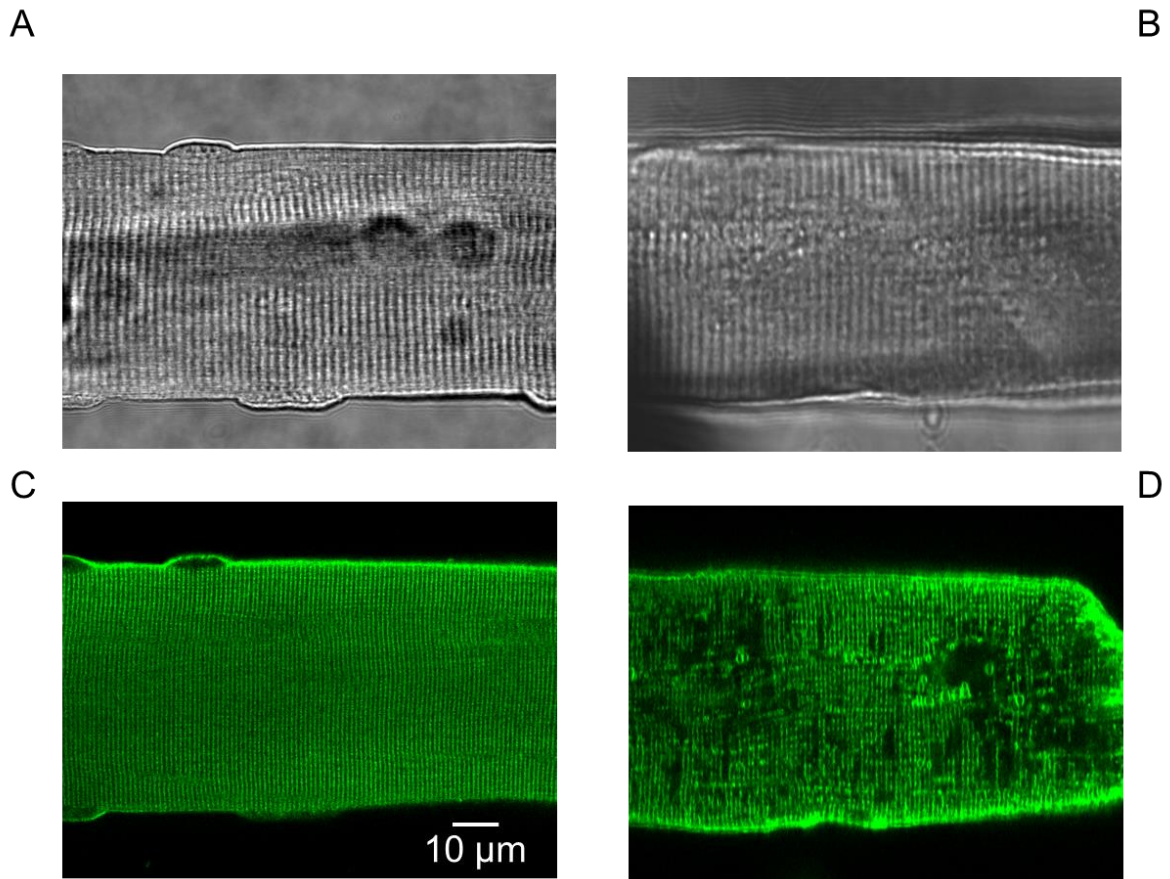
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901

902 **Figure 6.** Voltage dependence of RyR1 activation. (A) Time course of channel opening (dR/dt)  
 903 in response to 100 ms long depolarizing pulses to the indicated membrane potentials (V<sub>m</sub>).  
 904 Traces were normalized to the number of channels within the junctional space. (B) Membrane  
 905 potential dependence of the relative number of open release channels. Superimposed is the best  
 906 fit of Eqn. 1a to the data points. The parameters of the fit are R<sub>max</sub>=48.90, V<sub>50,a</sub>=-18.12 mV, and  
 907 k<sub>a</sub>=11.30 mV The voltage dependence of Q taken from Fig. 1B is also shown (red curve). (C)  
 908 [Ca<sup>2+</sup>]<sub>junc</sub> dependence of the time constant for channel inactivation. Values on the x-axis are  
 909 expressed as relative to the maximal attainable [Ca<sup>2+</sup>]<sub>junc</sub>. The data points were fitted to Eqn. 5  
 910 (best fit is shown superimposed) with K<sub>d,rel</sub> = 25.6, k<sub>i</sub> = 0.0787. Parameters for the simulation  
 911 are given in Table 1. For further details see text.

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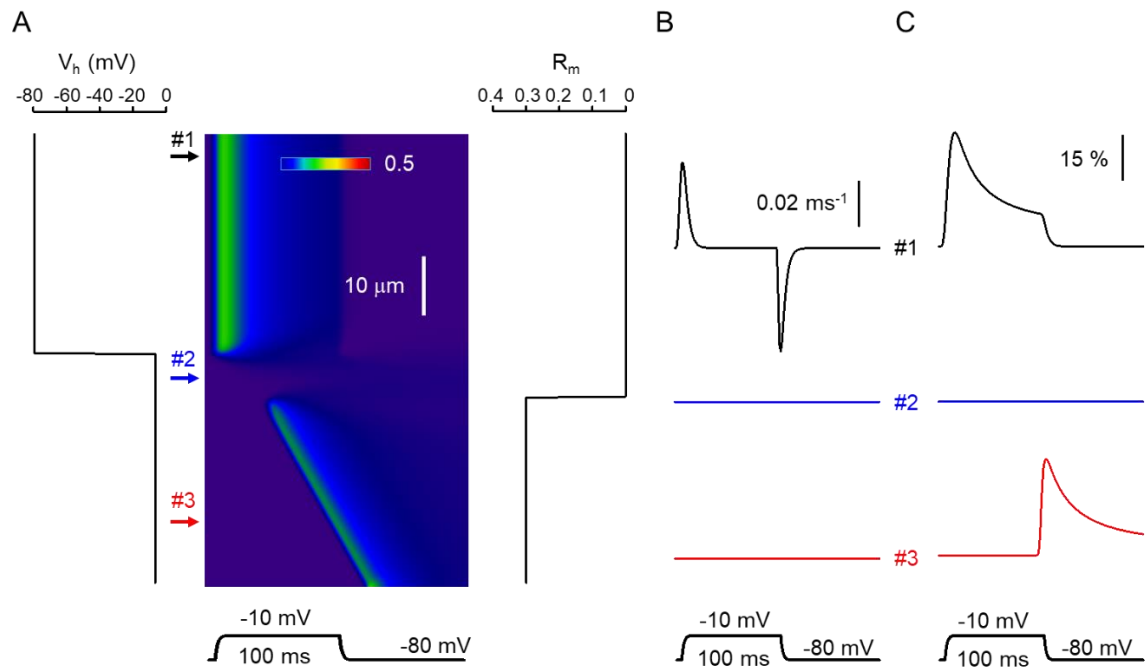


913

914 **Figure 7.** Disrupted T-tubular network in a fibre from an MTM1-deficient mouse. Transmitted  
915 light image of a wild type (A) and a MTM1-deficient (B) FDB fibre. Fluorescent image of a di-  
916 8-Anepps stained wild type (C) and a MTM1-deficient (D) FDB fibre collected with a confocal  
917 microscope. Note the regular and the incomplete patterns of the T-tubules in the wild type and  
918 the MTM1-deficient fibres, respectively.

919

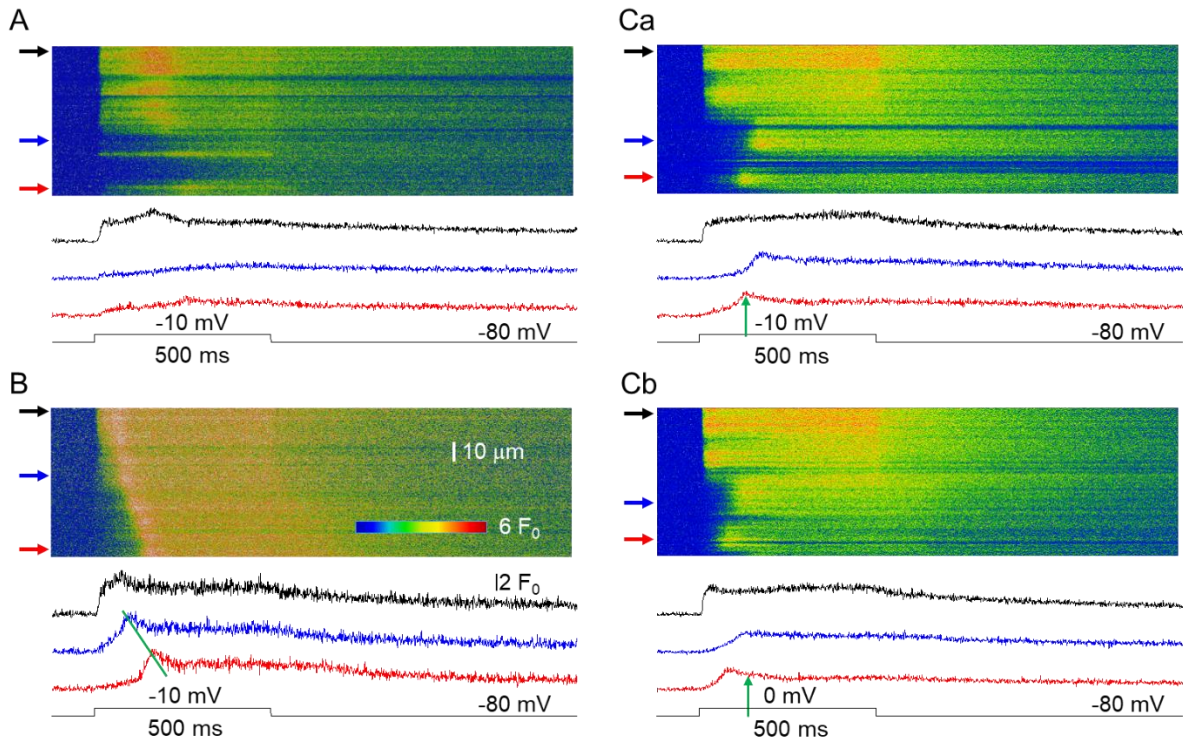




920

921 **Figure 8.** Simulated spatio-temporal pattern of intracellular calcium concentration in an  
 922 artificial muscle fibre. (A) Line-scan image showing the calcium concentration profile. The  
 923 assumed distribution of the holding potential ( $V_h$ ) and relative availability of RyRs that can be  
 924 activated by calcium ( $R_m$ ) along the fibre are presented next to the image (left & right,  
 925 respectively).  $V_h$  was determined by setting  $\rho = 5$  and 0.2 in Eqn. 4 at positions where the T-  
 926 tubules were assumed to be intact (black arrow) and affected (blue and red arrow), respectively.  
 927 The fibre was depolarized to -10 mV for 100 ms. (B) Non-linear capacitive currents  
 928 representing intramembrane charge movement at different locations along the fibre as indicated  
 929 by the respective color coded arrows in panel A. (C) Time course of open RyRs at the same  
 930 spatial positions. Same parameters were used in the simulation as in Fig. 1&6.

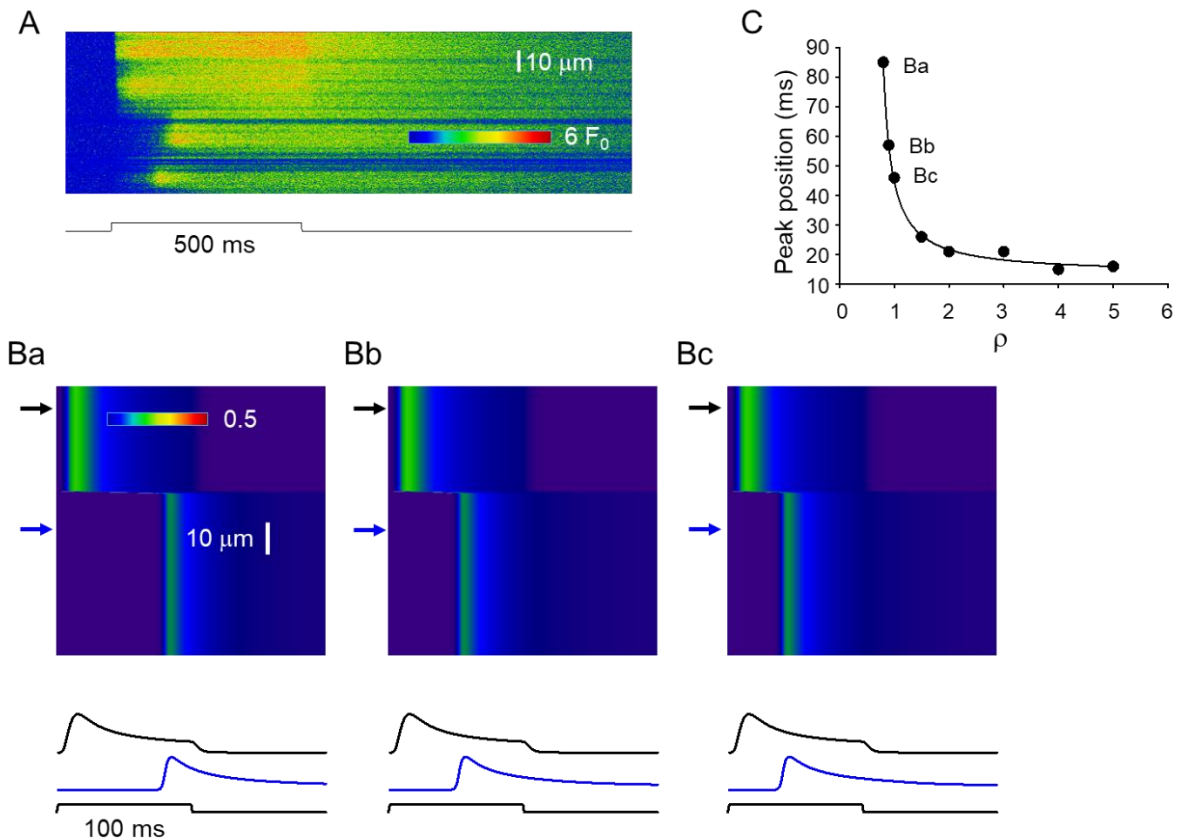
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932

933 **Figure 9.** Characteristic features of SR calcium release in MTM1-deficient mouse skeletal  
 934 muscle fibres as assessed by confocal line-scan imaging. (A) A fibre displaying areas where  
 935 normal (black arrow) or reduced (to a lesser, blue arrow, or to a more pronounced extent, red  
 936 arrow) calcium release is observed. Here, and in all other panels, traces below the image  
 937 represent the time course of the transient calculated by averaging 10 points in the spatial domain  
 938 at the corresponding (color coded) arrow. (B) A fibre displaying a propagating calcium wave.  
 939 The slope of the line connecting the peaks of the transients (green line) can be used to determine  
 940 the speed of propagation (570  $\mu\text{m/s}$  for the image in panel B). (Ca) A fibre displaying areas  
 941 where the start of the transient significantly lags behind that of the voltage command. Black  
 942 trace represents a non-affected area, whereas blue and red traces present examples for delayed  
 943 activation. The fibre was depolarized to -10 mV. (Cb) Same fibre as in panel Ca, but now  
 944 depolarized to 0 mV. The positions of the arrows next to the images and thus the time course  
 945 of the transients correspond to identical spatial positions in panels Ca&Cb. Note that the delay  
 946 for activation in the affected area is less in the fibre with the greater depolarization as indicated  
 947 by the green arrows pointing to the peak of the red trace in panel Ca (the arrow in panel Cb is  
 948 positioned at the same time point as that in panel Ca). The resting membrane potential was set  
 949 to -80 mV and the depolarization was 500 ms for all fibres in the figure.

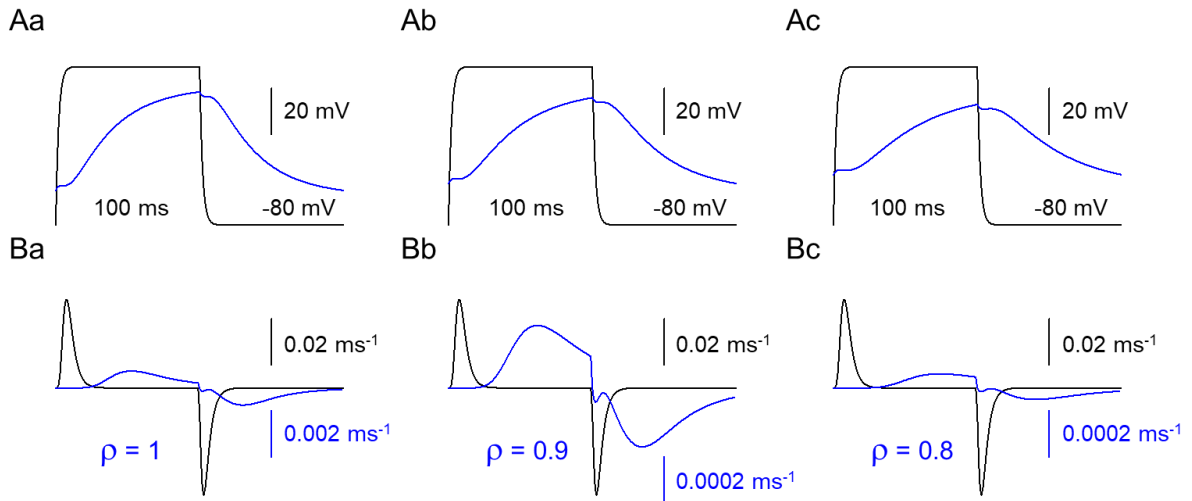
950



951

952 **Figure 10.** Simulation of delayed activation in MTM1-deficient muscle fibres. (A) Same line-  
 953 scan image as presented in Fig. 9Ca. (B) Simulated line-scan images (simulation done as  
 954 described in Methods and presented in Fig. 8 and Figs. 2-4) using two areas with different  
 955 parameters. Black arrows point to areas where activation is normal (for normal parameters see  
 956 Table 1) while blue arrows point to affected areas where  $\rho$  and  $\tau$  (Eqn. 4) were altered.  $\tau$  was  
 957 increased to 200 ms from its normal value of 20 ms, while  $\rho$  was decreased to 1, 0.9, and 0.8  
 958 from its normal value of 5 for panels Bc, Bb, and Ba, respectively. Traces below the images  
 959 represent the time course of the transient at the corresponding (color coded) arrow. (C)  
 960 Dependence of the activation delay (as assessed by the position of the peak of the transient  
 961 compared to the onset of the depolarizing pulse) on the selection of  $\rho$ . Points labeled Ba, Bb,  
 962 and Bc correspond to the blue traces in panels Ba, Bb, and Bc, respectively. In the simulation  
 963 the fibre was depolarized to -10 mV for 100 ms from a holding potential of -80 mV to mimic  
 964 the depolarization of the fibre in panel A. Note that the simulated images, here and in all  
 965 subsequent figures, display the time course of the calcium transient in the triadic junction (see  
 966 Methods).

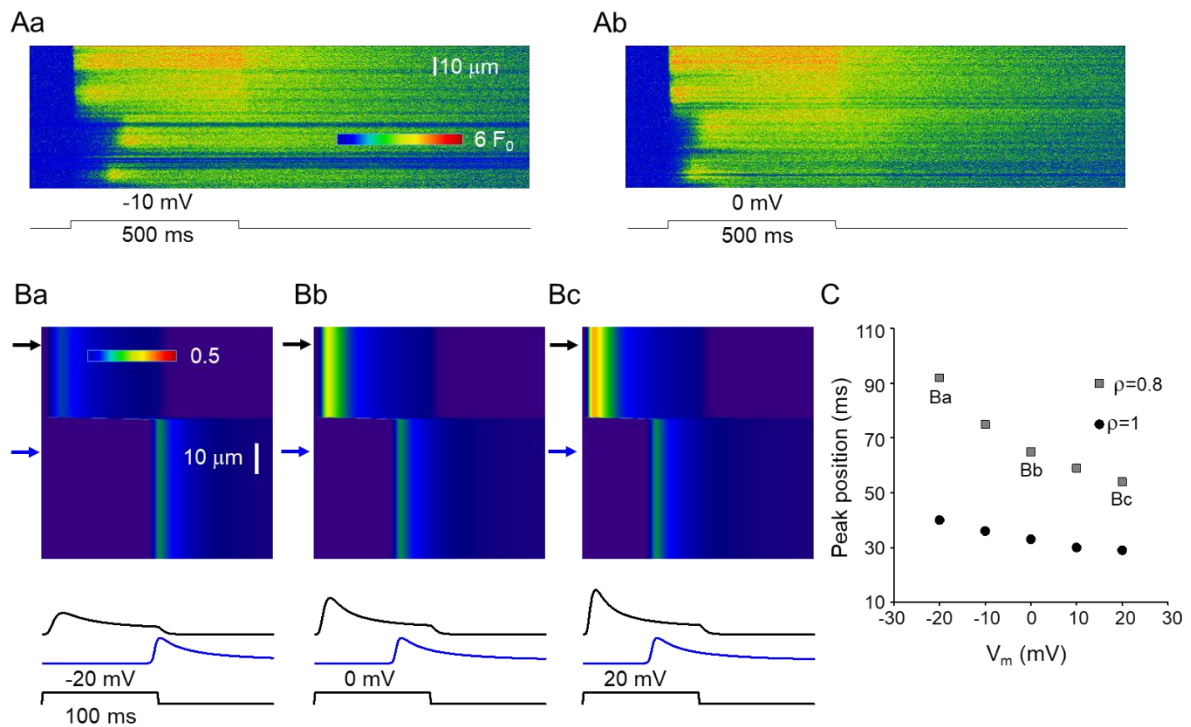
967



968

969 **Figure 11.** (A) Membrane potential changes at the arrows corresponding to panels Ba, Bb, and  
 970 Bc in Fig. 10. Scale bars are valid for black and blue traces as well. (B) Charge movement  
 971 currents ( $I_Q$ ) calculated at the same position on each panel. Note the different scales for the blue  
 972 traces. Black traces were calculated with  $\rho=5$ .

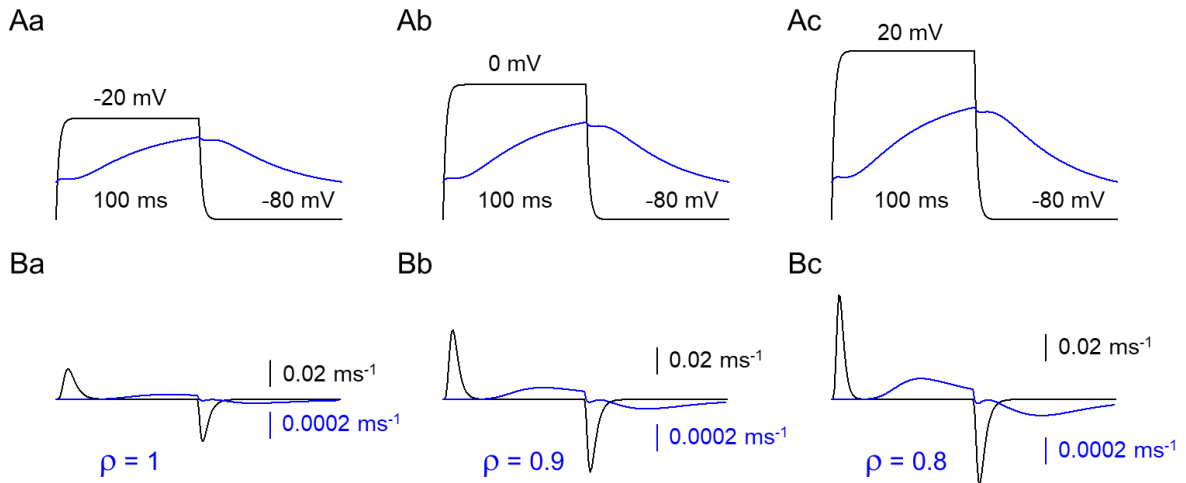
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974

975 **Figure 12.** Simulation of the voltage-dependence of delayed activation in MTM1-deficient  
 976 muscle fibres. (A) Same line-scan images as presented in Fig. 9C. (B) Simulated line-scan  
 977 images using two areas with different parameters. Black arrows point to areas where activation  
 978 is normal while blue arrows point to affected areas where  $\rho$  and  $\tau$  (Eqn. 4) were altered.  $\tau$  was  
 979 increased to 200 ms from its normal value of 20 ms, while  $\rho$  was decreased to 0.8 from its  
 980 normal value of 5. The depolarizing pulse brought the membrane potential ( $V_m$ ) from a resting  
 981 value of -80 mV to -20, 0, and +20 mV for 100 ms for panels Ba, Bb, and Bc, respectively.  
 982 Traces below the images represent the time course of the transient at the corresponding (color  
 983 coded) arrows. Note that the activation of the blue trace is faster if the membrane is more  
 984 depolarized. (C) Voltage dependence of the activation delay (as assessed by the position of the  
 985 peak of the transient as compared to the onset of the depolarizing pulse) at different  $\rho$  values.  
 986 Points labeled Ba, Bb, and Bc correspond to the blue traces in panels Ba, Bb, and Bc,  
 987 respectively.

988



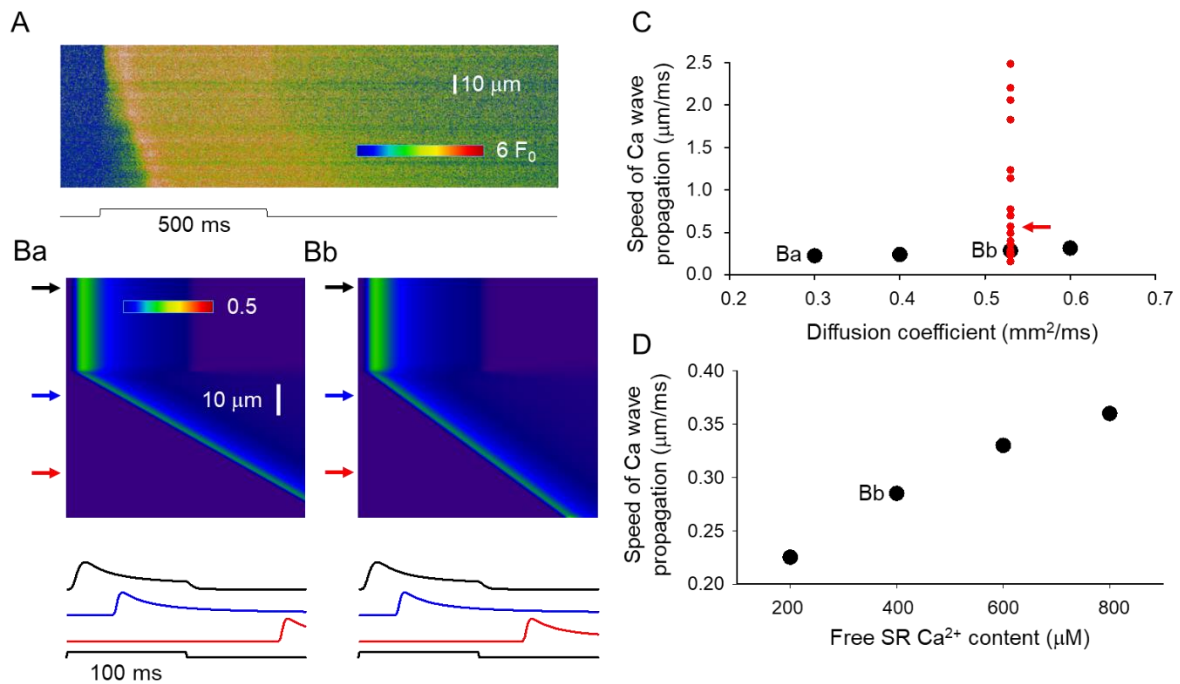
989

990 **Figure 13.** (A) Membrane potential changes at the arrows corresponding to panels Ba, Bb, and

991 Bc in Fig. 12. (B) Charge movement currents ( $I_Q$ ) calculated at the same position on each panel.

992 Note the 100 times smaller scale for the blue traces.

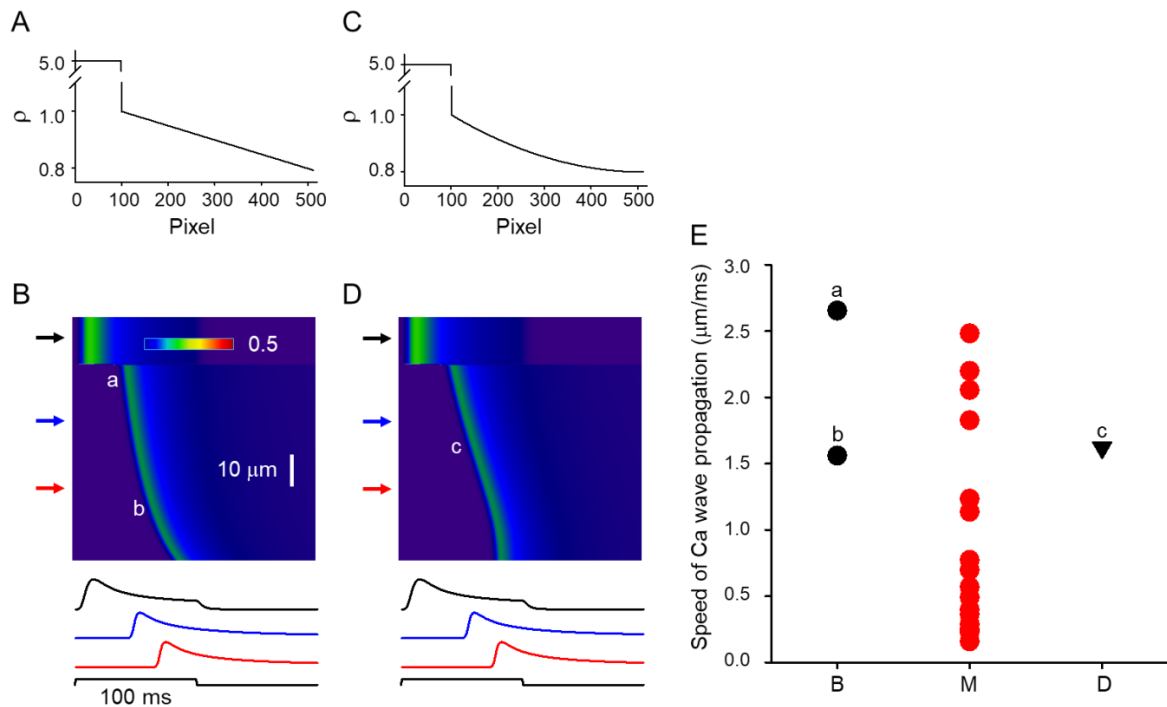
993



994

995 **Figure 14.** Simulation of calcium wave propagation in MTM1-deficient muscle fibres. (A)  
 996 Same line-scan image as presented in Fig. 9B. (B) Simulated line-scan images using two areas  
 997 with different parameters. Black arrows point to areas where activation is normal (for normal  
 998 parameters see Table 1) while blue and red arrows point to affected areas where the presence  
 999 of calcium activatable RyR-s were introduced (30% of all channels in the affected area) while  
 1000  $\rho$  was set to 0.2. This allowed calcium-induced calcium-release (CICR) to initiate a propagating  
 1001 calcium wave. The diffusion coefficient for calcium was set to 0.53 (see Donahue and  
 1002 Abercrombie, 1987) and 0.3  $\mu\text{m}^2/\text{ms}$  in panels Bb, and Ba, respectively. Traces below the  
 1003 images represent the time course of the transient in the spatial domain at the corresponding  
 1004 (color coded) arrows. (C) Dependence of the speed of the propagation of the calcium wave on  
 1005 the selected diffusion coefficient for calcium (black circles). Points labeled Ba and Bb  
 1006 correspond to images in panels Ba and Bb, respectively. Red circles represent actual values  
 1007 measured in line-scan images (as that in panel A), arrow points to the data (0.57  $\mu\text{m}/\text{ms}$ )  
 1008 obtained from the image in panel A. Note that the measured values cover a much broader range  
 1009 that those obtained in the simulations, in many cases far beyond what would be expected for  
 1010 CICR. (D) Dependence of the speed of the propagation of the calcium wave on the SR Ca  
 1011 content. Point labeled Bb correspond to images in panel Bb. In the simulation the fibre was  
 1012 depolarized to -10 mV for 100 ms from a holding potential of -80 mV to mimic the  
 1013 depolarization of the fibre in panel A.

1014



1015

1016 **Figure 15.** Simulation of calcium wave propagation without calcium-induced calcium-release  
 1017 in MTM1-deficient muscle fibres. (A&C) Spatial profiles of  $\rho$ . Graphs present the change in  $\rho$   
 1018 along the spatial domain (512 pixels) of the simulation. Two cases were considered;  $\rho$  either  
 1019 changed linearly (A) or following a second order polynomial (C) from 1 to 0.8 in the affected  
 1020 region. (B&D) Simulated line-scan images using the  $\rho$  profiles from A and C, respectively ( $\tau =$   
 1021 400 ms). Black arrows point to areas where activation is normal while blue and red arrows point  
 1022 to affected areas. Traces below the images represent the time course of the transient at the  
 1023 corresponding (color coded) arrows. Note the appearance of a clear wave front in both images.  
 1024 Note also that the wave front seems to „curve” in panel B (i.e. the calculated speed of  
 1025 „propagation” would be different at different spatial positions) while it is linear for most part  
 1026 in panel C (i.e. the calculated speed of „propagation” would be constant at different spatial  
 1027 positions). In the simulation the fibre was depolarized to -10 mV for 100 ms from a holding  
 1028 potential of -80 mV (E) Red circles represent actual values measured (M) in line-scan images  
 1029 (same as that in panel C in Fig. 14), a, b (black circles), and c (black triangle) label the data  
 1030 obtained from the image in panels B and D.

1031



1032 **Table 1.** Parameters used in the simulation.

#	Description	Value	Unit of measure	Reference
1	$V_{50,a}$ (for Boltzmann distribution)	-35	mV	7
2	$k_a$ (for Boltzmann distribution)	15	mV	7
3	$V_{50,i}$ of the inactivation site	-57	mV	13
4	$k_i$ of the inactivation site	8	mV	13
5	Maximal time constant for charge movement ( $\tau_{max}$ )	5	ms	7
6	Pixel size	0.15	$\mu\text{m}$	see Methods
7	$K_d$ of the activation site	10	$\mu\text{M}$	43
8	$k_{off}$ of the activation site	0.3	1/ms	11
9	$K_d$ of the inactivation site	200	$\mu\text{M}$	43
10	$k_{off}$ of the inactivation site	0.1	1/ms	44
11	Rate constant for the transition C1 -> A1	$10^{-6}$	1/ms	*
12	Rate constant for the transition A1 -> C1	1	1/ms	*
13	Rate constant for the transition C2 -> A2	3	1/ms	*
14	Rate constant for the transition A2 -> C2	0.01	1/ms	*
15	Rate constant for the transition N1 -> I1	$3 \cdot 10^{-8}$	1/ms	*
16	Rate constant for the transition I1 -> N1	0.001	1/ms	*
17	Rate constant for the transition N2 -> I2	0.3	1/ms	11
18	Rate constant for the transition I2 -> N2	2	1/ms	11
19	Maximal free SR $[\text{Ca}^{2+}]$	400	$\mu\text{M}$	55
20	Resting $[\text{Ca}^{2+}]$	0.1	$\mu\text{M}$	52
21	Diffusion coefficient of $\text{Ca}^{2+}$ in the myoplasm	0.53	$\mu\text{m}^2/\text{ms}$	10

1033 \* adjusted for best fit of the time course of SR calcium release