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"Functional ageing" of L-type Ca channels in dystrophin-deficient cardiomyocytes

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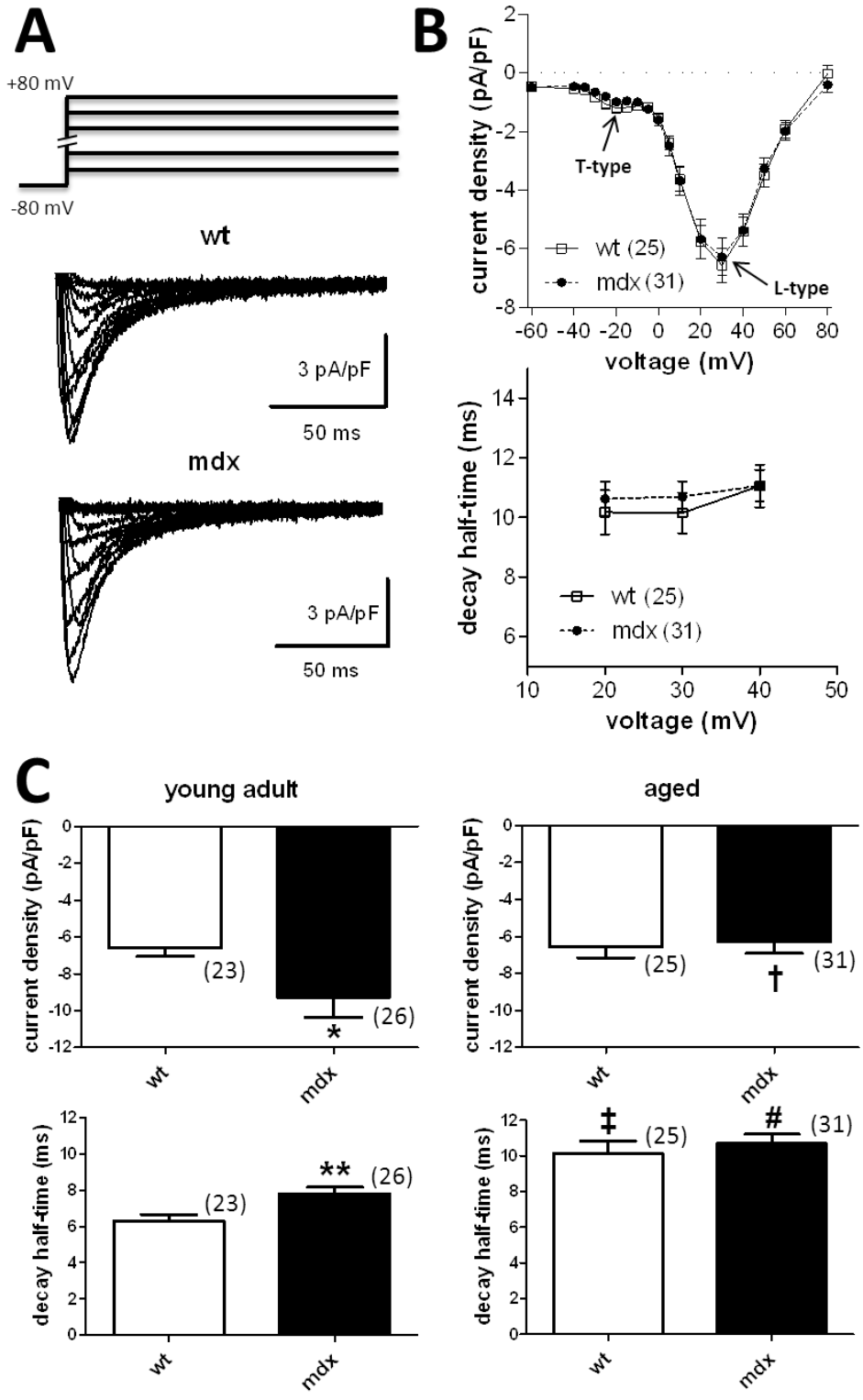
Project results

1) Characterization of potential L-type Ca current abnormalities in dystrophic ventricular cardiomyocytes isolated from "aged" mdx mice

Figure 1*A* shows typical original traces of calcium currents (I_{Ca}) recorded from a normal wild type (wt) and dystrophic (mdx) cardiomyocyte, which were derived from an aged (> one year of age) wt and mdx mouse, respectively. The currents were elicited by the pulse protocol displayed on top. A summary of the current density-voltage relationships, derived from a series of such experiments, is presented in Fig. 1*B* (top). It can be noticed that the current densities of wt and dystrophic cardiomyocytes were similar over a wide range of tested potentials. This suggests that dystrophin deficiency does not affect the I_{Ca} density in cardiomyocytes derived from aged mice. Analysis of the current decay after channel activation (Fig. 1*B*, bottom) revealed that the kinetics of inactivation was also comparable in wt and mdx cardiomyocytes. These data imply that, similar to current density in myocytes of aged mice, the kinetics of channel inactivation is independent of the expression of dystrophin. These results were unexpected since, in dystrophic cardiomyocytes derived from neonatal or "young adult" mdx mice, I_{Ca} is abnormally enhanced (Koenig *et al.*, 2014;Sadeghi *et al.*, 2002;Viola *et al.*, 2013;Viola *et al.*, 2014;Woolf *et al.*, 2006).

Figure 1


A
+80 mV
-80 mV
wt
3 pA/pF
50 ms
mdx
3 pA/pF
50 ms
B
current density (pA/pF)
T-type
L-type
wt (25)
mdx (31)
voltage (mV)
decay half-time (ms)
wt (25)
mdx (31)
voltage (mV)
C
young adult
aged
current density (pA/pF)
(23)
(26)
*
(25)
(31)
†
wt
mdx
decay half-time (ms)
(23)
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(26)
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(25)
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(31)

Figure 1. Ca current properties in wt and dystrophic (mdx) cardiomyocytes. (A) Original traces of Ca currents of a typical "aged" wt and mdx cardiomyocyte elicited by the pulse protocol displayed on top. (B) Current density-voltage relationships (top) and current decay kinetics (bottom) of "aged" wt and mdx cardiomyocytes. The numbers of cells tested for each population (n numbers) are given in brackets. The current density-voltage relations revealed two distinct negative peaks (indicated by arrows): one at -20 mV representative of T-type Ca channel activation, and the other at +30 mV reflecting L-type Ca channel activity. For comparison of the Ca current decay kinetics (bottom), decay half-times (representing the time period between the current peak and the time point at which the current had decayed to 50%) were plotted against voltage. No significant differences between wt and mdx cardiomyocyte parameters were found. (C) Comparison of current density (top panel) and decay kinetics (lower panel) at +30 mV between "young adult" (left panel) and "aged" (right panel) wt and mdx cardiomyocytes. A test pulse potential of +30 mV was chosen because at this voltage L-type Ca current was maximal. The respective n numbers are given in brackets. A significant number of the "young adult" cardiomyocyte data points were taken from our previous study (Koenig et al. 2014). *indicates a significant difference between the current density values of "young adult" wt and mdx cardiomyocytes with $P < 0.05$. **indicates a significant difference ($P < 0.01$) between the decay half-times of "young adult" wt and mdx cells. †, decreased current densitiy in "aged" versus "young adult" mdx cells ($P < 0.05$) (top panel); ‡, increased decay half-time in "aged" versus "young adult" wt cells ($P < 0.01$) (lower panel); #, increased decay half-time in "aged" versus "young adult" mdx cells ($P < 0.01$) (lower panel).

2) Characterization of potential T-type Ca current abnormalities in dystrophic ventricular cardiomyocytes isolated from "aged" mdx mice

Although normally hardly present in healthy adult cardiac ventricles, increased ventricular re-expression of T-type Ca channels may contribute to the progression of heart failure (Kinoshita *et al.*, 2009;Vassort *et al.*, 2006). Thus, it is possible that T-type I_{Ca} is enhanced in "aged" dystrophic ventricular cardiomyocytes, which may lead to abnormally increased Ca influx into dystrophic cells. Figure 1*B* (top) shows that we could only detect very tiny T-type I_{Ca} (see arrow "T-type") both in "aged" wt and mdx cardiomyocytes. Differences in current density or voltage-dependence of channel activation between normal and dystrophic cells were not detectable (Fig. 1B), and activation as well as inactivation kinetics of the currents appeared similar. This suggests that T-type I_{Ca} is neither upregulated nor dysregulated in "aged" dystrophic cardiomyocytes.

3) Comparison of the data from "aged" wt and dystrophic cardiomyocytes with the previously described Ca current abnormalities in cardiomyocytes derived from "young adult" wt and dystrophic mice

In order to allow for assessment of age-related changes in Ca channel properties, the current density and decay values at +30 mV (taken from Fig 1*B*) were related to the respective parameters we previously detected in cardiomyocytes derived from "young adult" (15-25 weeks

of age) wt and mdx mice (Koenig *et al.*, 2014) in Fig. 1*C*. It can be observed that current density (increased in “young adult” but not “aged” dystrophic (mdx) cardiomyocytes when compared to age-matched wt control) was not affected by ageing in wt but reduced in mdx cells (Fig. 1*C*, top panel). Moreover, ageing slowed the kinetics of inactivation both in wt and mdx cardiomyocytes, whereby this effect was more pronounced in wt cells (Fig. 1*C*, lower panel). Together the data suggest that ageing has a different impact on the Ca channel properties in wt and dystrophic hearts, finally resulting in similar properties at over one year of animal age. Thus “functional ageing” of Ca channels is different in normal and dystrophic cardiomyocytes.

4) Isoprenaline superfusion experiments with cardiomyocytes derived from aged wt and mdx mice

These studies- using the β-receptor agonist isoprenaline- should have clarified whether β-adrenergic regulation of the L-type Ca channel is abnormal in “aged” dystrophic cardiomyocytes. This experimental series failed, because we could not obtain reproducible results following isoprenaline application to our cardiomyocytes. It is possible that this can be explained by the existence of various cell populations in our preparations, which have responded differently to the drug.

5) Intracellular Ca transients in “aged” wt and dystrophic (mdx) cardiomyocytes

(additionally performed set of experiments not mentioned in original project application)

During the plateau phase of the cardiac action potential (AP), Ca influx through L-type Ca channels into the cell triggers Ca-induced Ca release from the sarcoplasmic reticulum, which finally initiates contraction. To check if normal L-type Ca channel function in “aged” mdx cardiomyocytes (see above) goes along with normal Ca release, we compared electrically evoked Ca transients between wt and mdx myocytes derived from aged mice (Fig. 2). Figure 2*C* (left) shows that the peak of the Ca signal was similar in wt and mdx cells, suggesting normal Ca release in “aged” dystrophic cardiomyocytes. The decay of the Ca signal after electrical stimulation, on the other hand, was significantly slowed in mdx myocytes (Fig. 2*C*, right). Finally, when caffeine was used to elicit Ca transients (Fig. 2*D*), both the peak of the Ca signal (left) and the decay of the Ca transient (right) were similar in “aged” wt and mdx cardiomyocytes.

Figure 2

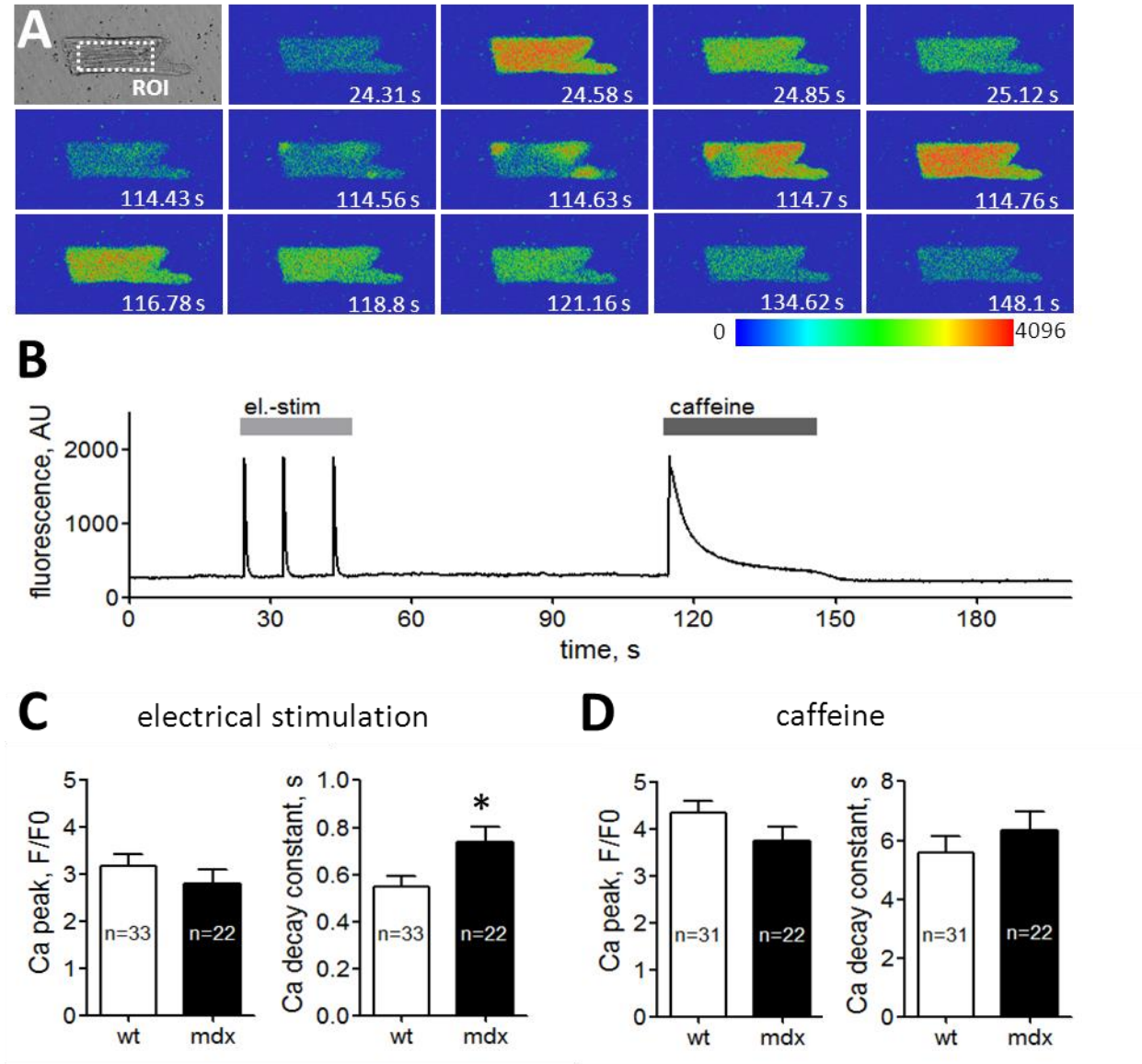

Figure 2. Electrically- and caffeine- induced cytosolic Ca transients in wt and dystrophic (mdx) cardiomyocytes. (A) Representative transmitted light image (top left) and image series of false color Fluo-4 fluorescence during the course of a typical experiment as shown in (B) xyt image series was acquired with a sampling rate of 90 msec frame^{-1}, and emitted Fluo-4 fluorescence was spatially averaged from a region of interest (ROI) drawn in the central region of a cardiomyocyte. (B) Time course of averaged Fluo-4 fluorescence reporting rises in cytosolic Ca concentration during electrical field stimulation (el.-stim, light gray bar) and application of 10 mmol/L caffeine (dark gray bar). AU, arbitrary units. (C) Mean Ca peak fluorescence relative to baseline (F/F0) (left), and Ca transient decay constant (right) elicited by electrical field stimulation for "aged" wt and dystrophic (mdx) cardiomyocytes. (D) Mean Ca peak fluorescence relative to baseline (F/F0) (left) and Ca transient decay constant (right) elicited by application of 10 mmol/L of the ryanodine receptor agonist caffeine for aged wt and mdx cardiomyocytes. The respective numbers of cells tested (n) are given within the bars. * indicates a significant difference in the el.-stim induced Ca transient decay between wt and mdx with $P < 0.05$. No other statistically significant differences were found.

Interpretation of the project results

Age-related Ca channel functional changes in normal wild type (wt) cardiomyocytes

In senescent cardiomyocytes, the amplitude of currents through L-type Ca channels is significantly increased. Because this typically occurs in parallel with an enlargement of the myocytes, the I_{Ca} density (current per membrane surface) remains unaltered (see (Zhou *et al.*, 1998;Feridooni *et al.*, 2015;Li *et al.*, 2014)). Furthermore, Ca channel inactivation is slowed in "aged" cardiomyocytes, resulting in an increased net Ca influx during each AP in senescent compared to young hearts. On the one hand, this augmentation of Ca influx may provide a compensatory mechanism to preserve cardiac function in the senescent heart in the basal state. On the other hand, enhanced Ca influx also raises the risk of Ca overload and Ca-dependent arrhythmias (Zhou *et al.*, 1998). Comparison of the I_{Ca} properties from "aged" wt cardiomyocytes (present study) with the data from our previous study (Koenig *et al.*, 2014) revealed unaltered current densities but markedly slowed inactivation in "aged" compared with "young adult" wt cardiomyocytes (Fig. 1*C*). These findings are well consistent with previous reports (see above; (Zhou *et al.*, 1998;Feridooni *et al.*, 2015;Li *et al.*, 2014)). Interestingly, the established age-related changes in L-type Ca channel function in wt cardiomyocytes are similar to Ca channel adaptations occurring in myocytes of diseased (hypertrophied or failing) hearts (see (Zhou *et al.*, 1998) and references therein). This suggests that the ageing process *per se* induces disadvantageous adaptations in cardiac Ca channel function.

Normal Ca channel function in "aged" dystrophic cardiomyocytes

Gain of function L-type Ca channel abnormalities in dystrophic cardiomyocytes (enhanced current density and impaired inactivation) are a hallmark of the disease phenotype in the dystrophic heart when studied in young dystrophic mice (between a few days and 25 weeks of age) (Koenig *et al.*, 2014;Sadeghi *et al.*, 2002;Viola *et al.*, 2013;Viola *et al.*, 2014;Woolf *et al.*, 2006). In the present study, to our surprise, we found that the I_{Ca} properties of dystrophic cardiomyocytes derived from aged mdx mice were similar to those of myocytes derived from wt mice in the respective age. This implies that, irrespective of the presence of a pronounced dilated cardiomyopathy in aged mdx mice (Quinlan *et al.*, 2004;Au *et al.*, 2011;Sarma *et al.*, 2010), the I_{Ca} properties in dystrophic cardiomyocytes are normal. Furthermore, the data suggest that dystrophin- regulation of L-type Ca channel function in the heart is completely lost during the murine ageing process. Possibly, this can be explained by a significant loss of

dystrophin protein in the senescent murine heart (Townsend et al. 2011). Besides the present study, to the best of our knowledge, only (Li *et al.*, 2014) have so far investigated L- type Ca channel properties in “aged” dystrophic ventricular cardiomyocytes (derived from mdx mice). These authors reported increased current densities in cardiomyocytes derived from 12 months-old mdx compared to age-matched wt mice. The apparent difference to our results (similar current densities in “aged” wt and mdx cardiomyocytes) may be explained by two circumstances: i) the use of a considerably older mouse population in the present study; ii) the use of male mice only (present study) versus both male and female animals in (Li *et al.*, 2014). In contrast to our paper, Ca channel inactivation was not studied in (Li *et al.*, 2014), because the I_{Ca} recordings were only a secondary aspect of this investigation.

Our study further suggests that normal Ca channel function in “aged” dystrophic cardiomyocytes goes along with normal intracellular Ca release. This can be deduced from similar electrically evoked Ca transient peaks in cardiomyocytes derived from aged wt and mdx mice, in agreement with a respective finding in (Li *et al.*, 2014). Ca release induced by caffeine application was also similar in “aged” wt and mdx cardiomyocytes. The significantly slowed decay of the Ca signal triggered by electrical stimulation in “aged” mdx myocytes we report coincides with similar findings in the literature (e.g. (Williams & Allen, 2007;Gonzalez *et al.*, 2014)), and can be explained by impaired Ca removal from the cytosol after release in dystrophic cells (Williams & Allen, 2007).

As opposed to similar I_{Ca} properties in “aged” wt and dystrophic cardiomyocytes under basal conditions (present study), a clear cut difference between wt and mdx was previously reported for Ca channel responsiveness to beta- adrenergic stimuli. Thus, I_{Ca} enhancement by isoprenaline, as observed in “aged” wt cardiomyocytes, was almost completely lacking in “aged” mdx cells (Li *et al.*, 2014). Reduced Ca channel responsiveness to beta- adrenergic stimuli in “aged” mdx cardiomyocytes may be caused by a marked age-related deterioration in β_1-adrenoceptor function (Lu and Hoey 2000). Irrespective of normal basal I_{Ca} properties in “aged” dystrophic cardiomyocytes, this represents a potential cause of reduced functionality and abnormal electrical properties in the aged dystrophic heart.

Shortened PQ and prolonged QTc intervals are hallmarks of the electrocardiogram (ECG) in old mdx mice (e.g. (Bostick *et al.*, 2010;Koenig *et al.*, 2014)). According to our finding of similar I_{Ca} properties in “aged” wt and mdx cardiomyocytes, we propose that other mechanisms than Ca channel abnormalities must generate these ECG deviations (and potentially associated cardiac arrhythmias (Fauconnier *et al.*, 2010;Colussi *et al.*, 2010;Gonzalez *et al.*, 2015)) in mdx mice. Among various potential candidates are abnormal

connexin 40 (Cx40) expression and impaired I_{K1} potassium channel function. First, consistent with a prolonged PQ interval in $Cx40^{-/-}$ mice (Simon et al. 1998), significantly increased Cx40 protein levels in the mdx heart (Colussi *et al.*, 2010) may contribute to the shortened PQ interval observed in ECGs from old mdx mice. Secondly, a decreased inward rectifier potassium current I_{K1} in dystrophic ventricular cardiomyocytes (Rubi *et al.*, 2017) may prolong the QTc interval in the "dystrophic ECG". These as well as potential other mechanistic considerations, however, remain speculative at present.

Finally, as gain of function Ca channel abnormalities do not exist in "aged" dystrophic (mdx) cardiomyocytes, they can also not account for the Ca overload, which was observed particularly in dystrophic cardiomyocytes from old mdx mice (Williams & Allen, 2007;Mijares *et al.*, 2014).

Summary and Conclusions

Here we have studied potential abnormalities in Ca currents and intracellular Ca transients in ventricular cardiomyocytes derived from aged dystrophic mdx mice. We found that both the L-type and T-type Ca current properties of mdx cardiomyocytes were similar to those of myocytes derived from aged wild type mice. Accordingly, Ca release from the sarcoplasmic reticulum was normal in cardiomyocytes from aged mdx mice. This suggests that, irrespective of the presence of a pronounced cardiomyopathy in aged mdx mice, Ca currents and Ca release in dystrophic cardiomyocytes are normal. Finally, our data imply that dystrophin- regulation of L-type Ca channel function in the heart is lost during ageing.

Future studies are needed to elucidate if a significant loss of dystrophin protein in the senescent mouse heart (Townsend et al. 2011) may account for this unexpected phenomenon. Further, it is currently unclear if normal Ca channel function in the "aged" dystrophic heart is also observed in other animal species and the human.

Resulting publications:

Original paper:

Rubi et al. (2018). Calcium current properties in dystrophin-deficient ventricular cardiomyocytes from aged mdx mice. *Physiol Rep*, **6** (1), e13567, https://doi.org/10.14814/phy2.13567

Planned presentations:

1) at Center for Physiology and Pharmacology, Dept. Neurophysiology and –pharmacology, Medical University of Vienna:

Talk: “Calcium current properties in dystrophin-deficient ventricular cardiomyocytes from aged dystrophic mdx mice” (April 2018)

2) at Europhysiology 2018 - Meeting: The Physiological Society (14. – 16. Sept. 2018)

The QEII Centre, London, United Kingdom

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