Final Report

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Title of Project: Identifying the pathways of excessive calcium leak in to dystrophic muscle.

The underlying cause of Duchenne muscular dystrophy (DMD) is a genetic one, but the progression of the disease is associated with contraction-induced damage to the muscles. There is significant muscle wasting as affected boys age, causing them to lose mobility and reduce their life expectancy through associated problems. Underlying the damage caused to the muscle is the entry of calcium that most likely activates deleterious pathways in the muscle, reducing normal function.

Calcium is dissolved at very high concentrations normally in the bodily fluids and has many important roles in the healthy body. Inside muscle (and all other tissues and organs) it is present at very low concentrations when the muscle is not contracting. In DMD too much calcium enters the muscle from that in the bodily fluids, upsetting the normal levels of calcium inside the muscle. What could further improve the outlook for DMD patients would be treatments or drugs that would redress the normal calcium balance across the surface of the muscle.

In the course of this project funded by The Brain Foundation, then PhD student Ms Tanya Cully and post-doctoral researcher Dr Isuru Jayasinghe have separately made important contributions to this problem. Tanya's work on the mouse model of DMD found that many of the proteins imbedded in the surface of dystrophic muscle that are responsible for determining calcium levels in the muscle change their expression levels compared to what is the case in healthy, non-dystrophic muscle. What was interesting was that it appeared that these changes may not be responsible for damage to the dystrophic muscle. If the dystrophic mouse muscle is 'compensating' for its pathophysiological state then this may be an important avenue for identify potential therapeutic targets for DMD treatment (Cully et al 2012). To move this work along we are currently measuring DMD and non-DMD human muscle samples for these calcium-transporting protein levels and continuing to assess the calcium handling of the muscles.

Measuring calcium entry into muscle is difficult. Muscle fibres have their surface membrane largely internalized, primarily to allow nerve impulses to travel throughout the muscle very quickly and evenly. Isuru has thoroughly described part of this membrane that lies close to the outermost surface of the fibre, obscuring it from the view of conventional imaging approaches. Isuru used 3D reconstructions of fluorescence images and other advanced techniques to show that this network is a mesh in rat and human muscle (Fig 1) (Jayasinghe et al 2013). We explored how to use this membrane network to assess calcium entry in to muscle and identify its normal role, which is unknown. This proved difficult and a new approach was taken, leading to a much more appropriate technique that is beginning to provide much more information of the Ca^{2+} movements across the surface and membranes of the muscle.

Moving to new techniques to calibrate $[Ca^{2+}]_{t\text{-}sys}$.

We realized during the course of the project that using the 'sub-sarcolemmal tubular network' (SSTN) to calibrate Ca^{2+} signals inside the t-system would only provide limited temporal information of such signals.

Recently we have calibrated t-system-trapped Ca^{2+}-sensitive dye fluorescence and $[Ca^{2+}]_{t\text{-}sys}$ to advance the methodology. We have found the relationship between t-system trapped rhod-5N fluorescence and $[Ca^{2+}]_{t\text{-}sys}$ could be fitted by a Hill curve with a β*K_D of 0.837 mM with a range that allowed fluorescence to be converted to $[Ca^{2+}]_{t\text{-}sys}$ up to ~3mM, which is appropriate for the expected physiological range of $[Ca^{2+}]_{t\text{-}sys}$ in the fibre (maximum expected $[Ca^{2+}]_{t\text{-}sys}$ of 1.5 to 2.5 mM).

We have now assayed $[Ca^{2+}]_{t\text{-}sys}$ to show *clear differences* in the ability of fast (FT) and slow-twitch (ST) muscle to extrude Ca^{2+} from the junctional space between the SR and t-system (Fig 2A). Also, steady-state $[Ca^{2+}]_{t\text{-}sys}$ is affected by the Ca^{2+}-buffer species, EGTA or Bapta (Fig 2B, FT shown). This novel result indicates that the RyR Ca^{2+} leak is critical to the ability of the t-system to control cell Ca^{2+} content. Note that Bapta is a *fast* buffer, capturing Ca^{2+} leaking through RyR within 5nm but EGTA is *slow*, with the longer Ca^{2+} length constant of 54nm, allowing leaked Ca^{2+} to reach the PMCA. This result colocalizes PMCA and RyR within 54nm and *importantly* show our ability to isolate the RyR Ca^{2+} leak in a skinned fibre preparation.

We suspect the major differences in dystrophic muscle form healthy is the ryanodine receptor causing an increased Ca^{2+} leak directly onto the t-system membrane and the increased expression and therefore average Ca^{2+} conductance of the store-operated channels.

Hypothesis vs. Findings: our original hypotheses were:

H1. There will be a greater resting leak of Ca^{2+} in dystrophic than healthy skeletal muscle (related to Aim 1).

H2. Specific t-system proteins will be responsible for the increased Ca^{2+} leak into dystrophic muscle across the t-system membrane (related to Aim 2).

Our original approach to use the SSTN to calibrate changes in the $[Ca^{2+}]$ of the rest of the t-system was fully explored. As mentioned above the SSTN was described in detail for the first time. However, it was found not to be the best reference point for calibrating $[Ca^{2+}]$ in this membrane system. Therefore we took on the large task of calibrating non-ratiometric Ca^{2+}-dependent fluorescent dyes and $[Ca^{2+}]_{t\text{-}sys}$ that would allows us to explore H2 of the grant. We achieved this goal (Fig 2). This method allowed us to realize that the Ca^{2+} leak at rest from the Ca^{2+} release channel (also known as the ryanodine receptor) of the sarcoplasmic reticulum, which abuts the t-system for signaling purposes, is responsible for maintaining the $[Ca^{2+}]$ gradient across the t-system (not previously known). This is significant as the ryanodine receptor is thought to be leaky in dystrophic muscle (Bellinger et al 2009). Furthermore, our new technique allows us to directly measure the effect of ryanodine receptor Ca^{2+} leak on the $[Ca^{2+}]$ at the t-system (Fig 2).

Unanswered questions:

What underlies Ca^{2+} dysregulation in the dystrophic muscle is more complex than thought. We are using the new method developed under the support of the Brain Foundation (Fig 2) to directly show the influence of ryanodine receptor Ca^{2+} leak and t-system Ca^{2+} leak on the final steady state $[Ca^{2+}]_{cyto}$ in the dystrophic muscle that is a major point of degradation of the dystrophic muscle.

What these research outcomes mean:

The major outcome is a method to measure ryanodine receptor Ca^{2+} leak in mouse muscle, which can be adapted to human muscle taken with a minimally invasive needle biopsy (Figs 2 & 3). **Note this will have major implications for directly testing drugs against ryanodine receptor mutations in human muscle that are the source of many muscle diseases/conditions.** We are currently analyzing data to determine the differences between dystrophic and healthy mouse muscle and will perform such experiments on human muscle very soon (human ethics application pending to work on biopsies from people with mild muscular dystrophy and myotonic dystrophy).

Photographs and diagrams:

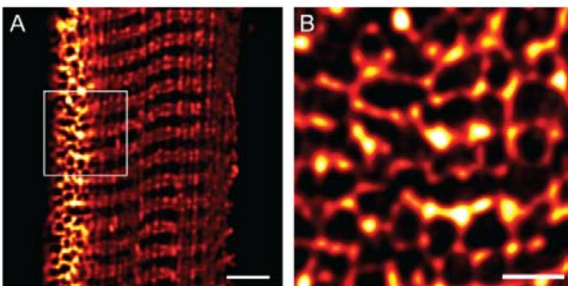

Fig 1: *Identifying complex structures in muscle fibres. Panel A shows the internalized surface membrane of a skeletal muscle fibre. The regular striations are well-known and required to transmit nerve impulse into muscle. The area covered by the white box is expanded in Panel B. This shows a complex membrane mesh, which has not previous been described in detail, called "sub-sarcolemma tubular network". Its function in muscle is not known.*

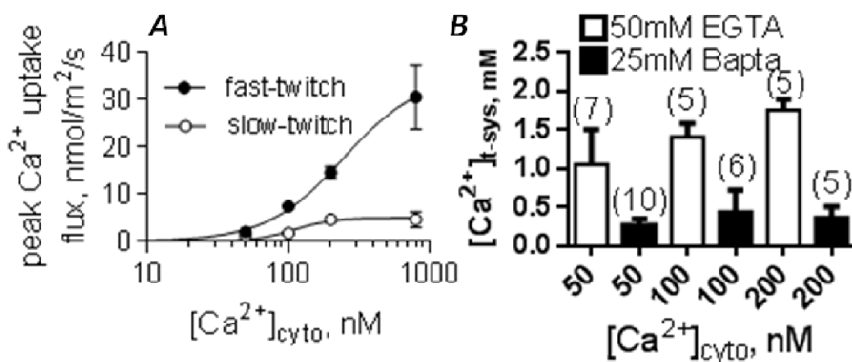

Fig 2. *A, rat fast- and slow-twitch muscle have different t-system Ca^{2+} uptake rates. B, steady-state $[Ca^{2+}]_{t\text{-}sys}$ is dependent on RyR Ca^{2+} leak reaching the t-system. RyR leak is restricted by Bapta but not EGTA.*

Fig 3. Ca^{2+}-sensitive fluorescence dye trapped in the t-system of a fibre isolated from a biopsy of myotonic dystrophy patient *(28 y.o. male). Note that we can work with diseased and atrophied human muscle and we are able to perform the same physiological experiments on human muscle as that summarized for in Fig 2 (not shown).*

Researchers:

Dr Tanya Cully

Dr Isuru Jayasinghe

References:

Bellinger AM et al (2009). Hypernitrosylated ryanodine receptor calcium release channels are leaky in dystrophic muscle. Nat Med 15, 325-330.

Cully TR, Edwards JN, Friedrich O, Stephenson DG, Murphy RM, Launikonis BS (2012). Changes in plasma membrane Ca-ATPase and stromal interacting molecule 1 expression levels for Ca^{2+} signaling in dystrophic mdx mouse muscle. American Journal of Physiology. Cell Physiology 303, C567–576.

Jayasinghe ID, Lo HP, Morgan GP, Baddeley D, Parton RG, Soeller C & Launikonis BS (2013). Examination f the sub-sarcolemmal tubular system of mammalian skeletal muscle fibers. *Biophys J* **104** (11), L19-L21. *Cited by* **F1000*Prime*** *where it was highlighted for its: "advanced imaging, analysis techniques, and fibre manipulations to give extraordinarily clear visual evidence of this network".*