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General Introduction

1.1 General introduction

Skeletal muscle comprises a complex matrix of individual units of force generating protein, singularly ensheathed within tubular lipid membranes, providing tensile strength of protein, complimented by passive elasticity of lipid in a synergy of fast excitatory stimulation and dynamic adaptive plasticity. The muscle fibre’s compressive force generation potential is however not a reflection of toughness or resilience of tissue and, when separated from one another, muscle fibres are indeed brittle and easily damaged by bending and compression forces. Moreover, significant damage to the sarcolemmal membrane surrounding individual fibres is sufficient to cause necrotic death of the fibre. Hence, the majority of research in the field of muscle diseases focuses on the proteins located at the interface of membrane and force generating protein. The mechanisms underlying the dynamic adaptability of fully differentiated adult skeletal muscle remain largely unknown, providing an attractive arena for the investigator. The discoveries that have been made largely stem from the fundamental theory that differentiated muscle arises from the fusion of mononucleate cells during development, giving rise to the classical multinucleate fibre structures, commonly associated with muscle. Indeed, it was the discovery of the satellite cell (Mauro, 1961) that underpinned the portrayal of differentiated muscle as maintained and plasticised through the dedicated currency and conveyance services of a resident population of mononuclear myogenic precursor cells located beneath the basal lamina of mature muscle fibres, at the interface of membrane and functional
protein. The complexity and diversity associated with this anatomically distinct and intricately dynamic population of cells has belayed the occupations of the skeletal muscle biology field for nearly 50 years, and continues to feature heavily at the forefront of research into skeletal muscle disorders.

1.2 Historical perspective

‘Muscular dystrophy’, from the Latin, ‘dys’, meaning difficult and the Greek ‘trophe’, relating to nourishment, denotes a heterogeneous group of neuro-muscular diseases, the most common of which is Duchenne muscular dystrophy (DMD). Dr. Guillaume-Benjamin-Amand Duchenne, who’s name is born by more than 10 of the now recognised list of over 40 such conditions, was born Sept. 17, 1806, in Boulonge-sur-Mer (Rudolf Kleinert). As well as a medical practitioner, his hobby was studying the emerging sciences of neurology and electrical stimulation, for which he famously constructed an apparatus allowing the surface-electrode stimulation of bodily tissues from living subjects. His scientific persuasions were complemented by an interest in photography, and he was one of the first people to use photographs instead of drawings to document pathological conditions for medical reference. In a series of works published between 1861-1872 (Paraplégie Hypertrophique de l’enfance de cause cérébrale, De l’Electrisation localisée et de son application à la pathologie et à la thérapeutique and L’Electrisation Localisée; http://www.whonamedit.com), Duchenne described a collection of juvenile patients with conditions that he considered to represent a form of juvenile spinal muscular atrophy, characterised by increases in the fatty interstitial tissue surrounding the musculature. He is also thought to be responsible for devising the first implement and procedure for conducting muscle-derived biopsy from living patients without anaesthesia (Charriere and Duchenne, 1865), there by enabling him to demonstrate the progressive nature of
the disease. Although now widely regarded as the first person to document the conditions which now bears his name, Duchenne may actually have been studying a condition already described in detail by an English physician by the name of Edward Meryon (Meryon, 1852, reviewed by (Emery and Emery, 1993). Meryon described a condition which particularly impressed him due to its “predilection for males and its familial nature”, which presented the now familiar symptoms of difficulty in walking from an early age and later climbing stairs, with eventual loss of ambulation and death in the teens. Following necropsy of an affected 16 year old boy, he commented “The chief structural change existed in the system of the voluntary muscles, which was throughout the entire body atrophied, soft, and almost bloodless… the striped elementary primitive fibres were found to be completely destroyed, the sarcous element being diffused, and in many places converted into oil globules and granular matter, whilst the sarcolemma or tunic of the elementary fibre was broken down and destroyed” (Emery and Emery., 1993). The parallels with modern-day descriptions of dystrophic tissue are obvious and Meryon’s work was actually discussed academically by Duchenne, who failed/refused to recognise the similarities in his own work, instead choosing to consider Meryon’s studies to describe a more specific form of muscular atrophy than his own findings. Indeed, many medical descriptions exist preceding either man’s contribution but it is Duchenne who retains much of the credit for the early work on characterising this class of disease. These diseases are now collectively referred to as neuro-muscular disorders, or muscular dystrophies, of which DMD is the most common.

1.3 Clinical presentation

DMD patients typically present clinically at 4-5 years of age, with symptoms including difficulties in standing from a seated position, climbing stairs, and keeping
up with their peers during play. The muscle degeneration is progressive and replacement with fibrous/fatty tissue occurs, which ultimately results in muscle contractions and eventual death by cardiac or respiratory failure by late teens to mid twenties (Emery and Walton, 1967). The introduction of positive-pressure ventilation has since increased life expectancy into late-twenties and early thirties. Severe cognitive impairments such as reduced verbal skills and delayed reading learning are seen in approximately 30% of DMD cases (Moizard et al., 2000). Elevated levels of creatine kinase derived from muscle, in serum and amniotic fluid (Emery, 1977) remain the most widely used clinical markers in DMD diagnosis, combined with mutational analysis based DNA sequencing tests for patients and their families, where possible. DMD displays an X-linked inheritance pattern and appears in approximately 1 in 3,500 males (Emery, 1991). Females are usually heterozygous carriers. Their skeletal muscles are multinucleate mosaics of affected and unaffected cells due to random X-inactivation in muscle precursor cells, where compensatory dystrophin expression is sufficient to render a normal phenotype. However, female carriers have been shown to develop cardiomyopathy (Hoogerwaard et al., 1999a, Hoogerwaard et al., 1999b), which highlights a physiological difference between skeletal and cardiac muscle, where the latter is thought to comprise around 75% mononucleate post-mitotic cells (Olivetti et al., 1996) and the former contains a small, yet indispensable, population of resident stem cells/satellite cells [1-6% of total myonuclei (Allbrook, 1981)], possessing high myogenic potential (Jackson et al., 1999). Becker's muscular dystrophy (BMD) also arises from mutations in the DMD gene, although phenotypes are milder due to the in-frame nature of the mutations, which result in differing levels of expression of partially functional, truncated dystrophin proteins (Hoffman et al., 1987, Hoffman and Kunkel, 1989).
1.4 Molecular principles of DMD

Genetics and molecular biology studies have facilitated the elucidation of the DMD gene carrying the mutations responsible for the DMD phenotype (Monaco et al., 1986) and its encoded protein product, dystrophin (Hoffman et al., 1987). Loss or truncations of this protein are responsible for the onset of DMD, and the milder BMD, respectively (Hoffman et al., 1987, Hoffman and Kunkel, 1989). Sequence analysis of the dystrophin gene revealed homology to the spectrin super-family (Koenig et al., 1988) and using the best available spectrin sequence data available at the time, that of Drosophila melanogaster, the NH2-terminal domains of β-spectrin, α-actinin, and dystrophin were found to contain striking sequence conservation, thought to reflect conservation of structural and functional properties in these proteins (Byers et al., 1989). The structural similarities of spectrin and dystrophin, mainly the conserved actin binding NH2 domain, combined with the sub-membrane distribution of dystrophin (Zubrzycka-Gaarn et al., 1988, Beam, 1988, Arahata et al., 1988), lead to the idea that dystrophin may protect the integrity of the muscle cell sarcolemma against muscular contraction in a similar fashion to the manner in which spectrin allows erythrocytes to become elastic when passing through narrow capillaries (Byers et al., 1989).

Dystrophin is the largest known gene covering 2.6Mbp within the Xp21 and contains 79 exons - it represents some 0.1% of the entire human genome. In normal skeletal muscle the dystrophin protein derived from full length transcription of this gene (which results in the mature mRNA of 14 kb) has a molecular weight of 427kDa. However, since the initial discovery, it quickly became apparent that the dystrophin gene is expressed as several tissue specific isoforms from multiple promoters residing within the gene. In decreasing order of mRNA length, the nine known isoforms of dystrophin in the human genome are: Cortical or brain dystrophin; Dp427c (Nudel et al., 1989), Purkinje dystrophin; Dp427p (Gorecki et al., 1992), muscle dystrophin;
Dp427m (Koenig et al., 1987), lymphocyte dystrophin; Dp427l (Nishio et al., 1994), although the significance of this promoter has since been disputed (Wheway and Roberts, 2003), retinal dystrophin; Dp260 (D'Souza et al., 1995), CNS dystrophin; Dp140 (Lidov et al., 1995), Schwann cell dystrophin; Dp116 (Byers et al., 1993), and also the ubiquitously expressed apo-dystrophin isoforms - Dp71 (Bar et al., 1990) and Dp40 (Tinsley et al., 1993) (Figure 1.4.1; A). An additional level of complexity exists due to alternative splicing of all known dystrophin transcripts, where both the 5' (Surono et al., 1997) and 3' (Nakamori et al., 2007) regions have been shown to be alternatively spliced in human and mouse skeletal muscle (Figure 1.4.1; B and C, respectively).
Figure 1.4.1 Predominant promoters and products of the DMD gene. Schematic illustrating promoter locations (upper panel; bent arrows) and main protein products (lower panel) of the DMD gene in humans and mice (A) with splicing products identified in the 5'
Specific functions remain to be ascribed to these specific protein products derived from the dystrophin gene. Sequence-based structural predictions led to the now widely accepted proposal that Dp427m is a rod-shaped protein consisting of four domains: an N-terminal actin-binding domain, twenty four triple helix spectrin-like repeats separated by four hinge regions, a cysteine-rich domain containing two predicted calcium binding motifs, and a unique C-terminal domain (Koenig et al., 1988). It is perhaps more suitable to describe dystrophin in terms of its location within normal muscle, rather than to attempt to assign any defined function, as consensus opinion views dystrophin as a cytoskeletal linker molecule, located in distinct sub-sarcolemmal bands distributed evenly along differentiated muscle fibres, where it shares links with F-actin at its N-terminus, and proteins such as nitric oxide synthase (NOS) via the syntrophins, the dystrobrevins, and syncoilin at its C-terminus (Suzuki et al., 1994). However, perhaps the most well known link regarding DMD is that made between the cysteine rich domain and the dystrophin glycoprotein complex (DGC) or dystrophin associated protein complex (DAPC), where dystrophin appears to coordinate a link from the actin cytoskeleton right through to laminin-2 of the extracellular matrix (Ahn and Kunkel, 1993, Campbell, 1995) by way of β-dystroglycan (Jung et al., 1995), the sarcoglycans and sarcospan located in the sarcolemma, and α-dystroglycan on the external face of the membrane (Figure 1.4.2) (Yoshida and Ozawa, 1990, Ervasti and Campbell, 1991). Mutations within dystrophin or almost any other component of the DAPC complex have been shown to give rise to various types of muscular dystrophies (reviewed by Khurana & Davies, 2003), yet the precise mechanisms relating to each pathology remain largely elusive, as do the specific functions of the individual proteins involved. One such protein,
which has recently acquired evidence of function is \( \alpha \)-sarcoglycan (adhalin), a constituent of the four-membered sarcoglycan complex which is retained in the DAPC through its link to \( \beta \)-dystroglycan. Mutations of this gene in humans produce the phenotype referred to as Limb Girdle Muscular Dystrophy type 2D (LGMD 2D), which shares similarities with DMD. \( \alpha \)-sarcoglycan has been shown to not only bind ATP (Betto et al., 1999), but also to degrade it, and the kinetics for this event have been suggested to be evidence of its function as an ecto-ATPase enzyme (Sandona et al., 2004). Considering the loss of DAPC complex in the absence of dystrophin, this could suggest the loss of some degree of inherent ATP-hydrolysing potential from dystrophic muscle.
Figure 1.4.2 Structure of the Dp427 protein and its interactions with other components of the dystrophin associated protein complex (DAPC) at the sarcolemmal membrane of skeletal muscle. Illustration depicting the main structurally distinct domains of the full length Dp427 protein. N-terminal actin-binding domain: Calponin homology domain 1 (CH1); cyan, CH2; green. Central rod domain: 24 coiled-coil, triple helical spectrin-like repeats. Cysteine-rich region: WW domain (containing two highly conserved tryptophan residues); green, Calcium binding EF hands; red, orange, cyan and purple. Carboxy-terminal region: syntrophin-binding element; yellow, and leucine zipper domains; red (upper panel). Dystrophin (red) facilitates anchoring of DAPC complex members via laminin in the extracellular matrix, through the dystroglycan complex, to the intracellular contractile
machinery. DAPC members include: α- and β-dystroglycan (αDG, βDG), sarcospan (spn), α-, β-, δ- and γ-sarcoglycans (SG), syntrophin (syn), α- and β-dystrobrevin. Syncoilin, dysferlin, calpain-3, caveolin-3, filamin-c, Grb-2, and nNOs all interact with the DAPC orchestrating downstream signalling cascades.

Sequence homology searches using cDNA libraries from humans, sea urchins (Strongylocentrotus purpuratus), fruit flies (Drosophila melanogaster), electric rays (Torpedo californica) and nematode worms (Caenorhabditis elegans) have identified multiple genes showing relation to dystrophin, prompting investigations into the evolutionary origin of the gene, which is thought to be highly conserved throughout metazoans, implying a fundamental role in animal biology (Roberts and Bobrow, 1998). Evidence for this has been provided recently with the demonstration that the membrane-cytoskeletal function of dystrophin extends to maintaining neural integrity in C. elegans (Zhou and Chen, 2011). The gene displaying the highest degree of sequence similarity to dystrophin is utrophin from ubiquitously expressed dystrophin homologue or dystrophin-related protein 1 (DRP1), with around 51% amino acid homology, similar exon/intron composition (Love et al., 1989, Pearce et al., 1993), and smaller transcripts arising from internal promoters (Fabbrizio et al., 1995, Blake et al., 1995). Smaller ancestral homologues include ‘torpedo’ in electric rays (Carr et al., 1989), ‘MSP-300’ and ‘dah’ proteins in fruit flies (Zhang et al., 1996), as well as the ‘dystrobrevins’ (Sadoulet-Puccio et al., 1996), ‘dystrotelins’ (Jin et al., 2007) and the vertebrate specific ‘DRP2’ protein (Roberts et al., 1996) in humans and mice, all of which retain significant similarity with the C-terminal and Cysteine-rich domains of dystrophin.
1.5 Animal models of DMD

The elucidation of dystrophin’s evolutionary history has yet to facilitate the elucidation of a precisely defined function for these proteins, but disruptive mutations of early dystrophin homologues has provided useful information: Null mutations of dys-1 protein in nematode worms result in subtle behavioural defects consisting of hyperactive locomotion, exaggerated bending of the head, and a tendency to hypercontract (Bessou et al., 1998), and in flies, MSP-300 null mutants have shown that this protein contributes to muscle morphogenesis during development by providing a functional link to laminin in the extracellular matrix, supporting the integrity of the sarcolemma during or following myotubes extension (Rosenberg-Hasson et al., 1996). This suggests a dual functional role for the single dystrophin homologue found in invertebrates, as has recently been documented in C. elegans (Zhou and Chen, 2011).

Utrophin has been show to be an autosomal parologue of dystrophin, separated by duplication at some point in the early evolution of vertebrates (Roberts and Bobrow, 1998, Wang et al., 1998), and has received attention with regard to potential therapeutic strategies aiming to replace dystrophin loss in DMD by up-regulating utrophin expression. Indeed, utrophin expression has been found to be up-regulated in both human DMD patients (Khurana et al., 1990) and in the murine ‘mdx’ mouse model of DMD (Love et al., 1991).

The mdx mouse arose as a spontaneous mutation in a mouse colony of C57BL/10ScSn background (Bulfield et al., 1984) that results in premature termination codon in the full-length dystrophin transcripts (Sicinski et al., 1989) and is probably the most widely studied model of DMD due to its relatively low cost of housing combined with short gestation and rapid maturation periods. Although the limb muscle pathologies of humans and mice do differ, numerous similarities exist; Delayed onset of disease pathology is observed in the mdx mouse, where muscle
appears histologically normal until around one week of age and then between approximately weeks 3-8 the muscles undergo cycles of degeneration and regeneration with apparent synergistic interplay of the immune system and stem cell populations (Turk et al., 2005). The acute degenerative period appears to end at around 12 weeks of age, marked by the cessation of large scale immune cell infiltrations and associated necrotic lesions within the tissue, thereafter muscles are locked into a regenerative state. The condition changes once more, demonstrating progressive fibrosis and muscle necrosis in animals of >1 year (Villalta et al., 2010). Mdx animals do possess contractile and conductive deficits as well as cardiac dilation and fibrosis that increase with age (Quinlan et al., 2004, Lefaucheur et al., 1995, Pastoret and Sebille, 1995, Morrison et al., 2000), although the degree of fatty/fibrous tissue formation in limb muscles is reduced in the mdx mouse compared with the human pathology (Villalta et al., 2010). The mdx mouse does however, display abnormal electrocardiogram readings and severely affected diaphragm by 6 months of age (Bia et al., 1999), and it is the diaphragm muscle that is widely considered to be the most reflective and reproducible of the human pathology (Stedman et al., 1991), where dystrophy and fibrosis are both severe and progressive from an early age. Feline and canine DMD models also exist. Cats display a pathology more reminiscent of the mouse, and dogs that of humans (Khurana and Davies, 2003); hence golden retriever muscular dystrophy (GRMD) (Cooper et al., 1988) may have addition benefits for pre-clinical therapeutic assessment (Schatzberg et al., 1998).

1.6 Therapeutic approaches

Current therapeutic interventional approaches in DMD centre on efforts to re-express or replace the functional elements of the dystrophin protein in muscle (Amenta et al., 2010, Mendell et al., 2010). These therapies can be crudely grouped
into gene-therapies, stem cell-therapies and pharmacological therapies. The latter are also used in managing the condition. Gene-therapy based approaches seek to artificially introduce DNA sequences into diseased tissues, with a view to achieving tissue specific expression of normal or modified host-derived protein sequences able to fulfil, or partially fulfil functions of proteins lost in the disease processes. Stem cell based approaches share a similar rationale, seeking therapeutic gain through the introduction of modified, host-derived stem cells or undifferentiated progenitor cells to replace loss of function through their incorporation back into host tissues. Neither avenue is without its problems, chief of which are the size of the gene to be replaced, immunological rejection of foreign vectors/non-host derived cells, difficulties in tissue specific delivery, lack of stable expression sufficient to confer functional gain and possible toxicity/side effects.

Classical vector-borne gene therapy approaches for DMD were hindered by problems of transgene size, which have been addressed using mini- and micro-dystrophin cDNA constructs delivered most successfully via modified adenoviral in-vivo and lentiviral vectors ex-vivo (Kochanek et al., 1996, Chen et al., 1997); (Rodino-Klapac et al., 2011, Rodino-Klapac et al., 2010, Trollet et al., 2009). Clinical trials research into scalable production techniques and immune tolerance of these vectors remains ongoing in an effort to develop methods of supplying sufficient amounts of packaged vector for full body application in humans.

Advances in modern molecular biology have facilitated the expansion of gene therapy from the classical gene delivery-based systems into pharmacological based attempts to repair/replace genetic defects in-situ. The well documented ‘reading frame’ hypothesis proposed by (Monaco et al., 1988) can explain ~90% of the phenotypic differences observed between DMD/BMD patients (Prior and Bridgeman, 2005). The subsequent discovery of a DMD patient possessing a 52bp deletion in exon 19, resulting in the transcriptional ‘skipping’ of the entire exon 19 coding sequence (Matsuo et al., 1991), led to the proposal of using targeted ‘exon skipping’
as a therapeutic approach for restoring the dystrophin reading frame and hence its expression in DMD patients (van Deutekom et al., 2001). Exon skipping using specific antisense oligonucleotides (AONs) has been applied in both the mdx mouse and DMD patients, the therapeutic potential of local administration of such drugs has been demonstrated in both mdx mice (Gebski et al., 2003) and DMD patients (Aartsma-Rus et al., 2003) and clinical trials are currently ongoing (Kinali et al., 2009); AVI Bio Pharmaceutics plans phase II trials by the end of 2010 (AVI-4658) and Prosensa/GSK are currently running phase III trials (PRO51). Another promising pharmaceutical candidate has been PTC124, the aminoglycoside derivative known as gentamycin, capable of inducing ribosomal read through of premature, but not native, termination codons within the DNA sequence being transcribed (Welch et al., 2007, Barton-Davis et al., 1999, Malik et al., 2010). This type of mutation would be applicable to 10-15% of DMD cases. Phase II clinical trials using PTC124 (Ataluren; PTC Therapeutics) demonstrated some beneficial effect; slowing the reduction in average 6 minute walking distance over a 48 week period, although this effect was dosage-dependent (Action Duchenne) and statistical analysis is ongoing. Antisense-mediated exon skipping, together with forced stop codon read through currently represent the most advanced clinical trials in the area of gene/gene-derived-therapies for DMD (reviewed in (Aartsma-Rus et al., 2010, Sugita and Takeda, 2010, Guglieri and Bushby, 2010).

Stem cell-based approaches have gained significant notoriety in the last decade, paralleled by an increasing public awareness of their potential for major therapeutic gains in a plethora of genetic disorders. Early endeavours exploited the classical satellite cell population for subsequent myoblast re-implantation (Gussoni et al., 1997, Fan et al., 1996) but subsequent studies revealed a plethora of cells types and to date, more than a dozen different populations of myogenic precursor/facilitator cells have been reported to offer potential therapeutic benefit for muscular dystrophy patients including myoblasts, mesoangioblasts, pericytes, myoendothelial cells,
CD133+ cells, aldehyde-dehydrogenase positive cells, mesenchymal stem cells, embryonic stem cells, induced pluripotent stem cells and PICs (Price et al., 2007, Quattrocelli et al., 2010, Meng et al., 2011, Tedesco et al., 2010, Negroni et al., 2011). However, the dynamic nature of skeletal muscle represents a highly complex arena in which to introduce proliferating cell types, many of which being multipotent. Although stem cell based therapies do not share the capacity issues of viral or plasmid derived vectors, there remain several obstacles to their efficient use in-vivo; Cellular survival and host immune rejection have always been a consideration here (Beauchamp et al., 1997, Mendell et al., 2010), as have satisfactory levels of migration and transgene expression (Carnio et al., 2011, Skuk and Tremblay, 2003). Yet, with so many potentially beneficial cell types coming to the fore, and cellular delivery methods ever improving, the current ‘one cell to cure all’ philosophy may give way to cell therapies tailored to curing/prolonging the life of the individual and most vital muscle groups.

While the search for effective gene- and cell-based therapies remains on going, the currently available pharmacological interventions broadly aim to manage/improve the phenotype for the patient. Corticosteroids such as prednisone and deflazacort have long been used in DMD (Drachman et al., 1974, Barthelmai, 1965, Bonifati et al., 2000, Muntoni et al., 2002) Although their exact mechanism of action remains largely elusive and have been reported to be complex, they do confer limited benefits in slowing disease progression., However, such benefits are often to some degree offset by unwanted side effects such as osteoporosis, weight gain and cataracts (Fisher et al., 2005). As muscle wasting is characteristic of the DMD phenotype, light exercise programs are encouraged to delay these processes. The use of anabolic steroids (Zeman et al., 1994) and creatine-based supplements have also been shown to provide small improvements in muscle strength (Walter et al., 2000b).

The loss of muscle mass in DMD is commonly seen to represent a failure of the organ to regenerate itself under sustained pathological degradation of its
progenitor cell population through degradative exhaustion or premature aging (Chamberlain, 2010). This has led to the exploration of possible solutions via growth factor manipulation, where-by up-regulating muscle growth \textit{in situ} may sufficiently compensate for the degree of muscle loss seen in DMD to give some kind of therapeutic benefit (McPherron and Lee, 1997); (Lee and McPherron, 2001). Antibody-mediated myostatin inhibition has been shown to result in anatomical improvement in the \textit{mdx} mouse (Bogdanovich \textit{et al}., 2002), and \textit{mdx} mice with myostatin mutations also display similar beneficial characteristics (Wagner \textit{et al}., 2002). Phase II clinical trials using a myostatin inhibitor (MYO-29, Wyeth Pharmaceuticals) are still ongoing (Wagner \textit{et al}., 2008). Attempts have been made to enhance muscle mass in the dystrophic background by manipulating other growth regulatory factors, such as insulin-like growth factor-1 (IGF-1) (Barton \textit{et al}., 2002) and follistatin (Iezzi \textit{et al}., 2004). Furthermore, potential gene therapy- and gene transactivation-based approaches, specifically through \textit{in situ} manipulation of utrophin promoter activity, are being exploited (functional parallels with dystrophin underlying this approach have been discussed earlier). Should pharmacological induction of utrophin expression prove successful, then such therapies could negate the inherent difficulties of synthetic vector borne gene delivery (reviewed in Khurana & Davies, 2003) and of immunogenicity of dystrophin protein introduced into dystrophin-negative individuals. Unfortunately, so far BioMarin’s 2010 phase I clinical trial of compound BMN-195 has proved disappointing, with administrations of up to 400mg/kg BMN-195 resulting in plasma concentrations below that believed to be required for utrophin production (www.actionduchenne.org).

Calcium channel blockers such as nifedipine and diltiazam have also been trialled as therapeutics (Khurana and Davies, 2003), but whereas increased intracellular calcium concentration has been a recognised hallmark of the disease since the work of (Bodensteiner and Engel, 1978) over 40 years ago, therapies aiming at blocking Ca$^{2+}$ channels have had limited success, so far (Jorgensen \textit{et al}., 2011).
The pathology of the disease does offer inviting openings for therapeutic intervention; the delayed onset of the phenotype until around 5 years of age, the role of the inflammatory response in muscle degeneration, the somatic induction of revertant fibre expression in myonuclei (through restoration of the dystrophin reading frame) (Hoffman et al., 1990, Nicholson et al., 1989), and perhaps most intriguingly, the documented absence of disease pathology in smaller muscle groups such as the extraocular muscles (EOM) (Karpati and Carpenter, 1986), a phenomenon recently reported to relate to the more capable calcium handling properties of the EOM compared with TA muscle (Zeiger et al., 2010), highlighting a resurgence in efforts to elucidate the role of Ca\(^{2+}\) in dystrophic muscle.

1.7 Calcium signalling and DMD

The notion of Ca\(^{2+}\) acting as a second messenger in signal transduction is long established (Rasmussen et al., 1990, Ringer, 1883), and since much of the early studies were conducted in cardiac and skeletal muscle (reviewed in (Ebashi, 1991), a large body of work already existed for those seeking to explain the abnormalities in Ca\(^{2+}\)-mediated signalling so often reported to coincide with the degenerative pathology in DMD patients. Abnormalities in Ca\(^{2+}\) homeostasis have long been postulated as effectors of the dystrophic pathology in humans and animals (Bertorini et al., 1982b, Bertorini et al., 1982a, Turner et al., 1988, Hopf et al., 2007, Gailly, 2002, Batchelor and Winder, 2006, Jorgensen et al., 2011, Allen et al., 2010a, Morine et al., 2010, Zeiger et al., 2010), yet whether the mechanisms by which dystrophin loss results in abnormally high intracellular Ca\(^{2+}\) levels in dystrophic muscle have been fully elucidated remains the topic of much debate (reviewed in Allen et al., 2010; Batchelor & Winder, 2006; Hopf et al., 2007). The hypothesis relating to the dystrophin protein functioning as a shock absorber, tethering its associated protein
complex to distinct areas of the muscle membrane has been extended in relation to the DMD pathology, with the suggestion that loss of dystrophin, aside from resulting in loss of the functional link between the actin-cytoskeleton and laminin in the extracellular matrix, may actually result in contraction-induced damage and deregulation of mechanosensitive \( \text{Ca}^{2+} \) channels as the internal myosin machinery is left to move freely inside the sarcolemma. The notion of calcium abnormalities in dystrophic muscle was actually suggested prior to the identification of the DMD gene (Bertorini et al., 1982a, Bertorini et al., 1982b) and several routes of entry for the observed increased levels of intramuscular calcium were proposed; with increases in sarcolemmal fragility reported in both DMD patients and \textit{mdx} mice (Petrof \textit{et al.}, 1993, Watkins \textit{et al.}, 1988, Beam, 1988). Another opinion has evolved that, rather than contraction-induced necrosis of entire myofibres, the degree of survivable sarcolemmal disruptions is up-regulated in the dystrophic pathology (Alderton and Steinhardt, 2000b, Alderton and Steinhardt, 2000a), where increases in survival have been documented following reductions in extracellular calcium levels (Gailly, 2002). This hypothesis has itself evolved as other mechanisms for \( \text{Ca}^{2+} \) entry into dystrophic muscle have been proposed; micro membrane ruptures, aberrant activation of sarcoplasmic reticulum (SR) calcium channels, sarcolemmal leak channels and \( \text{Ca}^{2+} \) buffering mechanisms (reviewed in (Batchelor and Winder, 2006, Gailly, 2002, Allen \textit{et al.}, 2005), leading to the proposition that fast and localised changes in membrane integrity, permeability and therefore intracellular \( \text{Ca}^{2+} \) concentrations ([\( \text{Ca}^{2+} \)]\text{\textit{i}}) give rise to unscheduled \( \text{Ca}^{2+} \) sparks within muscle fibres (reviewed in (Niggli, 1999, Batchelor and Winder, 2006). Regardless of the route of entry, most researchers agree that the intracellular accumulation of \( \text{Ca}^{2+} \) can be toxic to all living cells and its role in the activation of cellular proteases and the apoptotic cascade has been well documented in normal and diseased muscle (Alderton and Steinhardt, 2000a, Mallouk \textit{et al.}, 2000). However, the suggestion has also been made that the increased [\( \text{Ca}^{2+} \)]\text{\textit{i}} in dystrophic muscles may actually be beneficial and involved in repairing the
damage to membrane ruptures via stress-induced membrane repair involving dysferlin and annexin recruitment to sites of membrane ruptures (Bansal et al., 2003, Lammerding and Lee, 2007). With a recent revival in interest surrounding Ca\(^{2+}\) signalling in DMD, possibly owing to the lull in new ideas surrounding the structural role of dystrophin, the search for a unifying hypothesis to explain the pathology has given way to a more combined rationale, where dystrophin is seen as a much more multifunctional protein able to combine interlinked faculties including dystrophin-associated complex retention, structural definition and support, signalling cascade governance and cellular fate determination. In studying the potential roles for Ca\(^{2+}\) in the dystrophic pathology, researchers have focussed largely on the detrimental effects of increases in [Ca\(^{2+}\)]\(_i\), largely due to the pre-existing and well documented role of SR-derived Ca\(^{2+}\) release in malignant hyperthermia in muscle (Lopez et al., 1985, Blanck and Gruener, 1983). More recent studies started to unravel potential mechanisms by which Ca\(^{2+}\) sparks could induce not only the apoptotic and necrotic signalling cascades, but also those resulting in growth, proliferation, differentiation and cellular survival (reviewed by (Batchelor and Winder, 2006).
Figure 1.7. Calcium abnormalities in DMD muscle. Illustration depicting possible origins of calcium signalling perturbations in dystrophic muscle resulting from loss of Dp427 expression (red). Dystrophin mutations result in loss of connectivity between extracellular matrix and cytoskeletal filaments (green) through DAPC complex members at the sarcolemmal membrane. Associated abnormalities in calcium homeostasis result in disruption of normal intracellular signalling cascades, with complex downstream consequences.

Elucidating the molecular mechanisms of Ca\(^{2+}\) signalling in a complex and plastic tissue such as muscle, is difficult. Adding to that the consideration that dystrophic muscle endures sustained and often highly localised cycles of degeneration and repair often associated with relentless host immune cell invasion, it becomes plain to see why the intricacies of Ca\(^{2+}\) role in DMD remain elusive. Yet there is one area of molecular biology that is well accustomed to such considerations; that of tumour biology. The life or death paradox surrounding Ca\(^{2+}\) signalling in cancer may lend
itself well to that of muscular dystrophy (reviewed by Monteith et al., 2007), more specifically the area of purinergic calcium channels could be of particular interest in the area of muscle diseases, where high ATP concentrations abound.

1.8 Purinergic signalling and receptors

Although purinergic responses were originally characterised using adenine compounds (Bennet and Drury, 1931, Drury and Szent-Gyorgyi, 1929) to stimulate the heart, the signalling role of the related compound, ATP, was not fully appreciated until much later. Geoffrey Burnstock is credited with having coined the term ‘purinergic transmission’ to describe the actions of ATP on the gut and bladder (Burnstock, 1972). It was he who postulated that membrane receptors for ATP must exist due to the universal nature of its action on multiple cell types (Burnstock, 1976), and later went on to describe the basis for distinguishing two separate families of ATP receptors; the cation channels and the G-protein coupled receptors. Both types are membrane bound and belong to large families of structurally and pharmacologically related ATP receptors. The currently accepted nomenclature describing purine receptors includes: P1 (A1, A2A, A2B, A3) and P2 (X and Y) (see Burnstock, 2007, for a review). All P1 receptors are membrane bound G-protein coupled receptors, mostly acting via the activation or inhibition of adenylate cyclase. Recognising adenosine as their principal agonist, P1 receptors share 39-61% sequence homology with each other and 11-18% with the P2Y receptors.

P2Y receptors share structural characteristics within the family and similarities with the P1 receptor subtype. They are also G-protein-linked, with seven transmembrane domains, an extracellular N-terminus and intracellular C-terminus but differ in agonist selectivities. Collectively, P2Y receptors recognise a host of purine and pyrimidine agonists, such as ATP, ADP, UTP and UDP, typically in the sub-
micromolar range, and signalling is generally conducted via IP₃ and cAMP induced mobilisation of intracellular Ca²⁺. Eight human P2Y receptors have been cloned to date (P2Y1, 2, 4, 6, 11, 12, 13 and 14) (Burnstock, 2007), differentiated by their unique pharmacological profiles in response to various concentrations of ATP, ADP, AMP, UTP, and UDP and synthetic agonists and antagonists.

P2X receptors are structurally distinct from the P1 and P2Y subtypes (Burnstock and Kennedy, 1985), forming non-selective cation channels consisting of homo and hetero-subunit trimers. Individual subunits comprise intracellular N and C-termini that serve as binding motifs in protein kinase mediated signalling, two membrane spanning domains, which collectively form the membrane channel of the trimer, and a large extracellular loop containing the ATP binding pocket orientated by a structurally conserved disulphide-bridge domain of 10 cysteine residues (North, 2002, Nicke et al., 1998). The pharmacological profiles of P2X receptors also set them apart from other related families, the principal agonist here being ATP, which binds all P2X receptors with varying affinities in the low µM to mid mM range. Seven P2X receptor subunits (P2X1-7) have been cloned and characterised, sharing 30-50% sequence homology at the peptide level (North, 2002). Collectively, this family of nucleotide receptors represents a staggeringly complex yet elegantly intricate matrix of signal transduction, conferring to the individual cell the power to exert exacting refinements in a myriad of metabolically driven reactions, and to achieve this in paracrine, endocrine and exocrine mode/fashion. Many of these receptors have become tissue specific through the course of evolution. The diversity of function within this receptor family is beyond the scope of this study, comprehensive reviews include Burnstock & King, 1996; Burnstock, 2007, (White and Burnstock, 2006, Ralevic and Burnstock, 1998, Burnstock et al., 2010, Surprenant and North, 2009).
Figure 1.8. Predicted architectures of purinergic receptor subtypes. Illustration depicting the main structural and functional features of purinergic receptors: P1 and P2Y receptors are G-protein coupled receptors comprising seven membrane spanning domains, intracellular C termini and extracellular N termini (A). P2X receptors comprise two transmembrane domains, with intracellular N and C termini (B). P2X receptors classically form hetero- and homo-trimeric, non-selective ion channels gated by amongst others, ATP (C; closed (left) and open (right); three predicted ATP binding sites are indicated (dark blue crescents)). P2X7 receptors are unique amongst other P2X receptors, possessing an extra long C-terminal tail, and also possess 10 conserved extracellular cysteine residues (red circles). Schematic also illustrates some functional implications of mutagenesis studies using P2X7 receptors expressed in HEK-293 cells (D).

1.9 Purinergic systems in DMD

Of the P2 receptors that may be of interest with regard to neuromuscular disorders, the P2X family stand out as the most likely to show pronounced alterations in response under disease conditions, due to its heightened specificity for the one principal agonist, ATP. ATP has long been known to exist at high concentrations (5-10mM) within skeletal muscle (Carlson and Siger, 1960, Walter et al., 2000a).
Moreover, it has been shown to be released into the extracellular milieu of multiple tissue types via lytic and non-lytic mechanisms. These were most extensively studied in epithelial and endothelial cells following shearing stresses, compression, stretching, hydrostatic pressure, hypotonic shock and necrotic cell death (Grierson and Meldolesi, 1995, Sauer et al., 2000, Ferguson et al., 1997, Hazama et al., 1999, Grygorczyk and Hanrahan, 1997, Idzko et al., 2007, Martinon, 2008). Such studies have shown extracellular ATP (ATP$_e$) release to reach concentrations of up to 500μM, which would be sufficient to activate all P2X receptor subtypes. However, there is some on-going debate as to whether the concentrations of extracellular ATP, which actually occur in-vivo are of sufficient level and duration to overcome the activity of nucleotide- metabolising enzymes (ecto-nucleotidases) located on the outer surface of the membrane - It has been suggested that at concentrations of >100μM, ecto-nucleotidases cannot degrade sufficient amounts of ATP, leading to a sustained high level of extracellular ATP (Bodin and Burnstock, 1996) resulting in tissue damage (Neary et al., 1994). It seems less likely that ecto-nucleotidases would be sufficient to entirely abate the effects of substantial cellular ATP release from skeletal muscles, especially in the case of the dystrophic pathology, where dystrophin absence results in the loss of α-sarcoglycan from the sarcolemma, which is itself an ecto-ATPase enzyme (Betto et al., 1999, Sandona et al., 2004).

The expression of P2X2, 4, 5, and 7 receptors has been independently documented in muscle development (Meyer et al., 1999, Ryten et al., 2001), muscle regeneration following dystrophic degeneration (Ryten et al., 2004, Yeung et al., 2004), and in myoblast lines (Ryten et al., 2002, Yeung et al., 2006). Of these, only P2X5 has been assigned any prospective function, with a proposed involvement in the differentiation of myoblasts to myotubes but this was based only on differences in antibody staining intensities (Ryten et al., 2002, Ryten et al., 2004). Furthermore, considering the hive of interest surrounding the elucidation of the molecular
mechanisms pertaining to myogenic precursor lineage and fate determination, it is surprising to note that this work was never followed up in the literature.

Although the concentrations of ATP found within normal skeletal muscles are more than sufficient to activate all P2 receptors, one receptor in particular, P2X7, stands out as a clear candidate for generating the most observable changes in diseased muscle. P2X7 is the least sensitive to its principal agonist (ATP) of all the P2 receptors, its EC$_{50}$ being $>300\mu$M, which is about 100 fold higher than other P2X receptors (North, 2002). It is also distinguished from other family members by its pore-forming ability, which has been ascribed to its long (240 amino acids), cytoplasmic C-terminal (Surprenant et al., 1996). While all P2X receptors have been implicated in many diverse processes; growth, proliferation, migration, apoptosis, cancer, epithelial transport, cytokine release, apoptosis and cancer (Ralevic and Burnstock, 1998) the orthodox opinion of P2X7 describes it as a ‘death-inducing receptor’. It is because in some cells, prolonged P2X7 stimulation leads to the formation of a lytic pore, in addition to the ion channel function described above. This pore, first characterised in rat mast cells (Cockcroft and Gomperts, 1980, Cockcroft and Gomperts, 1979), facilitates the permeation of large molecules ($<900$ Daltons) through the plasma membrane, as demonstrated using various dyes, such as ethidium bromide, lucifer yellow, and YOPRO-1. This permeation pathway can induce cytoskeletal rearrangements of actin and tubulin, translocation of phosphatidyl serine to the outer membrane leaflet, membrane ‘blebbing’ and microvesicle shedding, as well as changes in mitochondrial size, shape and efficiency (for reviews, see (North, 2002, Ralevic and Burnstock, 1998, Burnstock and Knight, 2004). Investigations into the mechanisms of pore formation in macrophages and transfected HEK cells have led to two potentially distinct mechanisms being proposed: firstly that P2X7 subunits are recruited in greater numbers following prolonged ATP stimulation, thus widening the pore, allowing for the passage of larger molecules (Di Virgilio et al., 1999). Secondly, that the covalent recruitment of another protein or complex of proteins
occurs, which creates the membrane pore when triggered to do so by P2X7 (Pelegrin and Surprenant, 2006). Here, the best documented candidate is the pannexin-1 hemi-channel (Pelegrin and Surprenant, 2006), although this could not be confirmed in cells from pannexin-1 knockout animals (James Elliot, personal communication). Neither hypothesis has been universally accepted or rejected (Yan et al., 2008). Moreover, it has been reported that the small cation channel pathway can be differentiated from the NMDG/YO-PRO permeability pathway, but that the NMDG and YO-PRO uptake mechanisms can themselves be different by some as yet undefined mechanism.

More recently, attention has shifted from the P2X7 lytic pore forming properties to the well known, yet comparatively little studied, ability of this receptor to control fast cation fluxes at the cell membrane, which serve to initiate a wealth of signalling cascades. It has become apparent that lytic pore formation is not a requirement for the induction of apoptosis/necrosis in P2X7-expressing cells; it has been shown that pore formation and apoptotic induction following P2X7 activation are indeed often reversible. Hence researchers have tentatively coined terms such as ‘pseudo-apoptosis’ (Mackenzie et al., 2005) and ‘aponecrosis’ (Elliott and Higgins, 2004) to describe these phenomena; the accuracy, relevance and true mechanism of which are still a mystery and often cell/tissue specific.

There has been an increase in attempts to characterise the intricate mechanics of intracellular P2X7-mediated signal transduction. The main focus has been on the conditions surrounding pathological/disease states, such as neurological/psychiatric disorders, cardiovascular disease, inflammatory diseases and cancer, as it is now widely accepted that conditions such as stress, trauma and inflammation can lead to the release of high concentrations of ATP from damaged tissues (for comprehensive reviews, see (Skaper et al., 2009, Di Virgilio et al., 2009). From these studies, an intriguingly complex, time-and concentration-dependent system of ATP release and signalling is emerging now. It appears that low level or tonic stimulation at low ATP concentrations (mid µM range) of P2X7 receptors may be beneficial to the cell
through stimulation of its growth, proliferation, metabolic, or migratory pathways. The most well documented are effects mediated by alterations to the Ras, Raf, MEK, ERK, Fos/Jun mitogen activated protein kinase (MAPK) pathway. Interestingly, the dependence of this kinase pathway on calcium influx has been shown to be cell type specific (Amstrup and Novak, 2003). As stimulation progresses from the low level, tonic to the sustained high concentration (low mM range), the cellular response then shifts to being detrimental and even excitotoxic. This phase can also be divided by its calcium dependence: the calcium mediates phosphatidyl serine translocation, membrane blebbing, microvesicle shedding, cytoskeletal disruptions, all of which seem reversible upon removal of stimulus, and the calcium dependent, caspase-3 mediated apoptotic pathway. The mechanisms of how this Ca\(^{2+}\) dependence is transmitted and whether this division can be uniformly applied to multiple cell types remain unknown.

The paradoxical nature of P2X7 up-regulation in tumours may potentially serve to provide analogies to the purinergic phenotype observed in dystrophic muscle cells. However, it is puzzling to consider that a tumour may overexpress a receptor so sensitive to excitotoxicity in an environment so rich in its stimulating agonist. This observation has led researchers to adjust their hypotheses on P2X7 roles in disease, and, in doing so, to provide what may become an interdisciplinary view of signalling integration. This view, when applied to DMD, may go some way to explaining the complex degenerative/regenerative stimuli underlying the dystrophic pathology, with a view to future therapeutic intervention. Abnormalities in ATP\(_e\) responses in dystrophic myoblasts have been documented previously (Yeung et al., 2006), necessitating the more in-depth investigations into the mechanism of this response that are described in this study.
1.10 Research hypothesis

Changes in P2X7 receptor function contribute to the pathology of DMD.

1.11 Thesis aims

- Characterise the pharmacological ecto-ATP hydrolysing profiles of normal and dystrophic myoblasts and myotubes and thus evaluate the purinergic significance of \( \alpha \)-sarcoglycan loss from the cell membrane of dystrophic cells.
- Investigate P2X7 expression in normal and dystrophic muscle cell cultures in vitro and muscles in situ; characterise mRNA levels, splice variants expression as well as protein profiles and localizations.
- Analyse functional differences in P2X7 responses between wild-type- and dystrophic- derived muscle cell cultures.
- Characterise using mass spectrometry the immediate proteome response of normal and dystrophic myoblasts following exposure to extracellular ATP.
- Determine the effect of P2X7 receptor blockade in the \( mdx \) mouse \( in vivo \) through pharmacological inhibition.
2

Materials & Methods

2.1 Chemicals and reagents

Table 2.1. Chemical and reagents used in this study

<table>
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<th>Product name (alphabetical)</th>
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<td>Acrylamide/bis</td>
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<td>Agar</td>
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<td>Agarose</td>
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<td>Anti-collagen IV antibody</td>
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<td>Anti-dystrophin antibody (2166)</td>
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<tr>
<td>Anti-mouse IgG, biotin-labelled</td>
<td>Sigma-Aldrich Ltd.</td>
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Anti-rabbit IgG, biotin-labelled                Vector Laboratories Ltd.
Anti-rabbit IgG, peroxidase-labelled          Sigma-Aldrich Ltd.
Adenosine triphosphate disodium salt         Sigma-Aldrich Ltd.
Avidin biotin blocking kit                   Vector Laboratories Ltd.

B
Bicinchoninic acid kit                      Sigma-Aldrich Ltd.
Betadine                                      Gift from Marta Onopiuk
Bovine serum albumin                        Sigma-Aldrich Ltd.
β-mercaptoethanol                           Sigma-Aldrich Ltd.
BzATP (2’(3’)-O-(4-Benzoylbenzoyl)adenosine-5’-triphosphate tri(triethylammonium) salt) Sigma-Aldrich Ltd.

C
Calcium chloride                            Sigma-Aldrich Ltd.
Chick embryo extract                        Sera Labs Ltd.
Collagenase, type 1                          Sigma-Aldrich Ltd.
Coomassie brilliant blue G                  Sigma-Aldrich Ltd.
Cryo embedding medium                       Raymond A Lamb Ltd.
Cysteine                                    Sigma-Aldrich Ltd.

D
dATP                                         Invitrogen Ltd.
dCTP                                         Invitrogen Ltd.
dGTP                                         Invitrogen Ltd.
dTTP                                         Invitrogen Ltd.
Distilled water, RNase DNase free           Invitrogen Ltd.
Dithiothreitol                               Sigma-Aldrich Ltd.
DMEM                                         Lonza Ltd.
DNA ladder; 100 bp                           Invitrogen Ltd.
DNA ladder; 1 kb                             Invitrogen Ltd.
DNase I; amplification grade                 Invitrogen Ltd.
Donor horse serum                            Sera Labs Ltd.
DPX mounting medium                         Fisher Scientific Ltd.

E
96% EtOH                                     Fisher Scientific Ltd.
100% EtOH                                    Fisher Scientific Ltd.
ECL Western blotting detection system        Cheshire Biosciences Ltd.
EcoRI
Ethylene-diaminetetraacetic acid (EDTA)
Ethylene glycol tetraacetic acid (EGTA)
Ethidium bromide solution

**F**
Faramount® aqueous mounting medium
Foetal bovine serum (FBS)
Formaldehyde

**G**
Glycerol
Glycine for electrophoresis
GoTaq polymerase

**H**
Haematoxylin and eosin kit
HEPES
Hind III
Hybond P membrane
Hydrogen peroxide

**I**
Isopropyl-beta-D-thiogalactopyranoside (IPTG)
Isopentane
Isopropanol

**J**
JM109 competent cell tranfection kit

**L**
Laemelli sample buffer
LB broth

**M**
Magnesium chloride
Methanol
Methyl green
Mini elute gel extraction kit

New England Biolabs Ltd.
Sigma-Aldrich Ltd.
Sigma-Aldrich Ltd.
Sigma-Aldrich Ltd.
Dako Cytomation Ltd.
Lonza Ltd.
Fluka Biochemika
Sigma-Aldrich Ltd.
Sigma-Aldrich Ltd.
Promega Ltd.
Sigma-Aldrich Ltd.
Sigma-Aldrich Ltd.
Amersham Pharmacia Biotech
Sigma-Aldrich Ltd.
Invitrogen Ltd.
Sigma-Aldrich Ltd.
Fluka Biochemika
Promega Ltd.
Bio-Rad Laboratories Ltd.
Sigma-Aldrich Ltd.
BDH Chemicals Ltd.
Fisher Scientific Ltd.
Vector Laboratories Ltd.
Qiagen Ltd.
$N$

$N,N,N',N''$-tetramethylethylenediamine (TEMED)  Sigma-Aldrich Ltd.
Normal chicken serum  Vector Laboratories Ltd.
Normal goat serum  Vector Laboratories Ltd.
Normal horse serum  Vector Laboratories Ltd.
Normal rabbit serum  Vector Laboratories Ltd.
NP-40  Sigma-Aldrich Ltd.
Nuclease free water  Fisher Scientific Ltd.

$P$

pGEM-T easy vector kit  Promega Ltd.
Paraformaldehyde  Sigma-Aldrich Ltd.
Penicillin  Sigma-Aldrich Ltd / Lonza Ltd.
Phosphate buffered saline tablets  Sigma-Aldrich Ltd.
PCR master mix  Promega Ltd.
Phenol:chloroform:isoamyl alcohol (47.5:47.5:1)  Sigma-Aldrich Ltd.
Poly-L-lysine  Sigma-Aldrich Ltd.
Potassium chloride  Fisher Scientific Ltd.
Protease inhibitor cocktail tablet; complete mini  Roche Diagnostics Ltd.

$R$

Ready gel 10% tris-glycine precast gels  Bio-Rad Laboratories Ltd.
RNaseOut  Invitrogen Ltd.
RNeasy minikit (50)  Qiagen Ltd.

$S$

Sodium acetate  Sigma-Aldrich Ltd.
Sodium azide  Sigma-Aldrich Ltd.
Sodium borohydride  Fisher Scientific Ltd.
Sodium chloride  Sigma-Aldrich Ltd.
Sodium dodecyl Sulphate  Sigma-Aldrich Ltd.
Sodium fluoride  Sigma-Aldrich Ltd.
Sodium hydroxide  Sigma-Aldrich Ltd.
Sodium phosphate, dibasic  Sigma-Aldrich Ltd.
Sodium phosphate, monobasic  Sigma-Aldrich Ltd.
Sodium orthovanadate  Sigma-Aldrich Ltd.
Sucrose  Sigma-Aldrich Ltd.
SuperScript III reverse transcriptase  Invitrogen Ltd.
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2.2 Animals

All research animals were supplied by Portsmouth University Bioresources Centre, where all animal procedures were performed in accordance with the Local Ethical Review Committee agreements and UK Home Office Animals (Scientific Procedures) Act 1986.

2.2.1 Mice

Mice were bred and maintained under an environment conditioned at 19-23°C (with humidity at 45-65%) with 12 hr light/dark cycle. They were fed a standard pellet diet (economy rodent breeder diet, special diet services, Witham, UK) and tap water ad libitum. The animal beddings (Goldmix) provided were further enriched with shredded papers and sunflower seeds.

**C57BL10 & mdx strains:** mdx and C57BL10 mice were used, the latter as the normal background to the mdx genotype. All strains are tested routinely for main pathogens and all were healthy.

2.2.2 *In vivo* injections:

C57BL10 and mdx male mice were used in accordance with approvals of the institutional Ethical Review Board and the UK Home Office. Mice (3-9 per group) received 125mg/kg b.w. Coomassie Brilliant Blue G 250 (Sigma) intraperitonealy (i.p.), in sterile saline, according to two dosage regimes: daily from weeks 3-6 and every three days from weeks 3 to 14. Control mice received the same volume of saline solution. At 16 weeks mice were killed and
organs immediately flash frozen in isopentane cooled in liquid N₂. Injections were carried out by Prof. Gorecki, dissections and sample preservations were a combined effort between Prof. Gorecki and the candidate.

2.3 Cell Culture

2.3.1 Immortalised H2Kᵇ-tsA58 and H2Kᵇ-tsA58/mdx myoblast lines

The Immortalized H2Kᵇ-tsA58 and H2Kᵇ-tsA58/mdx myoblast cell lines were a gift from Prof. Hans Lochmüller (University of Newcastle). Culture media for both lines comprised Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% (v/v) foetal bovine serum (FBS), 2mM L-glutamine, 100 U/ml penicillin, 100μg/ml streptomycin and 20 unit/ml murine γ-interferon. Cells were grown in T25 flaks (Greiner) and passaged using 1X Trypsin (Lonza) when approaching 70-80% confluency. Incubation conditions consisted of a humidified atmosphere (95% air, 5% CO₂) at 33°C for myoblast proliferation, switching to 37°C, with removal of γ-interferon and substitution of 20% (v/v) FBS for 10% (v/v) horse serum (HS) to induce myoblast to myotube differentiation.

2.3.2 C2C12 Immortalized myoblasts

C2C12 mouse myoblast cells purchased from the European Collection of Cell Culture (ECCC; #91031101) and were cultured in a medium comprising DMEM supplemented with 20% (v/v) FBS, 2mM L-glutamine, 100 unit/ml
penicillin and 100ug/ml streptomycin. Incubation conditions consisted of a humidified atmosphere (95% air, 5% CO$_2$) at 33°C for myoblast proliferation, switching to 37°C and substituting 10% (v/v) FBS for 10% (v/v) HS to induce myoblast to myotube differentiation.

2.3.3 **C57BL10-/-mdx-derived primary myoblast cultures**

**First generation primary myoblasts:** Freshly dissected hind limb muscle groups [tibialis anterior (TA), gastrocnemius (GC), soleus (Sol), and flexor digitorum brevis (FDB)] from male 4 month old C57BL10 and mdx mice were used to establish primary myoblast cultures as described by (Rosenblatt et al., 1995a). Briefly, muscles were dissected tendon to tendon where possible with great care taken to avoid stretching or compression induced damage. Dissected muscles were washed in 5ml 1X Hank’s Buffered Saline Solution (HBSS) containing 2 drops of Betadine (100mg/ml povidone-iodine) to sterilise the tissues and remove bacterial contamination from the surface of the skin. Sterilised muscles were then incubated in 0.1% (w/v) type 1 collagenase in a shaking water bath at low speed for 45 mins. Muscles were then transferred to a 6 well plate (Greiner) containing 5ml plating medium (DMEM supplemented with 10% HS, 0.5% chick embryo extract (CEE) and 2mM L-glutamine) using a 25ml pipette. Individual fibres were then dissociated through gentle pipetting with reducing diameter pipettes (25, 10 and 1 ml). Isolated single living muscle fibres were selected under a bright field microscope (Leica) and transferred to 5ml of fresh plating medium three times to wash the fibres and remove contaminating cell types prior to plating. Single fibres (in 20ul plating media) were plated in 24 well plates (Greiner) pre-coated with 100ul 1mg/ml Matrigel and left for 5 mins to adhere before the addition of a further 1ml plating media. After 3-5 days, mono-nucleate cells could be seen migrating off the fibres. At
around 7 days, medium was changed to proliferation medium (DMEM supplemented with 20% FBS, 5% CEE and 2mM L-glutamine) and cultures were observed to proliferate.

**Second generation primary myoblasts:** Due to the tendency of the above protocol to induce rapid differentiation of isolated myoblast cultures, a second primary isolation protocol was developed. This 2\textsuperscript{nd} generation protocol used the same method of isolating living myofibres as described above, but differed in the method of mononuclear cell culture establishment. The 2\textsuperscript{nd} generation myoblasts were cultured using novel growth media [20% Knockout Serum Replacement (KRS; Invitrogen), 10% Donor Horse Serum, sterile filtered (DHS; Sera Labs), 2mM L-glutamine]. This media was used throughout, ‘plating’ media was completely omitted from the 2\textsuperscript{nd} generation procedure. KRS media was observed to greatly reduce the tendency of isolated myoblasts to differentiate; myogenic cells retained a more spheroidal appearance and proved easy to separate from any contaminating non-myogenic cells, which adhered strongly and adopted a more elongated constitution. In contrast to the 1\textsuperscript{st} generation procedure, myofibres isolated using the 2\textsuperscript{nd} generation procedure did not adhere to the Matrigel substratum. This effect was thought to be due to differences in media composition. Indeed, the lack of adherent fibres proved crucial for the isolation of pure myoblast cultures; clonal colonies of both myogenic and non-myogenic cells were obtained from cleanly isolated myofibres, which deposited single cells randomly across the Matrigel substratum if plates were gently agitated twice a day for 2-3 days. This facilitated the subsequent purification of myoblast colonies from colonies of contaminating cell types, which actually had a strongly differentiation inducing influence over proximal myoblast or mixed myoblast/contaminating cell colonies. After 2-3 days or once 5-6 colonies of cells were observed per 3.5cm plate, myofibres were removed. Myoblast colonies were purified and separated
from contaminating cell types using a modified pre-plating technique: media was removed and cultures were washed with 1ml 1X Cell Dissociation Solution (CDS; Sigma) for 1 min. CDS was removed and the plates were gently tapped 5 times in the horizontal plane to induce cellular detachment. 2ml of fresh media was added and the isolated cells re-plated in fresh Matrigel-coated plates (100ul 2mg/ml Matrigel / 3.5cm plate; Greiner). This pre-plating procedure was repeated 3 times over the first week of culture to ensure purely myogenic cultures had been derived. Using this technique, pure myogenic cultures could be reproducibly obtained, based on their ability to form 100% contractile myotubes.

2.3.4 Human embryonic kidney 293 (HEK) cells

HEK cells and HEK cells transfected with P2X7 were a gift from Dr. Friedrich Koch-Nolte, Hamburg, Germany. Cells were cultured in a medium comprising DMEM supplemented with 10% (v/v) FBS, 2mM L-glutamine, 100 unit/ml penicillin and 100ug/ml streptomycin in a humidified atmosphere (95% air, 5% CO₂) at 37°C.

2.4 Molecular Biology

2.4.1 Total RNA extraction

Total ribonucleic acids were extracted from cultured cells according to the manufacturer’s instructions using Trizol (Invitrogen). Briefly, 50-100mg of tissue was weighed and added frozen to 1ml Trizol and crushed completely
using 20 passes of a 10ml Potter-Elvehjem homogeniser (Fisher). Cellular
debris was removed through centrifugation at 5,900g for 10 mins at 4°C in 1.5ml
Eppendorf tubes. Supernatants were transferred to new tubes containing 200μl
Chloroform and shaken vigorously for 15s then incubated at RT for 2-3 mins.
Samples were centrifuged at 5,900g for 15 mins at 4°C to separate phases. The
upper aqueous phase containing RNA was decanted into a fresh Eppendorf tube
and RNA precipitated through addition of 0.5ml isopropanol followed by
several inversions and incubation at RT for 10 mins. RNA was pelleted through
centrifugation at 5,900g for 10 mins at 4°C. Pellets were washed in 1ml ice-cold
75% nuclease-free EtOH, re-pelleted through centrifugation at 3,300g for 5 mins
at 4°C, then air dried for 5 mins and re-suspended in 20-30μl nuclease-free
water.

2.4.2 Determination of RNA/DNA concentration

Small aliquots of total RNA/DNA samples were diluted 1:800 with
ddH₂O and their absorbances measured at wavelength of 260 nm and 280 nm in
quartz cuvettes (Sigma-Aldrich Ltd) using a BioMate™ 3 Series
spectrophotometer (Thermo Electron Corporation Ltd). Total RNA
concentration was determined after taking into account the absorbance at 260
nm, the dilution factor, as well as the A₂₆₀ value of 1 corresponding to 40 μg/ml
(i.e. 50 μg/ml for DNA; see below). Purity of RNA could be ascertained based
on the ratio of absorbances at 260 nm and 280 nm (i.e. A₂₆₀/A₂₈₀).
2.4.3 cDNA synthesis

Complementary DNA (cDNA) was synthesised from total RNA according to manufacturer’s instructions using Superscript III reverse transcriptase (Invitrogen). Briefly, 2µg of RNA was incubated for 25 mins at 25°C in a 10µl reaction buffer containing 200 mmol/l Tris-HCl (pH 8.4), 20 mmol/l MgCl₂, and 500 mmol/l KCl, in the presence of 1U amplification grade exoyriboonuclease 1 (DNase1) (Invitrogen) to eliminate any remaining genomic DNA. DNase1 was inactivated by heating samples to 65°C in the presence of 2.5mM EDTA to protect the RNA from Mg²⁺-dependent hydrolysis. Half of the resulting sample was reverse transcribed at 42°C using 200 ng of random hexamer primers, 400 units of Superscript III reverse transcriptase (Invitrogen) and 40 units of RNaseOut (Invitrogen) supplemented with 2 mM dNTP, 0.1 mM DTT, and 1X first strand buffer (Invitrogen) in a final reaction volume of 50 µl. The remaining half of the RNA sample was prepared as a negative control without the addition of reverse transcriptase, following the same protocol. Finally, enzymatic reactions were stopped by sample incubation at 70°C for 15 min. The cDNAs were either used directly for PCR amplifications or stored at -20°C.

2.4.4 Polymerase Chain Reaction (PCR)

Primers: The primer sequences were designed to span exon-intron borders (where possible) and were between 20-30 bp. The annealing temperatures were 55-65°C (Table 2.4). 1-3 µl of each cDNA sample were included in 25µl GoTaq MM (Promega) containing 200nM of specific primers, 1.5mM MgCl₂, 200 µM dNTPs, 1.25 units Taq polymerase and loading dye (Promega). Samples were amplified using a thermal cycler (either Primus 25
Advanced® or Primus 96 Plus®; PEQLAB Biotechnologie GmbH, Erlangen, Germany) with PCR profile: 94°C for 2 mins, followed by 35 cycles of 94°C for 40sec, appropriate annealing temperature for 40sec, and 72°C for 60sec/kb of product size, with a final extension step of 72°C for 10min. In each case, the annealing temperature adopted was usually 1-3°C below the lowest melting temperature of the primer pair. The PCR products (10 µl) were resolved on agarose gels and the rest stored at -20°C. The size of PCR amplicons was compared against size markers and their specificity confirmed either by sequencing or following restriction enzyme digestion.

Control primers, like glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used to ensure equivalent amounts of first strands being used.

**Quantitative PCR (qPCR), and semi-quantitative ‘manual’ qPCR (mqPCR):** Quantitative (Taq man qPCR) and semi-quantitative (mqPCR) analyses were performed using an ABI Prism 7700 sequence detection system (PerkinElmer Life Sciences) with Taqman probes. Relative expression was calculated as a ratio of P2X7 to GAPDH. Primers (Mm01199501_m1 to mouse P2X7 exon 3-4 and Mm00440582_m1, exons 5-6; mouse GAPDH endogenous control; Applied Biosystems) were used according to manufacturer’s instructions. Semi-quantitative experiments to compare relative levels of P2X7(a) and P2X7(k) transcripts were performed by removing 5µl aliquots of PCR reactions at cycle # 14,17,20,23 and 26 for GAPDH, and 26, 29, 32, 35 and 40 for P2X7. PCR products were resolved by agarose gel electrophoresis and imaged following 2s UV exposure using CHEMI GENIUS2 BIO imaging system (Syngene). Bands representing PCR products were quantified using the integrated pixel density measurement function of ImageJ software. Integrated densities were plotted against cycle number to generate PCR profiles using non-linear curve fitting (Origin 7.0). C_t values for GAPDH and P2X7 were derived.
from a standardized base-line and relative expression calculated as above. All experiments were repeated at least 3 times and performed in triplicate.

2.4.5 Agarose gel electrophoresis

PCR products could be loaded directly into wells formed in 2% agarose gels containing 0.001% (v/v) ethidium bromide (EtBr) along DNA ladders (100 bp or 1kb, Invitrogen Ltd.) used for size reference. These samples were resolved by electrophoresis (5 V/cm) for ~30-50 min or until required separation of bands occurred. For testing Total RNA quality, 0.2-0.5μl was loaded into 1% (w/v) agarose gels containing 0.001% (v/v) EtBr with 1ul 10% (v/v) glycerol replacing the loading dye. Images of fully resolved agarose gels were captured using the CHEMI Genius² Bio imaging System (Syngene Ltd).

2.4.6 Restriction digests

Specific restriction enzymes for digesting the DNA fragment of interest were identified using the freeware, Sequence Manipulation Suite (SMS, Version 2.0), available from the website of Bioinformatics Organisation Inc (Massachusetts, USA). The chosen restriction enzymes (preferably single or double cutters) were added at concentrations of 2-5 units into an appropriate 1x buffer or directly into the PCR product (if a particular enzyme was active in the 1x PCR buffer). Otherwise, the PCR products were first purified using the MinElute PCR purification kit according to manufacturer’s instructions. Alternatively, the specific PCR product band in agarose gel slices was extracted with the MinElute gel extraction kit (50) Qiagen. A 0.5 μl from these purified samples was used for restriction enzyme digestion at 37°C for 2 hr.
Subsequently, the enzymes were inactivated at 70°C for 10 min before resolving the digested samples on 2% (w/v) agarose gels or using in cloning or other downstream applications.

2.4.7 TA cloning of PCR products

Fresh PCR products (3 μl) were ligated into 50 ng of pGEM®-T Easy Vector (Promega) in a reaction buffer containing 1X rapid ligation buffer, and 3 units of T4 DNA Ligase (Promega). The ligation process was performed either at room temperature for 2 hr or at 4°C overnight. PCR products kept for more than 3 days at -20°C need dA-overhangs refreshed prior to ligation. For this, 3 μl of PCR product were incubated at 70°C for 30 min in a 10 μl reaction buffer containing 1X PCR buffer, 5 units of Taq DNA Polymerase (Qiagen), and 0.2 mM dATP (Invitrogen).

Transformation of Ligated Vector into JM109 Competent Cells: Ligation reaction mixtures (10 μl) were introduced into 200 μl of JM109 competent cells (Promega), on ice. JM109 competent cell/ligation mixtures were heat-shocked at 42°C for 45-50 sec in 14ml polypropylene round bottom tubes (Falcon #352059) and returned immediately to ice for a further 2 min. Transformed JM109 competent cells were mixed with 500 μl of SOC medium (Promega) pre-equilibrated to room temperature. These mixtures were left in a shaking incubator (37°C, 150rpm) for 1.5 hr. 100 μl of transformed JM109 were spread (under aseptic conditions) onto LB agar plates supplemented with 50 μg/ml ampicillin, 0.5 mM IPTG, and 80 μg/ml X-Gal. JM109 competent cells from the remaining SOC mix were pelleted at 800g for 10 min. The resulting transformed cell pellets were re-suspended in 200μl of fresh SOC medium, of which 100μl was spread on LB agar plates, thereby creating a concentrated
version of the above. Inoculated LB agar plates were incubated upside-down at 37°C overnight.

**Blue/White Recombinants Colony Screening:** a few blue and white colonies on LB agar could be expected after an overnight incubation at 37°C. Those positive ones (white colonies; preferably surrounded by some blue) were picked up with the aid of sterile toothpicks, which were then briefly dipped in prepared PCR reaction mixtures (containing gene-specific primer pairs), and then dropped into 3 ml LB broth with added 50 μg/ml ampicillin. The inoculated LB broths were shaken (37°C, 220rpm) for 6-8 hr at 37°C and subsequently centrifuged at 2,300g for 5 min. Plasmid DNA were later extracted from the pelleted bacterial cells. In the meantime, the PCR amplification results would have indicated those colonies, which were likely to contain the plasmid clones of interest.

**Extraction of Plasmid DNA:** Plasmid DNA was extracted using the QIAprep® Miniprep Kit (Qiagen Ltd.), according to the manufacturer’s instructions. Briefly, pelleted bacterial cells were re-suspended in 250μl of buffer P1 (with the RNase A provided). Cells were lysed following the addition of 250μl of buffer P2 with gentle mixing. Unwanted cell debris were immediately precipitated by adding 350μl of buffer N3 and pelleted at 15,600g for 10 min. The resulting supernatants were transferred onto the QIAprep Spin Column (Qiagen) and passed twice through the silica-gel membrane within by centrifuging at 15,600g for 1 min. Plasmid DNA trapped on the membrane was washed with 750μl of buffer PE drawn through the membrane bycentrifugation at 15,600g for 1 min. A further 2 min centrifugation was used to remove any residual ethanol present in buffer PE. Purified plasmid DNA was eluted from the silica-gel membrane into fresh micro-centrifuge tube by soaking the membrane with 30μl of nuclease free water or TE buffer followed by centrifugation at 15,600g for 1 min. This step was repeated again for increased
DNA yield. A small aliquot of extracted plasmid DNA was digested with appropriate restriction enzymes to confirm that expected clones were obtained. 5μl of the plasmid DNA was sent for commercial DNA sequencing (Lark).

2.4.8 Protein extraction

**Adherent cell cultures:** Proteins were extracted on ice by scraping cells (Cell Scraper, Greiner) in a minimal volume of extraction buffer composed of: 1x LysisM extraction buffer (Cat. #04719956001, Roche), 1x protease inhibitor cocktail tablet (Roche) per 10ml LysisM, 2x phosphatase inhibitor cocktail tablets (Roche) / 10ml LysisM, 2mM sodium orthovanadate. Samples were homogenised by passing through a 23g needle 20 times, then stored at -20°C.

**Tissues:** Proteins were extracted from frozen tissues by crushing a known weight of sample into a fine powder in liquid nitrogen using a mortar and pestle (pre-chilled on dry ice). The tissue powders were transferred into a 15 ml centrifuge tube (Fisher), any excess liquid nitrogen allowed to evaporate on ice. A minimal volume of extraction buffer was added (composition as above) and samples were further homogenised by passing through a 23g needle 20 times, then stored at -20°C.

2.4.9 Protein concentration assay

Protein concentrations of extracted samples were determined using the bicinchoninic acid (BCA) method (Sigma-Aldrich Ltd.). 1-5ul of protein samples were used for each triplicate measurement. Protein standards comprised of bovine serum albumin (BSA) (Sigma-Aldrich Ltd.) in the range 0-10ug. Standards and samples were added to a 96 well plate (Greiner), individually
mixed with 200µl of BCA working reagent (prepared by mixing 50 parts of BCA solution and 1 part of 4% (w/v) CuSO₄·H₂O). The resultant mixtures were incubated at 55°C for 15 min to allow blue colouration to develop. Absorbance was then measured at 550 nm using a plate reader (Antos 2001). Protein concentrations (µg/µl) were obtained by deriving the equation of line of best fit from average standard values according to y=mx+c.

2.4.10 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Gel casting: Mini-PROTEAN 3 casting frame, casting stand, and gel cassette (Bio-Rad Laboratories Ltd.) were assembled according to manufacturer’s instructions. Resolving gels were of composition 6-12% (v/v) acrylamide/bisacrylamide, 0.375 M Tris-HCl (pH 8.8), 0.1% (v/v) sodium dodecyl sulphate (SDS), 0.05% (v/v) ammonium persulfate (APS), and 0.01% (v/v) tetramethylethylenediamine (TEMED). Gels were poured, over-laid with water and set for ~30 mins, before the water was removed and stacking gels of composition 4% (v/v) acrylamide/bisacrylamide, 0.125 M Tris-HCl (pH 6.8), 0.1% (v/v) SDS, 0.05% (v/v) ammonium persulfate (APS), and 0.01% TEMED (v/v) were poured on top of the resolving gel. Combs (Bio-Rad Laboratories Ltd.) were inserted and gels set for ~5 mins. Gels were usually used immediately but sometimes stored for up to 5 days at 4°C wrapped in wet paper towels and Clingfilm.

Sample preparation for electrophoresis: Protein samples were mixed with Laemmli sample buffer (62.5 mM Tris-HCl, 2% (v/v) SDS, 25% (v/v) glycerol, and 0.01% (w/v) bromophenol blue) at 1:1 protein/buffer ratio. Occasionally, a 2:1 protein/buffer ratio was adopted to further maximise the amount of proteins being loaded in a well. Subsequently, these mixtures were supplemented with
2.5% (v/v) β-mercaptoethanol and boiled for 5 min at 95°C. Denatured protein samples were briefly chilled on ice before gel loading.

**Electrophoretic separation of proteins:** Mini-PROTEAN 3 Gel Cassette and electrophoresis module (Bio-Rad Laboratories Ltd.) was assembled according to the manufacturer’s instructions. 20-40μl of each denatured protein sample were loaded and resolved at 150-200V, 100mA in 6-12% (w/v) SDS-polyacrylamide gels for ~120 min in electrophoresis buffer (25 mM Tris, 192 mM glycine, and 0.1% (w/v) SDS, pH=8.3). 5-10μl of prestained molecular weight markers (Lonza Ltd.) was resolved alongside the protein samples serving as a reference for approximate protein size determination. Resolved protein gels were either transferred to polyvinylidene fluoride (PVDF) membranes (Amersham Biosciences Ltd) or stained with coomassie brilliant blue G (CBB) solution (Sigma-Aldrich Ltd.) to assess protein sample quality / amount / quality of gel running.

**Coomassie Brilliant Blue G staining of gels:** Resolved protein gels were submerged in CBB solution [0.1% (w/v) CBBG, 25% (v/v) methanol, and 5% (v/v) glacial acetic acid] for 30 min. The stained protein gels were washed three times in de-stain solution (7.5% (v/v) methanol and 10% (v/v) glacial acetic acid) for 15 min. With the final change of fresh destain solution, the protein gels were left to destain overnight covered with a piece of paper towel to soak up the released dye.

**2.4.11 Electrophoretic protein transfer**

**Trans blot assembly:** Mini trans-blot cell and gel holder cassettes (Bio-Rad Laboratories Ltd.) were assembled according to the manufacturer’s instructions. PAGE protein gels were placed onto PVDF (Hybond P, Amersham Bioscience Ltd) membrane pieces cut to the same size as the gel. PVDF
membrane, was pre-treated in absolute methanol for 5 sec, then washed in 1x Transfer buffer (25 mM Tris, 192 mM glycine, and 20% (v/v) methanol; pH 8.3) for 5 min before use. Both gel and membrane were then sandwiched between buffer-soaked filter papers, fiber pads, and gel holder cassettes. Proteins were transferred onto the blotting membrane through electrophoretic transfer (conditions: 100 V; 350 mA) for 2 hrs at 4°C using pre-chilled transfer buffer. Following electrophoretic transfer, blots were trimmed to size and submerged in blocking buffer [5% (w/v) non-fat milk powder in 1x PBS and 0.01% (v/v) Tween-20 (Sigma-Aldrich Ltd.)] for 1 hour prior to probing with antibodies.

2.4.12 Antibody immunoblotting

Blots were probed with relevant primary antibody diluted at the optimal concentration in the same blocking buffer overnight at 4°C or at RT for 2 hrs. Blots were then washed three times with 1x PBST for 10 min, and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences Ltd.) for 45 min. Following a further three, 10 min washes in 1x PBST, specific protein bands were visualized using luminol-based substrates (Uptilight/Uptilight US, Cheshire Sciences). Images were obtained using a G:BOX Chemi XT-16 system (Syngene). Used blots could be stripped by incubating in stripping buffer [50 mM Tris-HCl (pH 6.8), 2% (v/v) SDS, and 2 mM β-mercaptoethanol] for 10 min at 50°C, followed by 3 washes with 1x PBST for 5 min. Blots could then be re-probed with another antibody. The stripped blots could otherwise be sandwiched between filter paper sheets to store dry for later use.
2.4.13 Antibodies:

Primary antibodies, their sources and dilutions used are summarised in Table 2.3.

**Actin:** Anti-Actin (Sigma-Aldrich Ltd. Cat #A2066) is an affinity purified rabbit polyclonal antibody that has been designed to recognise the carboxyl terminal portion of almost all (α, β, and γ) actin isoforms. It recognises a 42 KDa actin band in different animal tissue extracts using WB (diluted 1:1000), this antibody was used for assessing the qualities and quantities of protein loadings.

**Desmin:** Anti-desmin clone D33 (Dako Ltd. Cat. #MO760) mouse monoclonal antibody labels the intermediate fiolament protein desmin in smooth and skeletal muscle cells. Used at 1:200 for immunocytochemistry.

**Dystrophin:** Anti-dystrophin 2166 (Gift from Prof Derek J Blake, Cardiff) is affinity purified goat polyclonal antibody which recognises a specific N-terminal sequence of the full length muscle-type dystrophin isoform (Dp427). The antibody was used at 1:400 dilution for immunohistochemistry. Anti-dystrophin 7A10 (Developmental Systems Hybridoma Bank, University of Iowa) is mouse monoclonal antibody recognising a specific C-terminal sequence of dytrophin present in all known dystrophin isoforms. The antibody was used at 1:100 for WB.

**ERK1/2 and phospho-ERK1/2:** Anti-ERK1/2 (Cell Signalling Cat. #9102) is a rabbit polyclonal and anti phospho-ERK1/2 (Cell Signalling Cat. #9106) is a mouse monoclonal antibody. These were used in WB (diluted 1:2000 and 1:1000 respectively) and recognised ERK bands of 44 and 42KDa. Phosporylated forms are undistinguishable by size and so antibodies were used separately to probe the same protein extracts.
**F4/80:** Anti-F4/80 pan-macrophage marker (Abcam Cat. #74383) is rabbit polyclonal antibody. In WB (diluted 1:500) this antibody recognises a specific band of ~130KDa plus an additional band of 30KDa of an unknown origin.

**P2X7:** Anti-P2X7 (Cell Signalling Ltd. Cat # 177003) is affinity purified rabbit polyclonal antibody raised against the C-terminal portion of the wild type variant. In WB (diluted 1:500) it recognises a specific 80KDa band and a possible un-glycosylated lower molecular weight variant of ~70KDa.

**Biotin-conjugated secondary antibodies:** Both biotinylated rabbit anti-goat IgG (Cat #BA-5000) and goat anti-rabbit IgG (Cat #BA-1000) were used at 1:200 dilutions. For IHC applications these secondary antibodies were used in conjunction with either VectaStain Elite ABC kit (peroxidase-based; Cat #PK-6100) or VectaStain ABC-AP kit (alkaline phosphatase-based; Cat #AK-5000), according to manufacturer’s instructions. Peroxidase (DAB or Vector VIP) and alkaline phosphatase (Vector Blue) substrates were applied to visualise the stains. For IF applications, Fluorescein avidin D (Ex/Em: 495/515 nm; Cat #A-2001) or Texas red avidin D (Ex/Em: 595/615 nm; Cat #A-2006) were used with those biotin-conjugated secondary antibodies (All from Vector Laboratories Ltd.).

**Fluorophore-conjugated secondary antibodies:** All fluorophore-conjugated secondary antibodies were obtained from Molecular Probes Inc., Eugene, OR. These included Alexa Fluor 488 goat anti-rabbit IgG (Ex/Em: 495/519 nm; Cat #A-11008), Alexa Fluor 488 chicken anti-mouse IgG (Cat #A-21441), and Alexa Fluor 546 donkey anti-goat IgG (Ex/Em: 556/573 nm; Cat #A-11056). These were used in IF at 1:200 dilutions for all applications including on cultured cells or tissues sections.

**Horseradish peroxidase-conjugated secondary antibodies:** Affinity purified horseradish peroxidase-labelled antibodies, goat anti-rabbit IgG (Cat #A0545)
and rabbit anti-goat IgG (Cat #A5420), were bought from Sigma-Aldrich Ltd., Dorset, UK. These secondary antibodies were used for WB at 1:10,000 dilutions.

2.4.14 Tissue processing techniques

Dissection of mouse tissues: Mice (4 months old) were killed by schedule 1 method and their tissues carefully dissected out. Excised tissues were either flash-frozen for proteins extraction by dropping into liquid nitrogen in sealed Eppendorfs for storage at -70°C or processed for cryo-sectioning. Processing tissues for cryo-sectioning: Freshly isolated organs were orientated and embedded in a dab of cryo-embed (R.A. Lamb Ltd.) on a 20mm diameter cork disc (R.A. Lamb Ltd.) prior to being inverted and submerged in a beaker of isopentane chilled in liquid nitrogen. Mouse brains were embedded in cryo-glue and left to slow-freeze on a flat metal surface placed on dry ice. Frozen tissues were then stored at -70°C until needed for cryo-sectioning. Samples on cork disks were transferred into a cryostat chamber (Bright OTF Cryostat; Raymond A Lamb Ltd. Cat No. E7.15/OTF). These were left to equilibrate at -20°C for ~1 hr before cryo-sectioning. Tissue samples were mounted and 10 μm thick sections cut. Sections were collected onto poly-L-lysine-coated slides, air-dried and stored at −70°C.

Fixation of tissue samples: 4% (w/v) paraformaldehyde in 1x PBST was the preferred fixative used for post-fixation of tissue sections. Slides with tissue sections were incubated with the fixative for 15 min on ice.
2.4.15 Immunolocalization

In order to aid antibody specificity high salt concentration (2.5% (w/v) sodium chloride) was employed during dystrophin antibody incubations, which had the effect of reducing non-specific background staining from the secondary antibody. Negative controls, performed using secondary antibody without primary antibody were utilised to assess the presence of any non-specific background.

**Immunocytochemistry:** Cells were fixed in either chilled absolute methanol or 4% (w/v) paraformaldehyde (PFA) in 1x PBS for 15 min at 4°C. The latter required the cells to be permeabilised, hence, 0.5% (v/v) Triton X-100 was added to the appropriate 10% (v/v) normal serum in 1x PBS, and the mixture was subsequently applied to the cells for 30 min. Further blocking with avidin and biotin blocking solutions (Vector Laboratories Ltd.), 15 min each, was necessary for colorimetric staining protocols.

Colorimetric immunocytochemistry: The cells were washed with 1x PBS for 5 min after every incubation step, then incubated overnight at 4°C or at RT for 2 hrs with the specific primary antibody (diluted using 10% (v/v) normal serum in 1x PBS). Cells were washed for 5 min each with 1x PBS prior to incubation with the respective biotinylated secondary antibody (diluted at 1:200 with 2% (v/v) normal serum in 1x PBS) for 45 min. The cells were visualised following incubation with Vectastain® ABC-AP kit (Vector Laboratories Ltd.) for 30 min, followed by its respective alkaline phosphatase substrate (Vector® Blue substrate kit; Vector Laboratories) until the desired colouration was achieved. A brief 5 min counterstaining in methyl green (Vector) was also used. Finally, the cells were coverslipped using Faramount® aqueous mounting medium (DakoCytomation).

Immunofluorescence: The cells were washed with 1x PBS for 5 min after every incubation step, then incubated overnight at 4°C or at RT for 2 hrs with the
specific primary antibody (diluted using 10% (v/v) normal serum in 1x PBS). Cells were washed three times for 5 min each with 1x PBS prior to incubation with the respective Alexa Fluor (Invitrogen) 488-conjugated chicken anti-rabbit IgG (1:200), 488-conjugated goat anti-rabbit IgG (1:200), or Alexa 546-conjugated donkey anti-goat IgG (1:200), plus Hoechst (1:1000) as a nuclear counterstain. Sections were mounted in non-fluorescing Vectashield® mounting medium (Vector Laboratories Ltd). Labelling was imaged using an Axioplan 2 imaging MOT microscope (Carl Zeiss, Jena, Germany) and specific sections were further analysed using the confocal laser scanning module, LSM 510 META (Carl Zeiss).

**Immunohistochemistry:** Tissue sections obtained by cryosectioning were post-fixed with cold 4% (w/v) PFA in 1x PBS for 15 min. Sections were treated with 0.3% (v/v) hydrogen peroxide in methanol for 30 minutes to eliminate any endogenous peroxidase activity, then blocked in appropriate 10% (v/v) normal serum in 1X PBS for 30 min and subsequently with avidin/biotin blocking kit (Vector Laboratories Ltd.) for 15 min per step if colorimetric staining protocols were used. Brief 5 min washes in 1x PBS were incorporated between each step. Next, tissue sections were incubated overnight at 4°C or at RT for 2 hrs with the specific primary antibody at optimised dilution prepared using the same blocking solution as above. Appropriate biotinylated secondary antibody (Vector) diluted at 1:200 with 2% (v/v) normal serum in 1x PBST, and subsequently Vectastain ABC reagent (Vectastain® Elite ABC Kit; Vector Laboratories Ltd.) were applied (30 min each). Sections were rinsed three times for 5 min each in PBS after each incubation step. Staining was visualised by incubating the sections in a peroxidase substrate (Vector® VIP or DAB substrate kits, Vector Laboratories Ltd.) until the desired colour intensity was reached. Sections were then counterstained with methyl green (Vector), dehydrated in ascending ethanol series (50-100%) into xylene, and mounted in dipthyline.
xylene (DPX). For immunofluorescence staining, biotinylated antibodies were exchanged for Alexa Flour (Invitrogen Ltd) 488-conjugated chicken anti-rabbit IgG (1:200), 488-conjugated goat anti-rabbit IgG (1:200), or Alexa 546-conjugated donkey anti-goat IgG (1:200), plus Hoechst (1:1000, Sigma) nuclear counterstain. Sections were mounted in non-fluorescing Vectashield® mounting medium (Vector). Labelling was imaged using an Axioplan 2 imaging MOT microscope (Zeiss) and specific sections were further analysed using the confocal laser scanning module, LSM 510 META (Zeiss).

2.4.16 Histological staining

**Haematoxylin and eosin staining:** Tissue sections were fixed on ice in cold 4% PFA for 15 min, and then incubated in haematoxylin solution (Sigma) for 1 min. Acid alcohol (1% (v/v) HCl in 70% ethanol) was used for the differentiation of tissue sections before finally counterstaining them with the eosin solution (Sigma) for a further 1 min. The sections were dehydrated through ascending ethanol series (50-100%), cleared in xylene and mounted in DPX under coverslips.

**Immunolocalisation of the revertant fibres:** Two 10 µm thick muscle cryostat sections were taken from approximately equal isobaric locations (one third and two thirds longitudinal distance from ankle to knee) in each muscle examined. Sections were dried onto poly-L-lysine coated glass microscope slides (Menzel Gläser), fixed with ice-cold 4% paraformaldehyde in PBS for 10 min and blocked in 10% v/v normal goat serum (NGS) in PBST (0.01% v/v Tween-20) for 30 min at RT. After blocking, the sections were incubated with the dystrophin antibody in PBST containing 2% v/v NGS, washed extensively in PBS and visualised either via immunofluorescent detection with Alexa Fluor 488-labelled secondary antibody and confocal analysis performed using an LSM
710 microscope (Zeiss) or via the histochemical detection using Vectastain® ABC-AP kit (Vector). In the latter method, secondary antibody incubation for 30 min was followed by its respective alkaline phosphatase substrate (Vector® Blue substrate kit; Vector), until the desired colouration was achieved. Centrally nucleated fibres were visualised by DAPI and methyl green staining for confocal and histochemical staining, respectively. For negative controls the primary antibody was omitted in the staining procedure. Labelled muscle sections were imaged using LSM 710 (Zeiss) with HD colour camera, then assembled as macro-images (Corel PHOTO-PAINT X3), which were used for binary label based counting (Origin 7.0, cell-counter plug-in). Data was presented as number of centrally nucleated / revertant fibers as a percentage of total fiber number per stained cross sectional area.

2.4.17 ATP₆ Hydrolysis assay - Measurement of inorganic phosphate (Pi) release

The following protocols are based on the colorimetric shift of phosphomolybdate complexes upon their reduction in acidic solutions. Two such methods were tested here, using different reducing agents, which included Malachite green hydrochloride and L-ascorbic acid. The latter was rejected in this instance; although this assay proved more sensitive over a greater linear range than the Malachite green-based method, a greater dependence upon the correct emission filter was encountered when using the L-ascorbic acid method. In this instance, the only available suitable emission filter was 600nm. With both methodologies trialled here requiring a 655nm emission filter, the flexibility of this parameter proved the deciding variable in assay selection, which led to the adoption of the Malachite green-based Pi detection assay. Should the correct 655nm emission filter be available in microtiter plate format,
the L-ascorbic acid method would be considered a more accurate and adaptable assay for small volume, low concentration Pi detection.

**Malachite Green-based assay:** The extracellular ATP-hydrolysing potentials of C2C12, IMO and SC5 cell cultures were assessed using a modified version of the Malachite green method (Lanzetta et al., 1979). Myoblasts were cultured in 12-well plates as described above; cells were seeded at 50% confluency and experiments conducted on the second day of culture, when cells were 70-80% confluent, or where myotubes were used, after a further 4 days in differentiation medium. Cells were washed twice with 500µl of phosphate-free buffer (140mM NaCl, 20mM Hepes, 4mM MgCl₂, 2mM CaCl₂, pH7.8) then incubated in 500µl of the same buffer containing 0–4mM ATP. Aliquots of the incubation medium (20µl for myotube, 50µl for myoblast cultures) were removed at time T=0 and T=30 mins following treatment with ATP for inorganic phosphate (Pi) detection. Samples were transferred to 150ul solution A (Two parts 0.045% w/v Malachite green and 0.002% v/v Triton-N101 in ddH₂O to 1 part 4.2% w/v ammonium molybdate in 4M HCl), which was mixed by rotation for 1 hour and filtered to remove debris prior to use (11µM, Whatman Grade 1). After 1 min, 50µl 2% sodium citrate was added to halt further colour development. Solutions were incubated for a further 15mins at RT, allowing for colour stabilisation prior to reading of absorbance values at 600nm using a POLARstar Optima microplate reader (BMG Labtech). Triton-N101 replaced Sterox (Lanzetta et al., 1979) as the Pi solubilising agent here due to the lack of commercially available Sterox. Triton-N101 proved to contain the lowest level of Pi of the laboratory grade detergents tested, e.g. NP-40, SDS, CHAPS, Triton-X-100, most of which contained sufficiently high levels of Pi to quench any ATP derived Pi. Phosphate standards (0-500µM KH₂PO₄ in ddH₂O) were prepared fresh and assayed alongside treated samples. Extracellular ATP hydrolysis was calculated by subtracting Pi at t = 0 from Pi at t = 30 min and
values were normalised against the protein content of each preparation (BCA protein assay, Sigma – see 2.4.9). Extracellular ATP hydrolysing potentials were compared by determining $K_M$ and $V_{max}$ values were calculated from the fitting of saturation curves to Pi release data in response to 0-4mM ATP using a one-site curve fitting function (Microcal Origin7.03). Differences between $K_M$ or $V_{max}$ values were tested for significance by two-way ANOVA and Tukey’s post hoc test.

**L-ascorbic acid-based assay:** The culturing, preparation and treatment of cultured cells did not differ between Malachite green- and L-ascorbic acid-based protocols – these protocols differed only in the reducing agent used for Pi detection. See the above Malachite green-based assay methodology for detailed protocol relating to the culturing of cells for this assay. As with the above protocol, 50ul aliquots of incubation media were removed at time $T=0$ and $T=30$mins following treatment with ATP for inorganic phosphate (Pi) detection. Aliquots were transferred to 150ul solution A (Two parts 12% w/v L-ascorbic acid in 1M HCl to one part 2% w/v ammonium molybdate tetrahydrate in ddH$_2$O – this solution was prepared fresh and not kept longer than 1 hour at RT). Following 5min incubation at RT, 100ul solution B (2% sodium tribasic dehydrate and 2% acetic acid in ddH$_2$O) was added to prevent further colour development. Solutions were incubated for 15mins at RT to allow for colour stabilisation prior to reading of absorbance values at 600nm using a POLARstar Optima microplate reader (BMG Labtech). Kinetic parameters were determined as per Malachite Green-based assay protocol above.
2.4.18 ERK1/2 phosphorylation assay

Cells were seeded at 50% confluency in 6 well collagen (Sigma) coated plates (Greiner) in 2ml normal growth medium and left to proliferate overnight. 24 hours later, cells were washed three times in DPBS and growth medium was replaced with low serum medium (0.5% v/v serum). Cells were incubated in this medium for a further 12 hours prior to treatment. Agonists and antagonists were applied for the stated time periods at the following concentrations: ATP, 500uM, BzATP, 300uM, Coomassie Brilliant Blue G (CBBG), 1uM, ivermectin (IVM), 0.25uM. Where used, antagonists were applied for a 10 min pre-incubation prior to agonist addition. Following treatment for the indicated time periods, cells were lysed as described above and extracted proteins used for immuno-blot analysis of ERK1/2 phosphorylation status by probing with specific anti-p44/42 and anti-phosphop44/42 antibodies (see below).

2.4.19 PNGase F deglycosylation

Proteins were deglycosylated using PNGaseF (New England Biolabs) according to the manufacturers’ instructions. Briefly, 100μg of total protein lysate was denatured in denaturing buffer (5% v/v SDS, 0.4M DTT) for 10 mins at 100°C, then samples were deglycosylated in reaction buffer containing 2500 U PNGaseF, 0.5M sodium phosphate, 10% NP-40, for 1 hour at 37°C. 15μg of digested samples were used for immune-blotting with anti-P2X7 antibody as previously described. In some cases the heat step was omitted.
2.5 Proteomics

2.5.1 Phosphoprotein purification

5 mg of total protein extract was required for each sample to be enriched for phosphoproteins. Cells were cultured as described in 500cm² plates and proteins extracted by scraping in 5ml of phospho-protein lysis buffer (25mM 2-morpholinoethanesulfonic acid (MES), 1mM NaCl, 0.25% w/v CHAPS, 1x protease inhibitor tablet, 1x phosphatase inhibitor tablet, 250U benzonase nuclease, pH, 6.0). Samples were then homogenised by 20 passes through a 23g needle. Phosphoprotein purifications were carried out using PhosphoProtein Purification Kit (6) (Qiagen) according to manufacturer’s instructions. Briefly, extracted protein was left on ice for 30 min, and then centrifuged at 10,000g at 4°C for 30 min, during which time phosphoprotein purification columns were prepared to receive samples by allowing the storage buffer to flow out. Sample supernatants were harvested and the protein concentration determined by BCA method, as described previously. A volume of lysate containing 2.5mg of total protein was diluted to 0.1mg/ml with phosphoprotein lysis buffer containing 0.25% w/v CHAPS. Final volumes were between 30-40ml per sample. Lysates were applied to binding columns at RT. Columns with bound samples were washed twice in 3ml phosphoprotein lysis buffer containing 0.25% w/v CHAPS and samples eluted into Amicon ultra centrifugal filters (Ultracel-3K, Fisher) in 4ml phosphoprotein elution buffer (50mM potassium phosphate, 50mM NaCl, pH 7.5). Samples were centrifugally filtered at 3,100g until volumes had been reduced to 200µl, and then stored at -80°C.
2.5.2 Mass spectrometry

Mass spectrometry was carried out using phospho-protein enriched samples in collaboration with Dr. Philippe Chan (University of Rouen, France). Samples were de-salted using maxi dialysis tubes (3ml, 3.5KDa, Generon) floating in cold ddH$_2$O for 2 hours, then again in fresh solution overnight. Sample pH was reduced to $\sim$pH 6.0 by adding triethylammonium (Sigma) until Litumus test was satisfactory. Samples were then denatured in 5mM tris(2-carboxyethyl)phosphine (TCEP) in triethylammonium at 60°C for 1 hour, then cystein residues were blocked in 55mM iodoacetamide at RT for 30 mins. Trypsin (V5111, Promega) digestion of samples was carried out using 1μg trypsin per 1μg protein at 30°C for 15 mins. Samples were labelled with iTRAQ labels from 114-117 (Applied Biosystems) dissolved in EtOH for 1 hour at RT. Labelled samples were subjected to isoelectric focussing in a 24 well 12.5% w/v poly-acrylamide gel strip (Agilent Technologies) placed in an OFFGEL fractionation system (Agilent 3100 Fractionator, Agilent Technologies) for ~48 hrs. Separated samples were analysed in an ‘ESI-LC-MS’ (Agilent 6200 TOF, Agilent Technologies) and phosphopeptide fragments identified by interrogating the ‘Mascot’ database (Matrix Science) using ‘Spectromill’ (Agilent Technologies). Fold changes in phosphoprotein levels between treated, normal and dystrophic myoblasts were calculated using the ‘iQuantitator’ package (2009) in the ‘R’ statistical environment (R Development Core Team, 2010).
2.6 Statistical analysis

Statistical significance of data was assessed using one- or two-way analysis of variance (ANOVA) with post-hoc Tukey’s test (Origin 7.0) or Bonferonni test (Prism 4) respectively. P<0.05 was considered statistically significant, data are reported as means ± SE, where n=3-5 for pharmacological assays, plus PCR and Western data, and n=3-9 for histological analyses of muscles. ‘Degrees of freedom’ (df) is used throughout in place of ‘n’, where df=n-1.
<table>
<thead>
<tr>
<th>Buffer/Stain/Solution</th>
<th>Composition</th>
</tr>
</thead>
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<tr>
<td>Acid Alcohol</td>
<td>1% (v/v) HCl in 70% ethanol</td>
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<tr>
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<td>5% Low fat dried milk powdered</td>
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**Primers used as controls**

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3

Characterisation of normal and dystrophic myoblast cell lines

3.1 Introduction

Homeostasis of atrophic/hypertrophic processes is central to the maintenance of correct mass and functioning in muscle, illustrated by the ability of adult skeletal muscle to regenerate itself in a manner recapitulating earlier stages of development (Allbrook, 1981). Attempts to elucidate the intricacies of this system have largely focussed on a population of muscle resident stem cells; originally identified by Mauro (1961), based on their anatomical localisation between sarcolemma and basal lamina, as well as their distinct morphology. These cells are now commonly referred to as satellite cells; mononuclear cells remaining quiescent in normal adult muscle pending stimulation to proliferate, whereupon their activated progeny (myoblasts) fuse with each other or pre-existing myofibres and contribute to the creation/regeneration of differentiated, multinucleate myofibres. Several satellite cell markers have been proposed, which delineate quiescent from activated myogenic lineage; the former include the receptor for hepatocyte growth factor c-Met (Tsukita and Yonemura, 1997), M-cadherin (Bretscher, 1999) and Pax7 (Seale et al., 2000), and the latter display Myf-5 and MyoD up-regulation prior to S-phase transition followed by proliferation and continued expression of Myf-5, MyoD and desmin through subsequent daughter generations (Bretscher, 1999). However, the satellite cell’s genetic profile/requirement has recently been proposed to be of age-dependent
character, inferring that extrapolations of the embryonic condition to the adult may hold yet unelucidated complexities (Lepper et al., 2009).

Figure 3.1. Satellite cell activation and division. Illustration depicting the asymmetric and symmetric division of the satellite cell population of skeletal muscle. Combinations of Pax7, Myf-5 and MyoD expression (amongst others) distinguish activated satellite cells from the quiescent progenitor population.

The *in vitro* culturing of myoblasts (activated satellite cells) has long strived to recapitulate the *in vivo* responses of these myogenic progenitors, and derived cultures have been widely used in research. The most well known perhaps being the commercially available C₂C₁₂ immortalised myoblast line (Schwacke et al., 2009), which is itself a subclone of the adult C3H crush-injured hind limb-derived C₂ cell line, created as a model of proliferating satellite cells (Yaffe and Saxel, 1977). In the context of this study, the widely used C₂C₁₂ immortalised myoblast line cannot
unequivocally be viewed as a true *in vitro* representation of the *in vivo* murine satellite cell; the early work of George Dickson’s group showed that dystrophin expression is almost absent in C2C12 myotubes whereas primary cultures of myotubes showed easily detectable levels (Noursadeghi et al., 1993). However, later work by Betto’s group showed that both full length dystrophin and other DAPC complex members are expressed and easily detectable in both 4 and 8 day C2C12 myotubes (Sandona et al., 2004), suggesting that clonal differences may exist within populations of immortalised cells in use throughout the research community. Although widely used in research, immortalised cell lines carry the perpetual burden of progressive distancing from the *in vivo* condition, hence this study utilises both immortalised and primary myoblast cultures, the source and characterisation of which are presented here.

A variety of techniques have been proposed for the isolation and *in vitro* culturing of myogenic precursor populations derived from the adult muscle of various animal models (Burton *et al*., 2000). These protocols have been adapted and optimised through many years of development by Bischoff’s group (Bischoff, 1997, Bischoff, 1990b, Bischoff, 1990a, Bischoff, 1986, Rosenblatt *et al*., 1995a) in Washington, who’s techniques have been subsequently adapted and re-coined by Partridge’s group in London (Rosenblatt *et al*., 1995a) to produce a widely accepted method of primary rat and mouse satellite cell isolation and culture, largely focussed on the use of the *extensor digitorum longus* (EDL) muscle group. Other researchers have explored the isolation of primary satellite cell cultures from adult murine muscle using techniques ranging from the differential sedimentation and pre-plating of tissue slurries (Goodell *et al*., 2005), as is common when using embryonic or postnatal tissue, to the cloning of single cell derived colonies from micro-dissected explants (Merrick *et al*., 2009). Having trialled several isolation methodologies, an adapted version of the Rosenblatt method was employed here for primary myoblast culture establishment (Rosenblatt *et al*., 1995b).
3.2 Myoblast lines

In the absence of human samples, this study aimed to compare ATP responses between normal and dystrophic murine myoblast cell lines *in vitro*. Normal and dystrophic immortalised myoblast cell lines were a gift from Prof. Hans Lochmüller (University of Newcastle) and represent clonally derived populations, isolated from mixtures of the TA, GC, Soleus and EDL muscles of 4-12 week male H-2K<sup>b</sup>-tsA58 and H-2K<sup>b</sup>-tsA58/mdx mice, respectively, according to protocols described by (Morgan *et al*., 1994, Springer and Blau, 1997). Genotyping of normal and dystrophic lines using the PCR based, *mdx*-amplification-refractory mutation system (ARMS) assay (Amalfitano and Chamberlain, 1996), confirmed the presence/absence of wild-type and *mdx* dystrophin alleles, respectively (Figure 3.2.1).

![Figure 3.2.1. Expression of wild-type/mdx dystrophin alleles in immortalised myoblast cultures. ARMS assay-based RT-PCR demonstrating expression of wild-type and *mdx* dystrophin alleles confined to normal and dystrophic lines, respectively. The upper band of >200 bp is a non-specific amplicon. Schematic below illustrates the technical principle of the ARMS method.](image-url)
Confirmation of myogenic lineage commitment was attempted by immunocytochemistry using anti-Myf-5 (Braun et al., 1989) antibody (Merrick et al., 2009). In both normal and dystrophic immortalised myoblast lines, 100% of cells stained positively for Myf-5 expression, although contrary to the published work of Janet Smith’s group at the University of Birmingham, this antibody (Santa Cruz sc-302) was deemed non-specific due to its propensity for cytoplasmic as well as nuclear staining in both normal and dystrophic myoblasts and myotubes. However, under reduced serum conditions, both normal and dystrophic myoblast lines displayed morphological characteristics of myogenic precursor cells; fusing to form differentiated, multinucleated, contractile myotubes, with an associated increased in desmin expression (Figure Appendix 1) (Tang et al., 2007). Immortalised cultures proliferated strongly in 20% FBS with a typical population doubling time of 2-3 days for stock flask cultures. Correct system operation was confirmed through the withdrawal of γ-interferon; culturing in proliferation media at 37°C in the absence of γ-interferon induced the cessation of proliferation within 1-2 days, where-by cells either came off the plate, or adopted a flattened, disc shape morphology, reminiscent of a cell having undergone senescence.

Although TA, GC, Soleus and FDB all proved suitable for use with the primary myoblast isolation protocols employed in this study, this study opted to use soleus muscles for such procedures due to the presence of distinct tendinous junctions, which aid the dissection of undamaged myofibres. Normal and dystrophic primary myoblasts were isolated from soleus muscles of male, 4 month C57BL10 and mdx mice, respectively, according to a modified version of the protocol described by (Rosenblatt et al., 1995b). Observations derived over a long period of method development led to the conclusion that the primary cultures derived using this method are not homogeneous (as will be discussed later). Collagenase digested, single living myofibres (both normal and dystrophic) retained numerous intricately associated mononucleate cells, appearing to occupy locations beneath the basal lamina in the
classically defined, satellite cell niche (bright field, phase-contrast derived observations only – basal lamina integrity not assessed) (Figure 3.2.2; A). Subsequent to plating, mononuclear cells were observed to migrate off *mdx*-derived myofibres in a seemingly contact dependent fashion (Figure 3.2.2; B, C and D).

**Figure 3.2.2. 1st generation primary myoblast cultures.** Representative brightfield images illustrating the migration and proliferation of myogenic precursors originating from single isolated living myofibres (A). Colonies derived from mononuclear cells were observed to originate from the ends as well as mid-sections of fibres (B-D), and displayed high degrees of proliferative potential upon removal of the original myofibre (E-F).
Satellite cells began to migrate off individually isolated, single living myofibres following approximately 4 days in culture, after which time cells were culture in ‘proliferation medium’ containing higher concentrations of serum, and cells were allowed to proliferate to approximately 60% confluency (over a further 10 days) with regular medium changes. Primary myoblasts isolated in this manner shall from now on be referred to as ‘first generation myoblasts’.

Repeated isolations using the same protocol, but substituting ‘serum-free’ growth factors (Knockout Serum Replacement; KSR, Invitrogen) for foetal calf serum (FCS) facilitated the elucidation of two morphologically distinct populations of mononuclear, myofibre-resident progenitor cells: The first population were observed to retain a spheroidal architecture, lightly adhering to Matrigel (2mg/ml). This population readily and consistently differentiated into multinucleate contractile myotubes (Figure 3.2.3; D), and shall here on be referred to as ‘second generation primary myoblasts’. Myogenic lineage confirmation was achieved by immunostaining of 2nd generation primary cultures for desmin the muscle specific intermediate filament protein, desmin (Figure 3.2.4). Conversely, the other distinct population of mononuclear cells isolated displayed a more protracted morphology, adhered both rapidly and tightly to the Matrigel substratum and, importantly, did not form multinucleate myotubes (Figure 3.2.3; E). Indeed, when approaching confluency this population rapidly and reproducibly induced the differentiation of proximal myogenic colonies, an effect which appeared to be, to a degree, contact-dependent (Figure 3.2.5; lower left panels). This non-myogenic population was observed to reproducibly give rise to two further morphologically distinct populations of mononuclear cells: One of a radiated, fibroblast-like appearance, and the other of a highly vesiculated, swollen cytoarchitecture. Subsequent immunohistochemical analysis confirmed the latter population to be 100% positive for the lipid stain, Oil Red O (Figure 3.2.5).
Figure 3.2.3. 2nd generation primary muscle cultures. Brightfield images illustrating two morphologically distinct populations of mononuclear cells generated from single isolated living myofibres (A); when isolated, cells of spherical shape - seen in the left hand side colony in (A), became highly proliferative (B) and consistently and uniquely differentiated into myotubes in reduced serum media (D). Cells displaying a more elongated morphology as seen in the colony on the right in (A), were also observed to proliferate rapidly, and consistently and uniquely differentiated into two different morphologies: a basal layer of fibroblast-like cells and an upper layer of a different, unidentified cell type (C+E).
Figure 3.2.4 Desmin expression in wild-type/mdx-derived 2nd generation primary muscle cultures. Brightfield images depicting representative colourimetric staining of desmin expression over 8 days differentiation of normal- and dystrophic-derived 2nd generation primary myoblast cultures, visualised by peroxidase staining (dark pink/purple). Primary
antibody was omitted from negative staining controls which represent secondary antibody staining only.
Figure 3.2.5. Adipocyte differentiation in 2nd generation primary muscle cultures. Brightfield images illustrating the differentiation of non-myogenic cell fractions of primary cultures into highly vesiculated mononuclear cells staining positively with lipid stain, Oil Red O. Upper panels of both genotypes are counterstained with haematoxylin. Lower left panels of both genotypes clearly display myotube-contact-dependant inhibition of adipocyte proliferation/migration.
3.3 Discussion

Muscle groups of differing developmental lineages have been shown to contain heterogeneous populations of satellite cells in terms of their proliferation and differentiation potentials (Ono et al., 2010). Indeed, soleus muscle has been reported to contain higher numbers of mononuclear cells expressing classic satellite cell markers than those found in EDL muscle (Schultz et al., 2006), and this heterogeneity within satellite cell populations also extends to sub-populations within individual muscle groups (Schultz, 1996, Zammit et al., 2004). It has been shown previously that satellite numbers present in soleus muscle do not significantly differ between C57BL10 and mdx in adult animals although satellite cells from mdx soleus muscle display reduced regenerative capacity compared with those of age-matched C57 animals (Reimann et al., 2000). This is contrary to observations made in this study, where once isolated, dystrophic myoblasts displayed equal, if not superior proliferative capacity compared with wild-type myoblasts, although this has not been quantitated here, and may be dependent on the fibre-type of origin.

In addition to myogenic progenitors, the method development described here also demonstrated the presence of a population of fibro/adipo-type progenitor cells in intricate association with isolated myofibres. A similar population has recently been described in normal skeletal muscles, during the characterisation of a population of Sca-1^ve/CD34^ve fibro/adipogenic progenitors (FAPs), clearly distinct from known myogenic progenitor lineages. Upon muscle injury these cells have been observed to undergo proliferation and, when cultured, were found to be a concentrated source of differentiation signals for primary myoblasts (Joe et al., 2010, Natarajan et al., 2010), alluding to have a potential role as an interacting partner of myogenic progenitors in muscle diseases, and a candidate for the non-myogenic component observed during primary culture development. Characterisation and enumeration of both the levels of these cells adhering to normal and dystrophic myofibres and the levels of P2X7
expression within normal and dystrophic populations of these cells remains ongoing. Interestingly, another study has described a population of muscle-resident non-satellite cell-derived myogenic precursors, identifiable by their PW1⁺/Pax7⁻, interstitial localisation (termed PICs) and described as enriched in the Sca-1⁺/CD34⁺ cell pool (Mitchell et al., 2010), (marker profile that would also describe the aforementioned FAP population), which display very different lineage characteristics in their differentiation. This suggests that the two populations may potentially be related; perhaps differential signalling at the interstitial matrix-myofibre interface facilitates the transition from PIC to FAP or vice-versa. However, it should be noted that PICs have only been studied in neonatal mice to date and their existence in adult muscle has not been documented. FAP-type cells have been demonstrated in 4 month skeletal muscle (Starkey et al., 2011) where their lineage has been proposed to be distinct from that of a satellite cell. This is based on the observation that satellite cells cultured in normal myoblast growth media do not undergo adipogenesis and those cultured in adipogenic media do accumulate cytoplasmic lipid, but do not fully undergo adipogenic differentiation (Starkey et al., 2011). These observations would suggest that the FAP-type cells observed in this study rather than originating from satellite cells themselves are derived from some unknown precursor cell type residing on isolated myofibres. The suggestion by Starkey that these adipocyte forming cells represent a contaminating interstitial population, which can simply be washed off isolated fibres proved not to be true for the soleus muscle fibres used here; moreover, these ‘contaminating’ cells were found so tightly attached to single isolated myofibres that they could not be removed by washing, vigorous pipetting or even through trypsinisation without inducing myofibre death. Starkey’s proposed distinction between satellite cell and adipocyte lineage, although most recent in the literature, is however not unequivocal. Many previous studies have previously documented satellite cells adopting adipogenic characteristics; by inhibition of Wnt signalling (Ross et al., 2000) or by growth in adipogenic media (Wada et al., 2002)
where increased age has been suggested to be a factor (Taylor-Jones et al., 2002). Therefore, it can be concluded from the current literature that although myofibre derived satellite cells and adipocytes are of separate lineage under normal growth conditions, the potential does exist for this relationship to differ in the dystrophic environment. The relevance of this point to the dystrophic phenotype, where fibrosis and fatty infiltration are well-documented patho-histological hallmarks of aging and degenerating skeletal muscle in humans (Lefaucheur et al., 1995, Pastoret and Sebille, 1995, Delmonico et al., 2009) requires further investigation.

In conclusion, the cell lines used in this study can be considered in vitro representations of activated satellite cells in vivo. Due to the lack of a specific marker to label all activated myoblast populations, immortalised and primary myoblast cultures have been isolated and selectively purified by established pre-plating techniques, to yield cultures with the potential to uniquely form 100% multinucleate, contractile myotubes (immortalised myoblast lines were isolated and characterised by Morten Riso; see Appendix 1, the primary myoblast cultures were isolated and characterised by the candidate). Non-myogenic adipocyte-generating populations characterised during primary culture establishment are thought to derive from non-satellite cell origins and were not present in the purified primary myoblast cultures described here.
4

Extracellular ATP hydrolysing potential of immortalised normal and dystrophic myoblasts and myotubes

4.1 Introduction

The consensus opinion that dystrophin protein expression is very low or even absent in undifferentiated myoblasts (Houzelstein et al., 1992, Yeung et al., 2006, Trimarchi et al., 2006, Scott et al., 1988) is reflected by the fact that the majority of work on DMD is being conducted in fully differentiated myotubes or myofibres. Myoblasts are not expected to dysfunction due to the absence of dystrophin. However, contrary to this view, an increased purinergic sensitivity in dystrophic lymphoblastoid cells has previously been described (Ferrari et al., 1994); also cells that contain dystrophin mRNA but have undetectable levels of protein. Moreover, our lab has described a similar phenotype in immortalised dystrophic myoblasts (Yeung et al., 2006). These mdx cells do not express protein or full-length dystrophin mRNA due to a premature stop codon occurring through point mutation at exon 23 (Sicinski et al., 1989) and the resulting nonsense-mediated decay (Buvoli et al., 2007, Sedlackova et al., 2009). Purinergic responses in these cells have been shown to be significantly altered when compared to their normal equivalent (C57BL10) myoblasts, which do express full length dystrophin mRNA but no detectable protein (Yeung et al., 2006). Importantly, altered sensitivity to extracellular nucleotides has been demonstrated for P2X and also P2Y receptors in these cell lines (manuscript in preparation), and this prompts the question as to whether the effects are specific to altered subunit expression of
individual purinergic receptors and their splice variants, or whether there is a more global alteration of nucleotide metabolism in the vicinity of these receptors.

Extracellular ATP can produce various effects acting via P2-purinoceptors and it is rapidly broken down by ecto-ATPases that limit its effect (Lavoie et al., 2011). Ecto-ATPases are ubiquitous in eukaryotic cells (Plesner, 1995) and have been shown to be involved in modulation of P2X receptor responses in muscle (Sneddon et al., 1999). The expression of ecto-nucleoside triphosphate diphosphohydrolases (NTPDases) and other more atypical ATP-hydrolysing proteins, such as α-sarcoglycan has been documented in muscle (Betto et al., 1999). The α-sarcoglycan (α–SG) protein is a component of the sarcoglycan complex of dystrophin-associated proteins, all of which are severely reduced at the sarcolemma in the dystrophic condition (Ohlendieck and Campbell, 1991, Ohlendieck et al., 1993). α–SG ecto-ATPase activity has been characterised in the well studied C2C12 mouse muscle cell line, where in myotubes it was predicted to provide about 25% of the overall ATP-hydrolysing activity (Martinello et al., 2011) and therefore play an important role in the modulation of purinergic receptor signalling (Sandona et al., 2004). The exact functional role of any of the SG proteins has yet to be established, but it is known that mutations in the α–SG gene result in Limb-Girdle Muscular Dystrophy type 2D (LGMD-2D), which shares a similar phenotype with DMD (Duclos et al., 1998). This lead to the hypothesis that the abnormalities in purinergic signalling described in dystrophic myoblasts may result from the inability of these cells to metabolise high [ATP]e (0.25-4mM), arising from both a release of lots of ATP from damaged dystrophic muscle fibres (Bodin and Burnstock, 1996) combined with the reduced ecto-ATPase potential due to loss of α–SG from its normal extracellular location.
4.2 Results

In order to test the hypothesis that loss of α-SG ATPase activity from the extracellular face of the DAPC complex in some way confers heightened purinergic sensitivity to dystrophic myoblasts, ecto-ATPase activity was analysed in immortalised cultures of normal and dystrophic myoblasts. Initially, RT-PCR analysis was used to confirm α–SG mRNA expression in normal and dystrophic myoblasts (Figure 4.2.1; A). Both cell types showed expression of this transcript however no estimation of the levels of expression were made as this PCR data cannot be considered quantitative. Western blot analysis showed that α–SG protein levels were below the detection threshold (data not shown). To make a functional assessment ATP hydrolysing activity has been compared in control and dystrophic cells. Several methods were trialled for the live-cell quantification of ectoATPase potential; luciferase based detection methods (Promega) proved too insensitive for the detection of small changes within a broad [ATP]e, range. Similarly, an ascorbic acid-based inorganic phosphate detection method (Gawronski and Benson, 2004) was also tested and actually proved to be the most accurate method of all, yet could not be scaled up to microplate format due to the lack of the correct 655nm filter wheel in the plate reader. A useable methodology was devised, adapted from the Malachite green based inorganic phosphate detection method described by (Sandona et al., 2004), itself a modification of the assay described by (Lanzetta et al., 1979). The malachite green-based assay also demanded microplate format absorbance values to be read at 655nm, yet this assay proved more flexible than the ascorbic acid assay, hence the available 600nm filter provided an acceptable degree of accuracy. No significant difference was found between the $K_m$ or $V_{max}$ values of normal and dystrophic myoblasts (P>0.05; Figure 4.2.1; B and C, respectively), although there appeared to be a trend towards a reduced $K_m$ value in both dystrophic myoblasts and myotubes compared with their normal counterparts. As expected, significant increases in $V_{max}$
were observed upon differentiation of the cells into myotubes (P<0.0001; Figure 4.2.1; C) - increases from 0.007 to 0.14 and 0.015 to 0.12 IU/mg of protein were observed for normal and dystrophic lines, respectively. However, no significant differences were found between $K_m$ or $V_{max}$ values relating to normal and dystrophic 4 day myotubes, or indeed between HK2$^b$-tsA58-derived and C2C12 wild-type myotubes (P>0.05; Figure 4.2.1; C).

**Figure 4.2.1.** ATP hydrolysing potential of wild-type- and $mdx$-derived immortalised myoblast cultures. $\alpha$-Sarcoglycan ($\alpha$-SG) mRNA was detected in both normal and dystrophic immortalised myoblast lines by RT-PCR (A). Myoblast/myotube ATP hydrolysing potentials were assessed through determinations of $K_m$ (B) and $V_{max}$ (C) – ANOVA revealed no significant differences between normal and dystrophic cells in terms of either parameter (P>0.05). Significant increases in $V_{max}$ were recorded for myotube cultures (4 days in differentiation media) of both genotypes compared with their respective myoblast
cultures. Error bars represent SE, df=4 (C; *P<0.0001). C2C12 cells represent a positive experimental control (see text).

4.3 Discussion

The proposed functional link between α–SG and LGMD2D stems from the observation that nearly all known mutations resulting in this phenotype can be mapped to the extracellular domain of the α–SG protein, which has been shown to demonstrate ecto-ATPase activity in vitro (Betto et al., 1999). Due to the potential for loss of ecto-ATPases such as αSG from the extracellular membrane of dystrophic cells, it was hypothesised that differences in the [ATP]e hydrolysing potential of myoblasts could be responsible for the purinergic signalling abnormalities observed in immortalised dystrophic myoblasts (Yeung et al., 2006). Expression of ecto-ATPases, such as αSG, have previously been characterised in C2C12 myoblasts. It was suggested to play a role in P2 purinergic signalling through limiting ATP accessing receptors present on the extracellular membrane of these cells (Sandona et al., 2004). Importantly, although the assay employed here was not α-SG specific, pharmacological profiles indicative of αSG activity were recorded despite the fact that only αSG mRNA and not protein have been detected. Here, immortalised cultures of normal and dystrophic myoblasts were shown to express αSG mRNA (Figure 4.2.1; A). In agreement with the work of (Sandona et al., 2004) in C2C12, the immortalised cells used here also displayed an approximately 10-fold increase in ecto-ATPase activity upon differentiation (Figure 4.2.1; C).

Characterisation of the ATP-hydrolysing potential of normal and dystrophic myoblast cultures at high [ATP]e was achieved, and kinetic profiles were derived from dose response curves from five independent experiments comprising triplicate samples. Non-linear curve fitting (Origin 7.0) showed that no significant alteration in extracellular nucleotide hydrolysing potential existed between normal and dystrophic
myoblasts in terms of $K_m$ or $V_{\text{max}}$, and moreover that any significant differences in purinergic signalling response induced through exposure to high [ATP]$_e$ (Yeung et al., 2006) were more likely due to differences in purinergic receptor expression and/or function at the protein level, potentially rendering the extracellular membrane more sensitive to purinergic stimulation, rather than due to the agonist degradation inadequacy that was hypothesised here. However, since the assay employed here was not specific to any individual ATPase enzyme, the observed trend displaying decreased $K_m$ values in dystrophic versus normal myoblasts and myotubes (Figure 4.2.1; B) could potentially indicate that different ATPase enzymes may be operating between the two genotypes. It is therefore possible that other ATPases (with different, in this case lower $K_m$ values) are actively compensating for the loss of $\alpha$-SG in the dystrophic cell line, since the $V_{\text{max}}$ values appear similar (Figure 4.2.1; C). Testing this hypothesis would require the purification of $\alpha$-SG enzyme from both normal and dystrophic myoblasts and myotubes for pharmacological characterisation.

The demonstration of significantly increased $V_{\text{max}}$ values in both normal and dystrophic myotube cultures, compared with cultures of the same undifferentiated cells (Figure 4.2.1; C) served as a positive experimental control, and was in agreement with the finding that levels of $\alpha$SG expression increase with increasing confluency and the onset of differentiation (Sandona et al., 2004, Martinello et al., 2011). Sandona’s group has previously described an increase in $V_{\text{max}}$, from approximately 8.6$^{-4}$ to 0.04 IU/mg protein, using C2C12 myoblast cells grown under differentiation conditions for 4 days (Sandona et al., 2004), and more recently provided a revised value of 0.03 to 0.09 IU/mg protein (Martinello et al., 2011). The changes in $V_{\text{max}}$ observed here for immortalised normal and dystrophic myoblasts and myotubes cultured in the same manner were 0.007 to 0.14 and 0.015 to 0.12 IU/mg of protein, respectively and thus higher than C2C12 values. For further clarification, $V_{\text{max}}$ has been calculated for C2C12 myotubes using the method described here and similarly
higher values were obtained. Interestingly, this difference represents an approximately 3.5 fold increase in $V_{\text{max}}$ in myotubes derived from the immortalised cells used here compared with the C2C12 values published by Sandona’s group in 2004 but only an approximately 2 fold difference compared with the recent data from the same group (Martinello et al., 2011) for, presumably, the same C2C12 cell line. The reason for these discrepancies is unknown and possible sources of variability may be due to different types of spectrophotometric instrumentation, sources of reagents, sample incubation times and temperatures, or indeed, cell lines of differing origin. $K_m$ values obtained for these cells could not be compared with Sandona’s data, as this group elected to determine their $K_m$ values from transfected HEK-293 cells, where they found $K_m = 3.71$ mM ATP and $V_{\text{max}} = 0.005$ IU/mg protein. $K_m$ values for the immortalised normal and dystrophic lines were found to be much lower; 1.04 and 0.68 mM ATP, respectively. Reasons for this again could relate to differences in curve-fitting approaches used, or possibly that $\alpha$–SG displays alternate substrate specificity dependent on the cell type by which it is expressed. However, close examination of Sandona’s group's data does suggest a biphasic dose response, not observed in the experiments described here. Such a response is uncharacteristic of a *bona fide* ecto-ATPase, and would certainly affect the derivation of a true $V_{\text{max}}$ value. Indeed, perhaps clarification of Sandona’s groups claims would be better achieved through the demonstration of heightened ATP-induced sensitivity in primary myoblast cultures derived from the $\alpha$–SG-deficient mouse model of LGMD2D (Duclos et al., 1998).

To conclude this chapter, it has been shown here that ectoATPase activity and therefore ATP$_e$ hydrolysing potential, does not significantly differ between normal and dystrophic myoblasts or myotubes. Should a difference have been found then further investigations into the component of such an effect specific to a particular ectoATPase such as $\alpha$-SG would have been further explored. However, the data
presented here does not unequivocally exclude the possibility for modulation of the ATP\textsubscript{e} hydrolysing reaction in dystrophic cells; the trend (although not statistically significant) towards lower \( K_m \) for this reaction in dystrophic versus normal cells may indicate a compensation by some unidentified ectoATPase which is may be masking the effect of losing \( \alpha \)-SG activity from the extracellular face of the sarcolemma.
P2X7 splice variant analysis in normal and dystrophic cells and tissues

5.1 Introduction

Nine splice variants of the P2X7 receptor have been cloned in humans (P2X7 ‘A’-‘J’) (Cheewatrakoolpong et al., 2005, Feng et al., 2006), the expression levels/patterns of which have been little explored. The main variant, P2X7A encodes the classical 595aa subunit with the N-terminal region, first transmembrane domain, large extracellular loop, second transmembrane domain and the long C-terminal tail. P2X7B transcript retains the intron separating exons 10 and 11 which generates a premature stop codon resulting in P2X7 isoform with 249 C-terminal amino acids following residue 346 replaced by unique 18aa generating a novel C-terminus, as well as a modified transmembrane domain II.

P2X7B variant has been shown to be ubiquitously expressed in human tissues with highest levels found in the brain, spinal cord, lymphocytes and lymph nodes. This receptor variant displays similar responsiveness to ATP and BzATP as the P2X7A variant, but is deficient in large pore formation and caspase-mediated apoptotic cascade induction (Cheewatrakoolpong et al., 2005). P2X7B was also shown to form functional homo- and hetero-oligomeric trimers when co-expressed with P2X7A in HEK-293 cells, serving to potentiate wild-type P2X7A receptor responses, and its expression was up-regulated upon mitogenic stimulation in peripheral blood lymphocytes, with accompanying increases in ER Ca²⁺ content,
NFAT nuclear translocation and cellular ATP content, suggesting a role for this variant in tumour progression (Adinolfi et al., 2010).

P2X7J, another C-terminally truncated variant (lacking the entire C-terminal tail, the second transmembrane domain and the last third of the extracellular loop) was cloned from, and so far has only been shown to be expressed in, cervical epithelial cancer cells. P2X7J has been shown to be a non-functional receptor variant but able to negatively regulate the apoptotic cascade-inducing properties of the P2X7A receptor variant through direct hetero-oligomerisation (Feng et al., 2006). The subtleties of such an interaction conferring potentially dramatic changes in disease pathology are currently being explored for therapeutic manipulations, with a monoclonal antibody against the P2X7J variant pending evaluation for its potential as a growth inhibitor in cervical neoplasia (Adinolfi et al., 2010). Such developments highlight both the intricacies of P2X7 signalling and the potential for the abnormal functioning of this receptor in other pathologies, including muscle disorders.
Adapted from Adinolfi et al., (2010)

Adapted from Feng et al., (2006)
Figure 5.1.1 P2X7B and P2X7J splice variant structures. Schematics depicting P2X7B (A) and P2X7J (B) sequence alignments with associated alterations in receptor structures. Lower case letters denote residues differing between P2X7A and P2X7B variants - Transmembrane domain 1 (TM1); yellow, TM2; green, novel C-terminus of P2X7B variant; red (A). Almost complete loss of exon 8 except for the final base pair (circled) results in frame shift-generated alternate C-terminus of P2X7J variant, which lacks the second transmembrane domain and entire C-terminus of the wild-type receptor (B).

Increasing interest in P2X7’s functional diversity and potential for therapeutic manipulations have led to investigations into the splicing patterns of this receptor in mice, where functional relevance in models of disease may be extrapolated more easily. Specific mouse splice variants have been identified: these include two transcripts encoding N-terminal isoforms: P2X7a, and P2X7k. P2X7k has a unique exon 1 encoding specific 42aa of alternative N-terminus and transmembrane 1 domain (Nicke et al., 2009). The P2X7k’ variant has been shown to form functional homotrimers when expressed in xenopus oocytes and HEK-293 cells, and is referred to as the “killer” variant, so named for its rapid activation and reduced deactivation kinetics compared with the P2X7a variant. Coupled to its proposed constitutive dilation and pore formation upon agonist application, the ‘k’ variant is deemed more sensitive to ATP than the ‘a’ variant. P2X7k has been shown to be expressed in all tissues expressing P2X7a.

More recently, two C-terminal variants: P2X7b and P2X7c have been identified as a result of a collaboration between Dr Murrell-Lagnado, Pharmacology Department, Cambridge University, The Babraham Institute Bioinformatics and our laboratory (see Results). Bioinformatic analysis of mouse P2X7 gene structure followed by searches of Ensembl predictions combined with analysis of expressions sequence tags (ESTs) over this region in mouse genome identified these two putative
alternative 3’ exons: P2X7b variant encodes an isoform with a significant truncation of the C-terminal tail, being 164 amino acids shorter than the P2X7a variant (431 & 595aa, respectively). P2X7c cDNA has been cloned as an element of this thesis and is described in the Results below. Protein sequence alignments comparing all known P2X7 splice variants indicate that a region of conserved homology exists in exon 2, proximal to the N-terminal portion of the extracellular loop in all rodent splice variants as well as the two currently characterised human splice variants; B and J (Figure 5.1.3). This region has been proposed to contain both sites of ATP binding (Worthington et al., 2002) and ADP-ribosylation (Adriouch et al., 2008). However, the same region of homology in the wild-type human P2X7A variant is found in exon 12, proximal to transmembrane domain II (Figure 5.1.3). Conservation of amino acid sequence homology between P2X7B and P2X7J and rodent P2X7a, k, b, c and d, could suggest conservation of functional site location between these variants. However, the reason for the alternate location of this site in the human wild type P2X7A variant remains unclear, yet the retention of this domain in different exons may be of functional significance in interpreting species specific responses.
Figure 5.1.2  **P2X7k, P2X7b and P2X7c splice variant structures.** Schematics depicting P2X7k (A), P2X7b and P2X7c (B) gene structures and protein sequence alignments. P2X7k exon 1 (1’) position is indicated in relation to other exons (numbered bars) and start and stop codons (A; upper panel). Differences in amino acid sequences between P2X7a and P2X7k are shown; boxes represent similar and identical residues, asterisks represent residues...
conserved between all rat P2X receptors, underlining denotes predicted TM1 domain (A; lower panel). The full length P2X7 receptor is encoded by 13 exons with the final exon encoding the long C-terminus relevant to P2X7b and P2X7c variants. These variants encode much shorter C-terminal sequences than the P2X7a variant. Variant P2X7b gives rise to a receptor that is truncated at position 430 and P2X7c has an alternative C-terminus with additional 11 amino acids beyond the common residue 430 (B; lower panel). The genomic organization of these two alternative transcripts is depicted in B; upper panel.
Figure 5.1.3 Multiple protein sequence alignment - Human and rodent P2X7 splice variants. CusfW2 multiple sequence alignment depicting the singular region of amino acid sequence homology conserved between all human (uppercase) and rodent (lower case) P2X7 receptor splice variant sequences. In the above section of sequence above ‘.’, ‘:’ and ‘~’ denote low, medium and high level sequence homology, respectively, with 100% conservation shown in red.
All splice variants form functional homotrimers when expressed in HEK-293 cells and *xenopus* oocytes, demonstrating both calcium channel activity and large pore opening, yet the kinetics of receptor activation for the three C-terminal variants is significantly altered in terms of Ca\(^{2+}\) currents; ‘b’ and, ‘c’ all display <10% of the BzATP-induced current density observed for the P2X7a variant, and reduced surface expression when compared with P2X7a in transfected cells. Moreover, the P2X7c variant actually displays a dominant-negative effect on the observed BzATP induced current density when co-expressed with the P2X7a variant. The possibility that these variants may hetero-oligomerise has been confirmed by coimmunoprecipitation pull down assay in the collaborating laboratory (Dr. Murrell-Lagnado; personal communication).

The significance of the existence of alternate P2X7 splice variants is manifested in the two currently available P2X7 knockout mouse lines: the GlaxoSmithKline (GSK)-generated line, which carries a LacZ transgene insertion in exon 1 (Solle *et al*., 2001, Sikora *et al*., 1999) and the Pfizer-generated line generated via a neomycin cassette insertion at exon 13 (Solle *et al*, 2001); The P2X7k variant escapes inactivation in the GSK P2X7 knockout mouse, where its expression has been shown to be ubiquitous and highest in spleen (Nicke *et al*., 2009). The functional importance of this variant is demonstrated by the increased P2X7 receptor-mediated T lymphocyte responses in the GSK P2X7 knockout mouse, where specific P2X7 receptor activation and large pore formation coincide with the induction of apoptosis (Taylor *et al*., 2009). Subsequent investigations in our laboratory have also rendered the Pfizer P2X7 knockout mouse a splice variant knockout, where P2X7 alternative splicing variants ‘b’ and ‘c’ escape inactivation (unpublished data).

Importantly, these observations may explain the antibody specificity issues that have long haunted the P2X7 receptor field, where different antibodies generate spurious staining patterns, some giving positive staining in knockout animals (Sim *et
al., 2004) and leading to the hypothesised existence of a ‘P2X7-like’ protein possessing similar immunogenicity. An examination of the P2X7 splice variant expression profile of skeletal muscle represents a novel undertaking and considering the specific properties of the different variants, it could potentially serve to explain the previously documented differences in P2X7-mediated pharmacological responses between normal and dystrophic immortalised myoblast cell lines (Yeung et al., 2006).

5.2 Results

5.2.1 Cloning of a novel C-terminally truncated P2X7c variant

The full length P2X7c transcript encodes a 442aa C-terminally truncated isoform with a novel 12aa C-terminus (Figure 5.1.2). Using specific primers based on mRNA sequences obtained from bioinformatic analyses the 1.8kb full length P2X7c cDNA was amplified from 4 month C57BL10 TA muscle and brain (Figure 5.2.1; A). The significant differences in amplification efficiency were found to be inherent to the properties of proof-reading DNA polymerase enzymes from different manufacturers - Takara’s ‘LA’ enzyme demonstrated far superior performance compared with Promega’s ‘Pfu’ enzyme (Figure 5.2.1; B). PCR amplicons were cloned into a standard PCT cloning vector (pGEM-T) using the T/A overhangs. Ligated plasmids were used to transform competent cells and positive colonies containing the correct cDNA insert were first identified using blue-white screening and by PCR amplification in crude bacterial extracts with the specific exon9 and exon13c primers producing 450bp amplicons. Specific colonies were picked, plasmid extracted and analysed by PCR (Figure 5.2.1; C) and restriction digestion with Not1 and BamH1. Expected restriction fragments of 3kb+1.4kb and 3.4kb+953bp were obtained (Figure 5.2.1; D). Cloned products were verified by sequencing (Pubmed entry P2X7 Variant 2; NM_001038839 and supported by ESTs: AK089434, AK144585, BB810482,
BY544874, BY548370, BY764453) and clones were sent to Dr. Murrell-Lagnado’s laboratory (Cambridge) for transfection and characterisation in HEK-293 cells.

**Figure 5.2.1. Cloning of full length P2X7c variant cDNA.** RT-PCR based amplification of full length P2X7a and P2X7c variants from total mRNA extracts of C57BL10-derived GC and brain tissues (A). P2X7c variant expression was lower and more difficult to amplify than P2X7a variant, although this effect was observed to be to a degree, DNA polymerase enzyme-dependent (B). Amplification of the specific P2X7 exon9a-exon13b region gave the expected 421bp band size in all of transfected bacterial colonies (C). Samples #1 and #4 were selected for further analysis, both displaying predicted band sizes following Not1 and BamH1 restriction digestion (D).
5.2.2 Characterisation of P2X7 splice variants expression in normal and dystrophic myoblasts and muscle groups

Semi-quantitative mqPCR was developed here as a method of quantitating RT-PCR data where primers for qPCR were too expensive. The aim here was to use manual sampling and plotting of PCR time courses to characterise muscle group specific patterns of P2X7 splice variant transcript up-regulation in mdx compared with normal age matched controls. The mqPCR technique allowed specific splice variant expression to be quantitated between different muscle groups, which could be compared with the more sensitive qPCR technique with primers spanning P2X7exon1-exon2 boundary, which would detect all P2X7 variants described here.

*Mdx* TA muscle demonstrated a uniform and significant 0.94-1.03 fold up-regulation (Figure 5.2.2; A; *P<0.001) in expression of all variants tested except P2X7k, although a similar average fold change was also observed for this variant (0.83). *Mdx* GC muscle demonstrated the largest significant fold changes in expression levels (Figure 5.2.2; A; *P<0.0001), again across all variants; from 3.3 fold for P2X7k to 6.9 fold for P2X7c. P2X7b variant showed an average 4.8 fold up-regulation in *mdx* GC, although this was not found to be significant (*P>0.05). *mdx* soleus muscle displayed 1.7, 2.3 and 3.2 fold up-regulations (Figure 5.2.2; A; *P<0.001) in the expression of P2X7a, k, and c variants, respectively, and although P2X7b was found to be an average of 2.6 fold up-regulated in dystrophic soleus muscle, this result was again shown to be not significant (*P>0.05). No significant differences were found in P2X7 splice variant expression levels between wild-type and dystrophic FDB muscles (*P>0.05). Interestingly, *mdx* diaphragm was the only muscle type to display significant differences in splice variant expression patterns in dystrophic vs. wild-type muscle, with a 2.0 fold up-regulation in P2X7a variant expression only (Figure 5.2.2; A; *P<0.001) and no significant differences in other variants (*P>0.05). Heart muscle results resembled those of FDB muscle, with no
significant differences in P2X7 splice variant expression found for any P2X7 variants (P>0.05).

Immortalised dystrophic myoblasts displayed significant up-regulations of 2.9, 1.8 and 3.4 fold for P2X7a, P2X7k and P2X7c variants, respectively (Figure 5.2.2; A; *P<0.001) – P2X7b variant expression did not significantly differ between wild-type and dystrophic immortalised myoblasts (P>0.05). Also the 2nd generation primary myoblast cultures demonstrated significant up-regulations in expression of 3.6, 2.4, 3.7 and 4.4 fold for P2X7a, P2X7k, P2X7b and P2X7c variants, respectively (Figure 5.2.2; A; *P<0.001).

Tacman primers for qPCR were available spanning P2X7 exon1-exon2 boundary, thus capable of amplifying all P2X7 variants discussed here. The qPCR for P2X7 showed higher levels of up-regulation in P2X7 expression than were found using mqPCR - actually demonstrating up to three times the expression levels described using mqPCR. Significant up-regulations in P2X7a variant expression were observed in all mdx-derived tissues tested using this more sensitive technique: 3.2, 13.1, 5.7, 2.5, 3.9 and 1.7 fold up-regulations were found in TA, GC, soleus, FDB, diaphragm and heart of mdx/wild-type, respectively (Figure 5.2.2; B; *P<0.0001). Indeed, a much higher level of P2X7 expression was observed in dystrophic immortalised myoblasts using this technique – where a 10.6 fold difference was observed in mdx/wild-type cells (Figure 5.2.2; B; *P<0.0001). 2nd generation primary myoblasts demonstrated a 3.5 fold up-regulation in P2X7 expression in dystrophic compared to wild-type cells (Figure 5.2.2; B; *P<0.0001), this figure was more similar to that seen using mqPCR.
Figure 5.2.2. **P2X7 splice variant analysis in cells and tissues.** Expression levels of four mouse P2X7 splice variants: ‘a’, ‘b’, ‘c’ and ‘k’ were assessed in mdx- compared with wild-type-derived muscle groups using semi-quantitative, mqPCR (A) and quantitative, qPCR (B). Values are shown as fold differences in expression – mdx vs. wild-type, where wild-type=0. Error bars represent SE, df=2 (*P<0.01-0.0001).
5.3 Discussion

Characterisation of the novel 5’ P2X7k or ‘killer’ variant, which displays faster activation and slower deactivation kinetics than the wild-type P2X7a variant (Nicke et al., 2009), together with a further two functional 3’ splice variants, P2X7b and P2X7c, suggested the potential for differential expression of these splice variants between normal and dystrophic muscle and cells, which may serve to explain the differences in purinergic responses previously observed between these cells (Yeung et al., 2006). Indeed, semi-quantitative mqPCR revealed significant up-regulations of this P2X7 receptor variant in whole muscle-derived RNA extracts of mdx GC and soleus muscle compared with age matched wild-type controls. The expression profiles described here for the P2X7k variant in particular, highlight the potential for dramatically altered purinergic signalling to be inherent in myogenic precursors present on myofibres of the mdx mouse at 4 months of age. As the only P2X7 splice variant in mice to be documented in the literature, the demonstration that P2X7k variant displays higher kinetic potential due to increased agonist sensitivities and retention (Nicke et al., 2009) suggests that similar, as yet uncharacterised differences in agonist sensitivities/selectivities may also exist for other variants. Moreover, this in turn suggests that antagonist profiles may also vary between variants. Antagonists were not analysed in relation to the P2X7k variant, which is surprising considering the potentially high therapeutic gains which could be envisaged through the pharmacological inhibition of such a potentially damaging splice variant. Indeed, considering the rapidly increasing diversity of P2X7 receptor mediated responses, the profiling of antagonist selectivities at mouse P2X7 variants would certainly be beneficial to the wider research community, facilitating more accurate interpretations of the role of P2X7 receptors in pathological environments.

P2X7 receptors are expressed on many different cell types in muscle, therefore, perhaps a more elegant future approach would be to employ one of the
myogenic regulatory factors such as Myf-5 or MyoD, together with immune cell markers such as F4/80 or CD11b as internal controls alongside GAPDH, thus allowing additional comparisons relating any observed differences in expression levels to the number of cells of myogenic/immune cell lineages. Moreover, counts comparing age-matched mdx- and C57BL10 derived soleus muscles have shown no significant differences in satellite cell numbers (Reimann et al., 2000), although heterogeneous populations of satellite cells have been reported in muscles of differing developmental lineage (Ono et al., 2010, Schultz et al., 2006), rendering comparisons between P2X7 expression levels in cells and tissues difficult without further characterisation of primary myoblast cultures. Tissues from 4 month animals were used here specifically to negate any problems associated with high levels of infiltrating cells as seen at earlier time points in mdx mice, and no significant differences in levels of immune cells were found between wild-type- and mdx-derived whole muscle extracts (see 8.2.1), implying that the observed increases in mdx compared with wild-type muscles are not the result of higher numbers of infiltrating immune cells in these tissues. The possibility that immune cells present in mdx muscle express higher levels of P2X7 receptors than those present in wild-type muscle cannot be ruled out, also the possibility exists that another un-quantified population of non-myogenic cells may be responsible for the differences in P2X7 expression levels seen here, however, both these points have been addressed through the demonstration of significantly up-regulated P2X7 expression levels in both immortalised and primary myoblast cultures, confirming the assertion that the effect is myoblast specific and that dystrophic myoblasts of this age display inherently heightened purinergic sensitivity.

The novel semi-quantitative mqPCR technique employed here proved a reliable relative quantitative measure, in that the pattern of P2X7a variant up-regulation observed in mqPCR was similar to that observed using the more sensitive technique of TaqMan qPCR - of the order GC>soleus~diaphragm>TA. However,
fold change values found to be $< ~2.5$ by qPCR, proved too small to detect using the mqPCR technique, rendering results obtained by mqPCR for FDB and heart as potentially less significant than they might have otherwise been. In the future, TaqMan analysis should be performed to answer this question in details.

The finding that all four P2X7 splice variants; ‘a’, ‘b’, ‘k’ and ‘c’, all share the same general pattern of tissue specific up-regulation (Figure 5.2.2; A) is in itself a novel proposal, the only marked inconsistency here being the lack of significant P2X7k variant up-regulation in mdx compared to wild-type diaphragm muscle. Such an observation may be of interest to immunologists, since the mdx diaphragm at this age would be expected to contain significant numbers of macrophages, where further characterisation of the potential for differential regulatory mechanisms of splice variant expression would be very interesting - although the lack of P2X7k variant specific antibodies would narrow the possibilities here somewhat.

The results presented in this chapter demonstrate significant upregulations in P2X7 receptor transcript expression in all dystrophic muscle groups tested, offering potential explanation for the heightened $\text{Ca}^{2+}$ influx response observed in these cells (Yeung et al., 2006). Chapter 6 will focus on whether the observed increases in P2X7 mRNA levels in the dystrophic myoblasts and muscles in situ result in increased P2X7 receptor protein expression levels and the possible functional consequences of this for dystrophic myoblasts. The data presented here indicate for the first time an apparent association between expression levels of all P2X7 splice variants, which in dystrophic skeletal muscle appears to be uniformly elevated, with the exception of the diaphragm where P2X7k variant appears differentially regulated. The mqPCR technique, although shown here to be a reliable quantitative methodology, did prove less sensitive than standard Taqman qPCR. Such error was however expected, and this work should be repeated using specific Taqman probes to confirm the data shown here.
P2X7 receptor expression and function studies in normal and dystrophic myoblasts

6.1 Introduction

P2X7 receptors are ATP-gated cation channels, facilitating fast excitatory responses to extracellular nucleotides and have been widely studied in inflammatory disorders and cancers. Orthologues have been cloned and characterised from humans (Surprenant et al., 1996), mice (Chessell et al., 1998b), rats (Rassendren et al., 1997), dogs (Roman et al., 2009), guinea pigs (Fonfría et al., 2008), and fish (Lopez-Castejon et al., 2007, Kucenas et al., 2003). Classical agonist sensitivities synonymous with P2X7 receptor activation differ significantly from other P2X receptors; EC$_{50}$ values for ATP at P2X7 receptors are >100 fold higher in mice, rats and humans [e.g. 2.3µM, 1.4µM and 5.5µM for P2X4 (Chessell et al., 1998a) and 936µM, 123µM and 780µM for P2X7 (Young et al., 2007), respectively]. The ATP analogue 2’,3’-O-(4-benzoyl-benzoyl)ATP (BzATP) is a well documented, specific agonist of P2X7 receptors displaying greater potency and current densities than ATP at the mouse orthologue and is less potent than ATP at other P2X receptors (North, 2002). Some significant species differences exist in the kinetics of both agonist and antagonist pharmacologies (Gever et al., 2006), exemplified by the finding that the guinea pig receptor orthologue displays reduced selectivity for BzATP over ATP (Fonfría et al., 2008). Rapid increases in [Ca$^{2+}$], follow agonist application, with agonist sensitivities of the order BzATP>ATP in mice, rats and humans; EC$_{50}$ values for BzATP at mouse, rat and human orthologues expressed in HEK-293 cells are
around 10 fold lower than those observed for ATP [285μM, 3.6μM and 52.4μM, respectively (Young et al., 2007, Chessell et al., 1998a)], hence the rat orthologue is considered more sensitive than that of human or mouse for its native agonist ATP, and more importantly here, the human receptor is actually considered more sensitive to ATP than that of the mouse orthologue. Certain antagonists are considered specific in inhibiting P2X7 receptor responses (Donnelly-Roberts et al., 2009); the most widely used being Coomassie Brilliant Blue G (CBBG) (at 1-2μM), which also evokes some low level inhibition at P2X4 receptors (North, 2002).

Moreover, sustained stimulation (>10s), or stimulation with high concentrations of ATP (1-10mM) in all species cells expressing P2X7 receptors induces the opening of a large non-selective pore, which facilitates membrane permeability to molecules <900Da. Such permeability was originally characterised in lymphocytes and since observed in multiple cell types. This event is usually coupled to membrane ‘blebbing’ and phosphatidyl serine translocation to the outer membrane leaflet (Skaper et al., 2010). The functional mechanisms and relevance of these seemingly dramatic cellular responses remain to be defined. Yet recently, P2X7a variant receptor expression has been shown to be up-regulated in the dysferlin knockout mouse model of LGMD2B, where a role in P2X7-induced, IL-1beta release and inflammasome activation have been proposed to contribute to the disease (Rawat et al., 2010). This is a functional effect more synonymous with macrophage activation (Di Virgilio, 2007) and highlights the potential role of P2X7 in muscle disease.

Growing commercial interest in developing more specific pharmacological inhibitors of the P2X7 receptor has facilitated the disentanglement of P2X7 receptor-mediated responses from those generated by other members of the extended family of membrane-bound purine receptors. It has also sparked great interest from the wider research community. However, the P2X7 receptor field itself is fast becoming
complex. In addition to ATP-induced P2X7 stimulation, NAD has also been shown to activate P2X7 receptors in certain cell types. The recently explored, NAD-induced cell death (NICD), occurring via ADP-ribosylation of P2X7 at Arg125 and Arg133 (Laing et al., 2010), has also been shown to play a significant role in cytolysis-related disorders; In cases such as bacterial infections, endogenous sources of NAD have been shown to be sufficient to induce both P2X7 activation and NICD (Seman et al., 2003b, Scheuplein et al., 2009). The proposed mechanism for the NAD-dependent P2X7 receptor response involves a functional interaction between P2X7 receptors and one of the members of the ADP-ribosyltransferase (ART) ectoenzyme family. The best documented in relation to P2X7 responses is the activity of ART2.2 in T lymphocytes and transfected HEK cells (reviewed in (Schwarz et al., 2009). NAD-mediated calcium fluxes through the P2X7 receptor channel have been reported following ART2.2 mediated ADP-ribosylation of residue R125K of the P2X7 receptor. The transfer of an ADP-ribose moiety from NAD$^+$ to P2X7 is proposed to effect the covalent retention of trimeric ligand binding (Adriouch et al., 2008, Schwarz et al., 2009), thus lower concentrations of NAD elicit activation of P2X7 receptors compared with those required for ATP-mediated activation (EC$_{50}$ for phosphatidylserine exposure being 2µM NAD, compared with 100µM ATP; Seman et al., 2003). ATP and NAD$^+$ both exhibit well documented characteristics of extracellular signalling molecules, and in an environment rich in ATP/NAD$^+$, such as degenerating muscle, it is not inconceivable that signalling mechanisms relating to both mediators may be perturbed.
Figure 6.1. Action of extracellular NAD and ATP on P2X7 receptors. Illustration depicting the ability of high, concentrations of ATP to activate P2X7 receptor ion channel activity. NAD indirectly affects P2X7 receptor function; acting as a substrate of ADP-ribosyltransferases (ARTs), which transfer an ADP-ribose moiety from NAD to covalently occupy the ATP binding sites of P2X7 receptor trimers, constitutively activating the ion channel.

ADP-ribosylation is not the only post-translational modification (PTM) of P2X7 receptors.; Glycosylation of extracellular domains is also a prevalent and important PTM, mediating both correct membrane trafficking of receptor subunits (Young et al., 2007) and the activation of the ERK signalling cascade following agonist-induced receptor activation (Lenertz et al., 2010). Moreover, tyrosine phosphorylation at Tyr343 of the intracellular domain has been proposed to be essential for linking P2X7 receptor activation to cytoskeletal rearrangements and signalling feedback (Kim et al., 2001), and palmitoylation of four regions of the C-terminal tail have been linked to the localisation of P2X7 receptors to lipid rafts (Gonnord et al., 2009).
Yet another layer of complexity in the study of P2X7 receptor-mediated responses stems from the existence of genetic polymorphism and alternatively spliced variants. Single nucleotide polymorphisms or point mutations have been well documented for P2X7 receptors, conferring a multitude of functional implications; such as an N187D mutation; a predicted hyposensitive facilitator of haematopoietic malignancy (Chong et al., 2010), and an A348T mutation linked to enhanced IL-1β secretion (Stokes et al., 2010). Mutagenesis studies have sought to elucidate residues of significant functional relevance in transfected HEK-293 cells and oocytes (Young et al., 2007, Liu et al., 2008, Schwarz et al., 2009) and many studies have indicated the importance of the C-terminal tail in membrane targeting, large pore formation and complex formation (Bradley et al., 2010, Lenertz et al., 2009).

One of the most widely documented functional consequences of P2X7 receptor activation in vitro is the induced Thr/Tyr phosphorylation of the mitogen activated protein kinase (MAPK) family proteins, extracellular signal regulated kinases 1 and 2 (ERK1/2). The mechanism of this event has been suggested to be independent of Ca\(^{2+}\) influx (Amstrup and Novak, 2003, da Cruz et al., 2006, Auger et al., 2005). This interdependence may be cell type specific, as CBBG (by inhibiting Ca\(^{2+}\) influx) has been shown to inhibit P2X7-induced ERK1/2 phosphorylation in microglia (Suzuki et al., 2004) but not in osteoblasts (Okumura et al., 2008). Another possibility is the potential for P2X7 splice variant-dependent regulation of the ERK1/2 signalling cascade. This signalling could theoretically be of significant relevance to the dystrophic pathology, having widely documented roles in the induction of both cellular proliferation and cell death in other systems (Keshet and Seger, 2010). ERK1/2 activation is itself characteristically biphasic, peaking strongly at 5-15mins following agonist application, followed by a lower intensity sustained activation lasting up to 6 hours (Schliess et al., 1996, Kahan et al., 1992, Meloche et al., 1992). Upon phosphorylation, ERK1/2 proteins migrate to the nucleus via integrin-mediated cytoskeletal re-organisation, where they promote cellular
survival/proliferation, facilitating the G1-S phase transition by relaying phosphorylation status to various substrates within the nucleus (Kahan et al., 1992, Meloche et al., 1992), such as c-Fos, Jun, and cAMP response element binding protein (CREB). In addition to the necessity for ERK1/2 phosphorylation and nuclear translocation during cell proliferation, the known functional repertoire of these proteins has recently been widened to include a more intricate balance between cell survival/proliferation and cell death/apoptosis, thought to occur via a coordinated integration of pro- versus anti-apoptotic stimuli. With obvious functional parallels between the documented effects of P2X7 activation and ERK1/2 signalling cascade induction, the P2X7-mediated component of the ERK1/2 phosphorylation response may in future prove to be a more ubiquitously conserved link between cellular life and death, making the environment of dystrophic muscle a very interesting arena for the study of mechanisms pertaining to the regulation of cell proliferation and differentiation in general. Consequently, therapeutic parallels may arise between DMD and other circumstances of abnormal ERK1/2 activation.

6.2 Results

6.2.1 C-terminal P2X7 antibody characterisation

This study has assessed P2X7 protein expression using a novel C-terminal antibody (Synaptic Systems Ltd), which has been extensively characterised by our laboratory. This antibody recognises a specific 78kDa band in lysates of P2X7a transfected HEK-293 cells, which is absent from moc-transfected cells. The same band is also present in brain and TA muscle lysates of mdx mice and and absent from both currently available P2X7 knockout mice, confirming specificity of the identified protein. Glaxo and Pfizer knockout animals express P2X7k and P2X7b, c and h, respectively, although levels are too low to be detectable in Figure 6.2.1; A.
Moreover, de-glycosylation of protein samples from both transfected HEK-293 cells and immortalised dystrophic myoblasts results in a characteristic shift from approximately 78-65kDa (Young et al., 2007), further confirming antibody specificity (Figure 6.2.1).

**Figure 6.2.1. Western blot-based characterisation of C-terminal P2X7 antibody.** The C-terminal P2X7 antibody used in this study demonstrates specific reactivity to the correct size 78kDa band corresponding with the expected size of P2X7 receptor in whole protein lysates of P2X7a transfected HEK-293 cells. Smaller bands may represent protein degradation products or partially de-glycosylated receptors. The same size band is recognised in mdx brain and TA, and absent from both mock-transfected HEK-293 cells and P2X7 knockout
(KO) tissues (A). Following de-glycosylation with PNGase F the specific P2X7 protein band recognised in immortalised dystrophic myoblast lysates demonstrated the expected molecular weight shift to approximately 65Kda (B). Identical band sizes and size shifts on deglycosylation were observed in whole protein lysates of 4 month mdx brain, diaphragm and GC muscles (C).

6.2.2 P2X7 receptor expression and function in immortalised dystrophic myoblasts

Following the demonstration of no significant difference in ectoATPase potential between normal and dystrophic myoblasts (Figure 4.2.1), and the subsequent and novel demonstration of uniformly upregulated P2X7 receptor mRNA transcript expression in dystrophic versus normal myoblasts (Figure 5.2.2; B), expression levels and potential functional consequences of P2X7 protein expression levels have been investigated in normal and dystrophic immortalised myoblast cell lines. Altered P2X7 receptor protein expression/function could potentially represent the source of the previously documented abnormal ATP{	extsubscript{e}}-induced Ca{	extsuperscript{2+}} influx response in dystrophic myoblasts (Yeung et al., 2006).

Significantly higher levels of P2X7 protein expression were found in immortalised dystrophic myoblasts compared with their normal counterparts, where P2X7 expression levels were undetectable using the immunoblot technique (Figure 6.2.2; B). Moreover, in response to high [ATP]{	extsubscript{e}} (500μM), dystrophic cells displayed a rapid and sustained increase in ERK1/2 phosphorylation status; from 40-100% (phospho-p42/p42) activation within 5-10min, an effect mirrored in the response of P2X7a variant transfected HEK-293 cells, but not observed in mock-transfected (P2X7{	extsuperscript{ve}}) HEK-293 cells, and only very weakly detectable in the wild-type immortalised myoblasts, where a small increase in ERK1/2 phosphorylation was observed in the normal immortalised myoblast cells, increasing to approximately 20% maximal activation within the 15min time course (Figure 6.2.2; B). Dystrophic
myoblasts however, actually displayed faster kinetics than transfected cells for the induction of ERK1/2 phosphorylation, achieving maximal activation within <1min, compared with a more gradual increase of induction in the transfected HEK-293 cells.

The hypersensitive ERK1/2 phosphorylation response seen in the dystrophic cells was consistent with significantly higher levels of P2X7 receptor expression in this cell line. ATP induced ERK1/2 phosphorylation in these cells was confirmed as P2X7 dependent through the use of specific agonists/antagonist combinations: a 10 min pre-incubation with CBBG (1μM) was sufficient to completely abolish the ATP-induced phospho-ERK1/2 response in dystrophic myoblasts. The levels of phospho-ERK1/2 response in normal and dystrophic myoblasts exposed to the P2X7 receptor agonist BzATP (300μM) mirrored the responses seen using ATP (500μM) and there were also inhibited by 10 min pre-incubation with CBBG (1μM) (Figure 6.2.2; D).

As P2X4 receptor expression have previously been demonstrated in the normal and dystrophic myoblast lines used here (Yeung et al., 2006), ivermectin (IVM) was employed as a well known positive allosteric modulator of P2X4 mediated ATP responses (Priel and Silberberg, 2004) However, no significant increase in ERK1/2 phosphorylation was observed in cultures of dystrophic myoblasts exposed to ATP (25μM) following pre-incubation with 0.25μM IVM (Figure 6.2.2; D). Moreover, ERK1/2 activation was not observed using 2.5μM ATP, further confirming that the more sensitive P2X4 receptor plays minimal if any involvement in the observed response.

Next, NAD⁺ was confirmed as a potential mediator of P2X7 signalling in immortalised dystrophic myoblasts: 2μM NAD⁺ induced a rapid and strong (yet transient) increase in ERK1/2 phosphorylation in dystrophic cells, whereas the normal myoblast line was completely unresponsive to NAD stimulation at this concentration. No induction of ERK1/2 phosphorylation was observed following NAD⁺ stimulation.
of HEK-293 cells or HEK-293 cells transfected with P2X7a or P2X7k variants (Figure 6.2.2; E), confirming the requirement for ART enzyme activity during P2X7 receptor stimulation via ADP-ribosylation (Seman et al., 2003a).
6.2.3 P2X7 receptor expression in wild-type- and mdx-derived primary myoblasts

In order to reinforce the physiological relevance the upregulations in P2X7 mRNA and protein expression (Figures 5.2.2; B and 6.2.2; B, respectively) found in immortalised dystrophic myoblasts and mdx muscles in situ, P2X7 protein levels were assessed in normal and dystrophic 1st generation primary myoblast cultures. Immunostaining using anti-P2X7 antibody (Synaptic Systems) was also carried out to determine the localisation of P2X7 expressing cells on isolated single living myofibres and derived myoblast cultures in vitro. Anti-P2X7 antibody staining revealed that primary myoblast cultures stained positive for P2X7 expression, again
with varying intensity between myoblasts and myotubes, and also with some degree of variation between individual myoblasts (Figures 6.2.3.2 and 6.2.3.3). Immunochemical staining was not easily quantifiable, but Western blot analysis of protein extracts of primary myoblast cultures confirmed a significant up-regulation in P2X7 protein expression levels in primary dystrophic myoblasts compared with age-matched controls (Figures 6.2.3.2), which was in agreement with the up-regulated mRNA levels observed in immortalised and primary cultures as well as muscle tissues by qPCR (Figure 5.2.2; B).

As has been previously discussed, the classic satellite cell is by no means the only myogenic precursor of adult skeletal muscle. Preliminary immunohistochemical localisation of P2X7 receptor expression in cells attached to single isolated living myofibres suggested that the majority of P2X7 positive cells were Pax7 negative, although their location relative to the basal lamina or indeed the integrity of the basal lamina could not be assessed. Immunocytochemical staining of primary myoblast cultures revealed that cells from both C57 and mdx mice were 100% positive for Myf-5 and P2X7 expression, though intensities varied amongst cultures and between myoblasts and newly formed myotubes, which expressed much higher levels of P2X7 expression.

Co-immunolabelling carried out using freshly dissected muscle groups, collagenase digested to release individual living myofibres, showed that P2X7 receptors were expressed by two populations of mononuclear cells, one positive and one negative for Pax7 expression.
Figure 6.2.3.1. P2X7 receptor localisation in muscle fibres *ex vivo*. Confocal microscope immunolocalisation: micrographs demonstrating P2X7 expression (red signal) in two distinct populations of mononuclear cells present on isolated myofibres: one Pax7 (green signal) positive (A), the other Pax7 negative (B). However another population of P2X7 positive, Pax7 negative cells was also observed, with morphology that could not clearly/consistently be described as mononuclear (C+D). Cell nuclei are visualised by Hoest counterstaining (blue signal).
Figure 6.2.3.2. P2X7 receptor expression in 1st generation primary myoblasts. Brightfield images depicting representative colorimetric immunocytochemistry staining of P2X7 receptor expression in wild-type- (A) and mdx- (B) derived 1st generation primary myoblast cultures (revealed by peroxidise). Primary antibodies were omitted for negative staining controls (C). Representative immunoblot and graphical representation of densitometric analysis demonstrating significantly increased P2X7 receptor protein expression levels in mdx- compared with wild-type-derived primary myoblasts (D); error bars represent SE, df=3 (F;*P<0.01).
Figure 6.2.3.3. **P2X7 receptor expression in 2nd generation primary myoblasts.** Colorimetric immunocytochemistry of primary myoblast cultures showing Myf-5 (upper panels) and P2X7 (middle panels) staining in wild-type- and mdx-derived 2nd generation primary myoblast cultures. Primary antibodies were omitted for negative staining controls (lower panels), cell nuclei were counterstained with Methyl green (blue).
6.2.4 Phosphoprotein analysis following P2X7 receptor stimulation in normal and dystrophic immortalised myoblasts.

Following the demonstration of significantly elevated P2X7 protein expression levels in dystrophic versus normal myoblasts in vitro, a wide aiming mass-spectrometry based approach was adopted to address the immediate functional relevance of the observed differences in Ca\(^{2+}\) influx and ERK1/2 phosphorylation following P2X7 stimulation in normal and dystrophic immortalised myoblast lines. Purified phosphoproteins from both cell types - treated or not with ATP, were separated by charge, iTRAQ labelled and analysed by ‘ESI-LC-MS’ (Agilent 6200 TOF, Agilent Technologies). In ATP-treated dystrophic compared with ATP-treated normal myoblasts, a total of 1272 unique phosphoproteins were identified. Of which, phosphorylation status was observed to be significantly higher for 143, and significantly lower for 121 individual phosphoproteins. In untreated samples, fewer significant differences were observed; from a total of 1272 uniquely identified proteins, 32 displayed significantly increased and 23 significantly decreased phosphorylation status (dystrophic vs. normal). The vast majority of significantly decreased phosphoproteins in ATP-treated dystrophic vs normal myoblasts involved proteins with functions relating to transcription, translation or splicing, such as FACT complex subunit SSRP1, Histone H1.5, Eukaryotic translation initiation factor 5B, 40S ribosomal subunit S6 and Poly(U)-binding-splicing factor PUF60 (2.9, 3.1, 2.5, 2.77 and 2.4 fold down regulated, respectively). In contrast, the majority of significantly increased levels of protein phosphorylation in ATP treated cells were seen in cytosolic proteins considered to be involved in a more immediate functional signalling cascade induction response, such as protein kinase C delta type (PKC\(\delta\)), ezrin, radixin, moesin and annexin A6 (2.7, 2.3, 1.4, 2.0 and 2.3 fold up-regulated, respectively). Significant ATP-induced phosphorylation effects (mdx/wild-type) on these and other proteins of particular functional interest are shown in Figure 6.2.4; B.
Figure 6.2.4. Changes in protein phosphorylation status in normal and dystrophic immortalised myoblasts in response to ATP. iQuantitator generated schematic illustrating positive (green) and negative (red) fold changes in protein phosphorylation status following treatment with ATP (500 μM). Large circles represent average values, extended arms linking
smaller circles represent SE, df=2. Significant and non-significant values are depicted as opaque and translucent bars respectively, all values shown represent mdx vs. wild-type. Top 30 average fold changes in protein phosphorylation status in ATP-treated (A) and un-treated (C) cells are shown, plus a selection of other significant differences in phosphorylation status in ATP-treated cells relating to proteins of specific interest (B).

6.3 Discussion

The demonstration that P2X7 receptor protein expression is significantly up-regulated in dystrophic myoblasts represents a novel finding. Western blot analysis demonstrated that this up-regulation coincided with a functional difference in the P2X7-mediated phospho-ERK1/2 signalling response following stimulation with high [ATP]_e, suggesting that the P2X7 receptor may have a significant functional role in the dystrophic milieu surrounding degenerating muscle where such high [ATP]_e may be common (Ryten et al., 2004). ERK1/2 are classically associated with cellular proliferation (Sharma et al., 2003, Cobb et al., 1991, Kim and Choi, 2010) and more recently, roles for ERK1/2 in mediating cell death, apoptosis, autophagy and senescence (Cagnol and Chambard, 2010) have been shown. Thus, the balance between a healthy proliferation and excitotoxic cell-death may depend on the intensity or duration of the pro- versus anti-apoptotic stimuli transmitted via ERK1/2 (Pearson et al., 2001). Indeed, it is now thought that constitutive ERK activation is sufficient to ultimately induce cell death in certain systems (reviewed in (Martin and Pognonec, 2010), although the intricacies of the functional mechanisms mediating this balance remain to be elucidated and are thought to occur via a coordinated integration of pro-versus anti-apoptotic stimuli. ERK1/2 proteins mediate the latter through phosphorylation/post-translational modification of several pro-apoptotic factors, such as the tumour suppressors FasL and BAD, which ERK/12 proteins inactivate through interaction with the FOXO and RSK families of transcription factors respectively.
The pro-apoptotic/autophagic/senescent routes are less well characterised. Here, ERK1/2 proteins are again thought to co-ordinate the relevant stimuli, but elucidation of the complete mechanism remains incomplete. P53 activation, DNA damage, ROS, death activated protein kinase (DAPK) phosphorylation, FasL cross-linking, and Akt repression, mitochondrial cytochrome c release and caspase-8 activation have all been implicated in this regard (comprehensively reviewed by (Mebratu and Tesfaigzi, 2009) and (Cagnol and Chambard, 2010).

The novel data demonstrate not only P2X7 induced ERK1/2 activation in myoblasts but also a specific functional difference in both basal and maximal levels of ERK1/2 phosphorylation in dystrophic immortalised myoblasts compared with their normal counterparts, suggests a possible route for therapeutic intervention in dystrophic muscle. However, the complexities of such mechanisms are exemplified by the observation that small molecule activators of ERK1/2 such as Resveratrol, appear to exert their effects in a cell/tissue specific manner, while reducing the invasiveness of some tumour types through apoptosis (Maher et al., 2011, Lin et al., 2011), yet suppressing chemically induced apoptotic cell death in other tumour types (reviewed in (Gupta et al., 2011) and actually inducing a protective effect in Huntington’s disease (Kim and Choi, 2010). Hence, any pharmacological strategy targeting the ERK1/2 mediated component of muscle cell death in DMD would have to carefully consider the particular target component and its effect on other aspects of cellular viability. From the currently available literature, inhibitors of Death Associated Protein Complex (DAPK) phosphorylation/cytoplasmic accumulation would be potential candidates (Mebratu and Tesfaigzi, 2009). However, whether DAPK phosphorylation/accumulation is up regulated in dystrophic muscle or muscle in vivo is unknown. ATP_e induced apoptosis was assayed for in immortalised normal and dystrophic myoblasts prior to primary cultures becoming available – as predicted, immortalised lines proved un-responsive to staurosporine or ATP_e induced apoptosis (Figure Appendix 2).
Functional hetero-oligomerisation has been documented between P2X7/P2X4 receptors following their co-expression in HEK-293 cells (Guo et al., 2007) and also as endogenous receptors in macrophages (Boumechache et al., 2009), and P2X4 has also been shown to induce phosphorylation of ERK1/2 proteins (Inoue, 2006). Therefore, dystrophic myoblasts were co-stimulated with ATP and IVM (as a positive allosteric modulator of P2X4) to elucidate any P2X4-mediated component of the purinergic response observed in these cells; low micro-molar concentrations of IVM (2.5μM), have been shown to increase P2X4-mediated Ca^{2+} influx by up to 10 fold (Priel and Silberberg, 2004, Khakh et al., 1999). Although low levels of ERK1/2 phosphorylation were induced by low [ATP]e [25μM; sufficient to fully activate P2X4 (EC_{50}, 2.3μM; (Jones et al., 2000), but not P2X7 receptors (EC_{50}, 936μM; (Young et al., 2007)] in immortalised dystrophic myoblasts, the magnitude of this response was approximately 5 fold lower than observed using higher concentrations (500μM), and the same cells proved unresponsive at lower concentrations of ATP (2.5μM) (data not shown). Moreover, the lack of a significant increase in Ca^{2+} influx (data not shown) or ERK1/2 phosphorylation in response to co-stimulation with ATP (25μM) and IVM (0.25μM) in our cells suggests that although a P2X4-mediated response may be present, it can be considered minimal in comparison to the P2X7-mediated response in these cells.

P2X7a and P2X7k variants have recently been shown to differ in their sensitivities to ADP-ribosylation; low micromolar concentrations of extracellular NAD trigger P2X7-dependent calcium influx and large pore formation in P2X7k variant transfected HEK-293 cells, but not cells transfected with P2X7a variant (unpublished data from Murrell-Lagnado, Cambridge). This difference in sensitivity has been shown in HEK-293 cells co-transfected with P2X7k and ART2.2, as these cells are deficient in the expression of ADP-ribosyltransferase enzymes (Schwarz et al., 2009). ART1 has been shown to be the predominant ADP-ribosyltransferase
variant expressed in skeletal muscle, (being originally cloned from rabbit muscle; (Zolkiewska et al., 1992), and although ART1 and ART2 genes are both expressed in mouse and human skeletal muscle (Glowacki et al., 2002), only ART1 expression has been shown to be expressed in cells of myogenic lineage, and only in vitro, where it proved undetectable in C2C12 and C3H-10T myoblast lines, yet was strongly up-regulated upon the induction of differentiation in the same cell lines (Zolkiewska et al., 1992, Friedrich et al., 2008). Considering the significant difference in P2X7k variant expression levels between normal and dystrophic immortalised and primary myoblast lines, and the NAD-induced ERK1/2 phosphorylation response specific to the dystrophic myoblast cell line it would seem a logical progression to quantitate any differences that might exist between the levels of ART1 expression in these cell lines. Direct measurements of ADP-ribosylation levels of P2X7 receptors in these cells could be obtained through treatment with etheno-NAD (a covalent antagonist of NAD-mediated ADP-ribosylation), followed by immunoprecipitation for P2X7 and immunoblotting using an etheno-adenosine specific antibody (Krebs et al., 2003, Friedrich et al., 2008).

Having demonstrated an abnormally high level of functional P2X7 expression in the immortalised and primary dystrophic myoblast cell lines by qPCR (Figure 5.2.2; B), this study has confirmed the potential for a specific purinergic phenotype to be operating in dystrophic muscle by demonstrating significant up-regulations in P2X7 receptor protein levels in both immortalised and primary dystrophic myoblasts (Figures 6.2.2 and 6.2.3.2, respectively). Results presented here not only report abnormalities in P2X7 receptor expression between normal and dystrophic myoblasts, but also question the findings of previous studies proposing a sovereign role for P2X5 in muscle development and myoblast differentiation (Ryten et al., 2002, Ryten et al., 2004). ATP induced ERK1/2 signalling has previously been demonstrated in myoblasts (Bennett and Tonks, 1997, Jones et al., 2001) and proposed to be P2X receptor dependent (Banachewicz et al., 2005). Burnstock’s group have proposed that
P2X5 is most salient in this regard, and a role in myoblast differentiation has been proposed (Ryten et al., 2002, Ryten et al., 2004). Surprisingly, this work has never been confirmed or extended, and indeed, several inconsistencies are present in the results presented by both Banachewicz and Burnstock: Firstly, the proposal by Banachewicz that C2C12 myoblasts display a specific \( \text{Ca}^{2+} \) dependent, P2X5-mediated response is unconvincing given the concentration of ATP (100\( \mu \text{M} \)) required to induce the documented effect, which is by far more reminiscent of a P2X7 than a P2X5 mediated response. Moreover, the ATP induced \( \text{Ca}^{2+} \) influx response they reported was actually activated by the specific P2X7 agonist BzATP (100\( \mu \text{M} \)) alone. Burnstock also chooses to use uncharacteristically high concentrations of ATP (100\( \mu \text{M} \)) for the induction of the proposed P2X5-mediated phosphoERK1/2 response, yet lower concentrations of ATP (10\( \mu \text{M} \)) were used in examining the more sensitive \( \text{Ca}^{2+} \) influx response. Moreover, ERK1/2 activation is itself not normally associated with P2X5 receptor activation and has only been well characterised following P2X4 and P2X7 stimulation. Therefore, it is unclear why these studies chose to ignore the potential involvement of P2X7 receptors, despite detecting its transcript in myoblasts (Banachewicz et al., 2005, Ryten et al., 2004) - it may be because the initial publications relating to P2X receptor involvement in muscle development focussed on P2X5 and P2X6 receptors (Meyer et al., 1999); In the light of the current study, a logical progression would be to characterise P2X7 receptor expression patterns in developing muscle. However, due to continuing difficulties with antibody specificity in immunohistochemical staining and the rapid advancements in the development of more specific P2X7 antagonists, investigation into the role of P2X7 in development and myoblast proliferation/differentiation would be better conducted using agonist/antagonist manipulation of specific cellular events. Importantly, P2X7 up-regulation in \textit{mdx}-derived primary myotubes has recently been documented (Rawat et al., 2010), alluding to the potential for a similar mechanism to be potentiating the pathology of DMD muscles and their mononuclear precursors.
As previously mentioned, a broadly aimed phosphoprotein characterisation was attempted using a mass spectrometry-based approach in an attempt to elucidate the immediate downstream functional consequences of P2X7 receptor activation in these cells. The rationale for this focussed on the possibility of elucidating other components of the P2X7-ERK1/2 signalling cascade. Maximal fold changes of ~4 were detected using this approach, which is in keeping with similar studies in the literature (Mayya and Han, 2009). The effect of charge bias affecting the mass spectrometry sample column seems more plausible, given the large bias in protein identification towards that of nuclear proteins. Yet this does not explain the identification of the ERM proteins being significantly (>1.5 fold) up regulated in dystrophic cells, unless the degree of phosphorylation of these proteins is large enough to remain undimmed by the issues of sensitivity. However, phosphoproteins are characteristically troublesome with regard to mass spectrometry due to their potential for multi-phosphorylation (ERK1/2 are dually phosphorylated) and their existence in low abundance of total protein samples. Hence, with the advent of more specific methodologies, perhaps a phosphoprotein based assessment of particular groups of related phosphate modifications, or use of more permanent modification techniques, such as Stable Isotope Labelling of cells (SILAC) (reviewed in (Mayya and Han, 2009) should be attempted. Moreover, full clarification of the significance of the “hits” would require Western blot based detection of these phosphoproteins detected by mass spectrometry and comparison of their phosphorylation status with that of the ERK1/2 proteins.

Of particular interest in the obtained phosphoprotein data are the significant up-regulations in phosphorylation status of the ERM proteins ezrin, radixin and moesin (Tsukita and Yonemura, 1997, Takeuchi et al., 1994, Bretscher, 1999). ERM represents a well characterised actin binding complex of proteins which has been implicated in the determination of cell shape, migration and proliferation (Denker et al., 2000, Ivetic and Ridley, 2004). Protein phosphorylations observed for ATP-
treated dystrophic vs normal myoblasts suggest that this protein complex may offer potential avenues for investigating some other rapid ATP<sub>e</sub>-induced responses observed in these cells - specifically that dystrophic cells detached from adherent surfaces within minutes of the addition of high [ATP]<sub>e</sub> (>5mM). The ERM proteins link integral membrane proteins to the actin cytoskeleton (Ivetic et al., 2004) and have been localised to focal adhesions and lamellopodia. Upon phosphorylation, ERM proteins undergo structural changes induced by relief of intramolecular interactions, generating an active conformation. In their active conformation, ERM proteins have been shown to bind actin through their carboxy-terminus and through their amino-terminus, to bind cell adhesion molecules (Integrin β2; (Tang et al., 2007), scaffolding molecules (EBP50; (Fouassier et al., 2000), and signalling transducers (Rho GDI; (Mammoto et al., 2000). The localisation of ERM proteins to focal adhesions and lamellopodia suggests that they may play a role in the ATP-mediated cellular detachment observed on exposure of dystrophic myoblasts to high [ATP]<sub>e</sub> (Yeung et al., 2006).

Another conspicuous phosphoprotein of potential significance is PKCδ - generally regarded as an inducer of cell cycle arrest or differentiation induction, PKCδ activation has also been linked to pro-mitotic stimuli, of particular interest here, is that mediated by ERK cascade induction, although such effects are very much cell type specific (Steinberg, 2004). Annexin A6 may also be of relevance in this context; a calcium dependent membrane protein expressed in skeletal muscle, where functional interactions with PKC have been proposed to act as a negative effector of MAPK signalling and calcium channel function by Ca2+ induced translocation of annexin A6 to the plasma membrane (Grewal et al., 2005, Grewal et al., 2010). Cofilin and destrin have been highlighted due to their proposed roles as actin depolymerisation factors (Liu et al., 2007) with documented roles in skeletal muscle in relating to potential abnormalities in actin depolymerisation in myopathies.
Specific functions for these phosphoproteins in myoblasts have not been documented, although coflin has previously been referred to as the generic “steering wheel” of the cell, defining cell motility (Ghosh et al., 2004). Nestin, a well known intermediate filament protein and stem cell marker has recently been ascribed a phosphorylation-dependent role in neuromuscular junction formation (Yang et al., 2011), and although nestin functions in mononuclear myogenic precursors are less clearly defined, a role in differentiation has been suggested (Cui et al., 2009, Kitai et al., 2010).

Interestingly, the actin binding protein zyxin was found to possess a significantly higher basal phosphorylation status in dystrophic compared to wild-type myoblasts (untreated). Zyxin phosphorylation has itself been linked to reduced cell-cell and cell-substrate adhesion through increased LIM domain availability (Call et al., 2011) in a similar manner to that involved in ERM protein activation.

In conclusion, this chapter presents the results of a mass spectrometry-based investigation into potential downstream consequences of P2X7 receptor activation in immortalised dystrophic myoblasts in vitro. Although the data have been generated following treatment with ATP rather than the more specific P2X7 agonist BzATP, sufficiently interesting ‘hits’ have been generated, which warrant further investigation to determine their role in the P2X7 specific response to ATP_e. In particular annexin A6, who’s membrane associated involvement in the IL-1β response in muscle may parallel that seen in macrophages (Grewal et al., 2010), which would be a novel finding and of potential therapeutic relevance to DMD pathology.
7

Effect of micro-dystrophin gene transfection on P2X7 receptor responses in immortalised dystrophic myoblasts.

7.1 Introduction

The issue of delivering the large (14kb) full length dystrophin cDNA to effect artificial expression of partially functional truncated dystrophin proteins has been addressed in two different ways; through the continued development of larger capacity vectors and the use of truncated dystrophin transcripts retaining the required functional elements. Reductions in transgene size have been facilitated following the discovery that large sections of the dystrophin transcript can be removed or altered without significant effect on its function. Several studies have sought to assess the impact of various dystrophin truncations; N-terminal deletions resulting in a BMD phenotype when expressed in the *mdx* mouse have been described (Corrado et al., 1996). It has been demonstrated that the C-terminal region is not required for the assembly of the DGC (Crawford et al., 2000) and that a truncated variant found in a BMD patient, lacking large parts of the central rod-domain retained most of its function (England et al., 1990). This work was subsequently extended to show that the presence and configuration of certain segments of the rod domain were required for functionality to be retained (Harper et al., 2002). The cysteine rich domain of dystrophin is thought to be crucial for functional retention of the DGC complex (Rafael et al., 1996), as demonstrated by DMD patients presenting with a single base pair deletion in this region (Tsukamoto et al., 1994). On the other hand, cDNA
constructs encoding the small, N-terminally truncated isoforms of dystrophin, e.g. Dp71 not only failed to improve the dystrophic pathology but had a detrimental effect (Greenberg et al., 1994, Cox et al., 1994). Therefore, therapeutic attempts to deliver dystrophin mRNA focus on internally truncated mini- or micro-dystrophin constructs lacking the majority of the central rod domain, only retaining the functional N and C termini, WW, EF and ZZ domains. Such constructs transfected into dystrophic progenitor cells have been widely used in attempts to revert the dystrophic pathology through in vivo/in vitro expansion of the dystrophin positive myofibre population (Acsadi et al., 1991, Vincent et al., 1993, Yang et al., 1998, Rodino-Klapac et al., 2010, Rodino-Klapac et al., 2011). In the context of P2X7 mediated signalling, myoblasts transfected with such constructs have been employed by our lab in order to elucidate the specific effect of dystrophin on P2X7 expression and signalling. Specific mini-dystrophin cDNAs and immortalised dystrophic myoblasts stably expressing these constructs were a gift from Dr Rolf Stucka (Friedrich Bauer Institute, Munich, Germany).

7.2 Results

7.2.1 Characterisation of micro-dystrophin expressing clones

Four micro-dystrophin transduced immortalised myoblast lines were available for analysis: clones # C8; containing 3759bp cDNA, 25-14a; 3435bp, 125-63; 3705bp and 24W57; 3696bp. Predicted molecular weights of resultant mini-dystrophin proteins were: C8: 127kDa, 25-14a; 115kDa, 125-63 and 24W57 both 125kDa (Figure 7.2.1; A). These established cell lines were characterised for the expression of their mini-gene constructs by PCR using primers specific for regions spanning those lost in truncated mini-dystrophin transcripts. PCR products were confirmed as specific to their region of interest, being absent from both the normal and dystrophic
immortalised un-transfected myoblasts, where the primers are separated by ~9kb of extra sequence that was removed when creating the mini-dystrophin transfectants. Multiple experiments demonstrated the C8 clone to be consistently positive for the presence of the construct in the genomic DNA (Figure 7.2.1; B) and for expression of mRNA (Figure 7.2.1; C) and the 25-14a and 125-63 clones both demonstrated lower levels of expression of their respective transgenes (Figure 7.2.1; B and 7.2.1; C, respectively) while the 24W57 clone was consistently negative for the presence and expression of the transgene.

Restriction digests were carried out to further confirm specificity of the PCR products amplified in the three positive clones: Sac1 digestion produced restriction products of the expected sizes (Figure 7.2.1; D). Both BamH1 and Xho1 produced the expected restriction products for the C8 clone but did not cut amplicons from the 25-14a clone, whereas BssS1 produced the expected band sizes in both the C8 and 25-14a clones. This is consistent with the sequence data, where the 25-14a is 324bp shorter than the C8 clone, and both BamH1 and Xho1 had predicted restriction sites within 200bp downstream of the deletion point, whereas BssS1, Sac1 and Hind3 all have their predicted restriction sites over 400bp downstream (and hence outside) of the deleted region (based on a restriction map generated from the known sequence of the 24W57 clone).

The clone expressing the largest mini-dystrophin gene; C8 was selected for further analysis. Interestingly, attempts to show transgene protein expression by immunoblotting using a C-terminal anti-dystrophin antibody to detect the transgene product in total protein lysates from this cell line proved not reproducible, whereas the same construct transiently expressed in HEK cells gave the expected size protein product (data not shown). Immortalised dystrophic myoblasts stably transfected with an empty plasmid vector were used as controls in all experiments.
Figure 7.2.1. Characterisation of micro-dystrophin transfected H2Kb-tsa58/mdx-derived myoblasts. Transgene expression levels were assessed in four stably transfected immortalised dystrophic myoblast lines containing slightly different, CMV promoter driven, micro-dystrophin constructs (A). PCR of specific micro-dystrophin gene region in gDNA (B) and RT-PCR of the same region demonstrated that three of the four clones expressed their transgene, albeit at different levels, with 24W57 being consistently negative for expression (C). Amplification product specificity was further confirmed by restriction enzyme digestion using Sac1. Restriction fragments of the expected sizes were obtained in all three of the four clones expressing detectable levels of transgene cDNA (D). Figure panel (A) adapted from a diagram kindly provided by Prof. Hans Lochmüller.
7.2.2 P2X7 expression and function studies in the C8 cells

The demonstration of abnormally elevated P2X7 receptor expression levels in dystrophic versus normal myoblasts and muscles in situ invites questions as to whether the phenomenon is functionally linked to the dystrophic phenotype, i.e. does dystrophin expression functionally affect P2X7 expression in the mdx mouse model of DMD? P2X7 protein expression levels were observed to be effectively normalised in the C8 micro-dystrophin transduced immortalised dystrophic myoblast cell line. P2X7 receptor expression was virtually undetectable in protein lysates from the mini-gene expressing dystrophic immortalised myoblast, a profile undistinguishable from the wild-type cells. At the same time immunoblotting using the same anti-P2X7 antibody demonstrated a significantly higher level of P2X7 receptor protein in the control- (empty vector transfected) dystrophic myoblasts. Thus the empty vector transfected dystrophic myoblasts displayed characteristics of the un-transfected dystrophic line (Figure 7.2.2; A). In the next step, immunoblot analysis using anti-ERK1/2 and anti-phospho-ERK1/2 antibodies revealed that the normalisation of P2X7 receptor expression levels in the C8 cell line coincided with the significant reduction of ATP induced ERK1/2 phosphorylation in the mini-dystrophin transfected cells, bringing it to levels more reminiscent of the wild-type myoblast line (Figure 7.2.2; B).

To further analyse the efficacy of various mini-dystrophins and variable expression levels, all stable transfectants were tested by a colleague for Ca\(^{2+}\) influx following ATP stimulation. This analysis demonstrated clear differences in response with the strongest Ca\(^{2+}\) influx effect in C8 cells and no normalisation in 24W57 cells, which were shown to have no detectable mini-gene expression (Wojtek Brutkowski; data not shown).

Further proof for the role of dystrophin in maintaining a proper P2X7 function was sought through shRNA mediated knock-down of dystrophin in wild type
immortalised myoblasts. However, despite using a commercial system consisting of lentivirus expressing shRNA, multiple attempts and modifications suggested by the manufacturer were unsuccessful and dystrophin mRNA levels remained unchanged in transduced normal myoblast cells (data not shown).

Figure 7.2.2. Normalisation of P2X7 expression and signalling in micro-dystrophin transfected H2K\textsuperscript{b}-tsA58/mdx-derived myoblasts. Representative immunoblots showing normalised levels of P2X7 receptor protein in H2K\textsuperscript{b}-tsA58/mdx immortalised myoblasts stably expressing the C8 micro-dystrophin construct (A). The effect of micro-dystrophin transgene expression also extended to the normalisation of the ATP\textsubscript{c}-induced phospho-ERK1/2 response in these cells (B). \textit{mdx\textsuperscript{C8}} and \textit{mdx\textsuperscript{ev}} denote H2K\textsuperscript{b}-tsA58/mdx myoblasts containing C8 micro-dystrophin and empty vector, respectively. All immunoblots represent triplicate experiments.
7.3 Discussion

The finding that the expression of a functional, albeit truncated, dystrophin mini-gene in an immortalised dystrophic myoblast cell line effectively reverted the hypersensitive purinergic phenotype of these cells to that of their normal counterparts is intriguing. PCR based analysis convincingly showed that the mini-gene construct had been stably inserted and was being expressed. It is possible that very low levels of dystrophin located in specific sub-membrane domains or even dystrophin mRNA directly are involved in some unknown regulatory mechanism in these cells. The first indication that dystrophin expression could be linked to P2 receptor function was the work of (Ferrari et al., 1994) who provided evidence that lymphoblastoid cells from DMD patients displayed a purinergic phenotype which rendered them highly sensitive to large increases in [Ca\(^{2+}\)], in response to high [ATP]. The important role for dystrophin mRNA and/or protein in myoblasts is again eluded to by the recent finding of metabolic abnormalities in dystrophic cells (Onopiuk et al., 2009) and by abnormally rapid differentiation of isolated dystrophic myoblasts in vitro (Yablonka-Rouveimi et al). The finding that P2X7 receptor protein expression is up-regulated in dystrophic myoblasts has parallels with previous studies; lymphoblastoid cells and myoblasts both express very low levels of dystrophin protein, yet the effect on the purinergic phenotype in both cell types is profound, suggesting the possibility for dystrophin protein or mRNA co-ordination/involvement in P2 receptor expression/recruitment and also potentially, for an important role for dystrophin in myoblasts.

Dystrophin isoform expression has been shown to be intricately regulated in both developing/regenerating and mature muscle tissue; Dp71, the shortest of the dystrophin isoforms comprising a unique seven amino acid N-terminal sequence followed by only the cysteine rich and C-terminal domains of dystrophin (Bar et al., 1990) has been shown to be ubiquitously expressed but excluding skeletal muscles,
where its expression is strictly confined to myoblasts (Rapaport et al., 1992). The regulatory role of MyoD expression on the efficacy of Sp1/3 transcription factor binding to Sp boxes upstream of the Dp71 explains Dp71 promoter down-regulation upon the induction of myoblast differentiation (de Leon et al., 2005). Regulation of Dp427, the full length dystrophin gene product transcribed from the M-type muscle-specific promoter, is achieved through the competitive binding of the zinc finger nuclear factor YY1 to CArG elements recognised by dystrophin promoter bending factor (DPBF) DNA binding protein (Galvagni et al., 1998) and this mRNA is mostly expressed upon myogenic differentiation and hence this isoform is the predominant variant found in mature myofibres (Lev et al., 1987). Interestingly, Dp71 is expressed in mdx myoblasts but it does not prevent the metabolic and purinergic abnormalities.

The mini-gene transduced cells used in this study utilised cDNA dystrophin isoforms truncated through deletion of large sections of the central rod domain but containing the N-, cystein-rich and C-terminal domain of the full-length isoform. It appears, therefore that it is the 5′-end or the N-terminal domain of the full-length transcript that may be essential for the maintenance of normal ATP responses in myoblasts. Surprisingly, following cell transfection, mini-dystrophin protein was not detectable in cellular lysates. Possible explanations for this could include low expression due to rapid promoter methylation, micro-RNA control mechanisms, premature mRNA degradation through immediate ubiquitination of the mRNA molecules, or the existence of an RNA storage mechanism for specific 5′-end containing sequences in these cells.

The full length dystrophin transcript requires ~16 hours to be transcribed and is co-transcriptionally spliced (Tennyson et al., 1995), so it would not be unreasonable to suppose that this process may begin in the myoblast some time prior to differentiation in order to optimise translation times upon differentiation. Indeed, perinuclear nuclear distributions of multiple DAPC complex components including dystrophin have been documented in undifferentiated human myoblasts, with
cytoplasmic localisation evident after 4 days of differentiation (Trimarchi et al., 2006). The storage of DAPC complex members in perinuclear locations prior to the onset of differentiation implies distinct functional relevance for other cellular pathways – for any storage mechanism must be monitored and regulated, thus it is not inconceivable that in the absence of dystrophin, such pathways would never reach a state of readiness, and any overriding inductions of differentiation would be accompanied by inappropriate signalling check point controls, potentially, in some way involving P2X7-mediated signalling cascades. However, in conclusion, insufficient literature is currently available to conclusively support a functional link between Dp427 and P2X7 expression, although the experiments using the C8 clone described here suggest some kind of mechanistic link.
Analysis of P2X7 receptor protein expression in 4 month wild-type and mdx muscle groups

8.1 Introduction

ATP has been known since 1983 to possess extracellular transmitter-like properties when Kolb & Wakelam demonstrated such action in 12-day cultures of chick derived myotubes (Kolb and Wakelam, 1983). ATP-induced cation influx in skeletal muscle was demonstrated soon after by (Haggblad et al., 1985) in similar chick derived myotube cultures. A role in the developmental initiation of myoblast fusion has been assigned to both P2X5 and P2X6 receptors who’s expression has been shown to disappear immediately prior to myotubes formation in developing chick and rat muscle (Meyer et al., 1999, Ryten et al., 2001, Ryten et al., 2002). The same group also conducted the first analysis of P2 receptors in mdx skeletal muscle, finding P2X5 to be expressed on regenerating fibres and in particular on a sub-population of activated satellite cells (myoblasts) (Ryten et al., 2004). Our laboratory also demonstrated localized expression of specific P2X receptors in DMD and mdx muscles with P2X1 and P2X6 co-expressed in small regenerating muscle fibres (Jiang et al., 2005). Moreover, previous work in our lab has shown the expression of all P2X mRNA transcripts at the 3 day, 4 week and 4 month stages of development in the mdx mouse (Yeung et al., 2006), and a significant increase in expression of P2X7 protein expression in 3 week mdx compared to normal (by quantification of immunohistochemical staining intensity) which were surmised to be in agreement with previous reports of P2X7 expression in dystrophic macrophage lines (Ferrari et al., 2005).
al., 1994) and their infiltration into degenerating dystrophic muscle (Kominami et al., 1987). The perplexing observation had been the lack of a significant difference in purinergic receptor protein levels, which could explain the significantly higher Ca\textsuperscript{2+} influx in response to high [ATP\textsubscript{e}] in \textit{mdx} myoblasts compared with their wt counterparts (Yeung et al., 2006). As described in Chapter 4, this, combined with increased P2Y-type responses led to the hypothesis that the purinergic phenotype may be due to lower ectoATPase activity and resulted in characterisation of the extracellular hydrolysing potential of these cells (see Chapter 4). This analysis however showed no significant difference that could explain the dystrophic purinergic phenotype in \textit{mdx} cells. It was only when quantitative RT-PCR and a novel C-terminal rabbit polyclonal P2X7 antibody (Synaptic Systems) were used that a significant up-regulation in P2X7 receptor expression was found in dystrophic myoblasts compared with their C57 counterparts (see Chapters 5 and 6). The use of immortalised myoblast cultures invites obvious questions as to the physiological relevance of any conclusions drawn from such a model. Hence the immediate requirement was to confirm that our observations could be extrapolated from our immortalised cell cultures to primary myoblasts and to the organs from which these were derived. Therefore, levels of P2X7 protein expression in C57 and \textit{mdx} hind-limb primary myoblasts and in muscle groups were analysed by Western blot using freshly dissected tissues. 4 months of age was selected as the time point for this analysis due to the absence of high grades of mononuclear (immune) cell infiltrations in muscles at this age, contrary to younger \textit{mdx} animals (Coulton et al., 1988, Carnwath and Shotton, 1987). At 4 months, the majority of infiltrating cells (and macrophages in particular) disappear from \textit{mdx} muscles (Yeung et al., 2004). This was imperative, as sub-populations of cells infiltrating dystrophic muscle have been shown to express both P2X4 and P2X7 receptors (Yeung et al., 2004, Yeung et al., 2006) which might influence our attempts to study P2X7 receptors in myogenic cells themselves. Hind limb muscle groups were selected for analysis due to the large body
of literature available describing the dystrophic phenotype in these muscles at varying ages. Therefore, P2X7 protein expression was assessed in TA, GC, Sol and FDB muscle groups, as well as in, diaphragm, as this muscle is known to show more continuous degeneration throughout mdx life.

8.2 Results

8.2.1 Analysis of P2X7, ERK1/2, phospho-ERK1/2, and F4/80 protein expression in normal and mdx muscle groups at 4 months

Abnormally high levels of P2X7 receptor expression have been so far show here to be upregulated at the mRNA and protein level in immortalised and primary myoblast cultures in vitro. This chapter serves to extend the PCR-based analysis of P2X7 receptor expression in mdx muscles in situ described in Chapter 5 by examining P2X7 receptor protein expression in tissues from the same animals. Western blot analysis of 4 month C57BL10 and mdx- muscle groups showed P2X7 receptor protein expression to be significantly up-regulated in four out of six muscle groups tested: TA, GC, soleus and diaphragm, displayed fold changes in mdx vs.wild-type expression of 2.4, 2.6, 2.2 and 2.5, respectively (Figure 8.2.1; A and C; *P<0.001). Co-existent, significant elevations in basal ERK1/2 phosphorylation status were observed in mdx TA and soleus muscle groups compared with those of age-matched wild-type animals, with fold changes of 1.6 and 1.4, respectively (*P<0.01) (phospho-ERK1/2 normalised to total ERK1/2; Figure 8.2.1; C). ERK1/2 levels did not significantly differ between wild-type and mdx GC muscle groups (P>0.05; Figure 8.2.1; C), although this would appear to be due to higher experimental variability rather than a tissue specific effect considering the SE variance involved. Significantly elevated P2X7 receptor expression was also detected in mdx diaphragm muscles of the same animals (Figure 8.2.1; B and C; *P>0.001).
No significant differences were found in pan macrophage marker F4/80 protein expression between individual muscle groups of age matched normal and dystrophic animals (P>0.05; Figure 8.2.1; E), and indeed, F4/80 expression proved undetectable in all muscle groups using the immunoblotting technique.
(A) | TA | GC | Soleus
---|---|---|---
mdx | WT | mdx | WT | mdx | WT
Dp427 | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) | 427Kda
P2X7 | ![Image](image5.png) | ![Image](image6.png) | ![Image](image7.png) | ![Image](image8.png) | 78Kda
Phospho p44/p42 | ![Image](image9.png) | ![Image](image10.png) | ![Image](image11.png) | ![Image](image12.png) | 44Kda
p44/p42 | ![Image](image13.png) | ![Image](image14.png) | ![Image](image15.png) | ![Image](image16.png) | 42Kda
β-actin | ![Image](image17.png) | ![Image](image18.png) | ![Image](image19.png) | ![Image](image20.png) | 42Kda

(B) Diaphragm
---
mdx | WT
---
P2X7 | ![Image](image21.png) | ![Image](image22.png) | 78Kda
β-actin | ![Image](image23.png) | ![Image](image24.png) | 42Kda

(C) Fold difference in P2X7 expression (mdx/C57)
---
TA | GC | Sol | Dia.
---
wild-type | ![Image](image25.png) | ![Image](image26.png) | ![Image](image27.png) | ![Image](image28.png)
mdx | ![Image](image29.png) | ![Image](image30.png) | ![Image](image31.png) | ![Image](image32.png)

(D) Fold difference in phospho-p44/p42 (mdx/C57)
---
TA | GC | Sol
---
wild-type | ![Image](image33.png) | ![Image](image34.png) | ![Image](image35.png)
mdx | ![Image](image36.png) | ![Image](image37.png) | ![Image](image38.png)

(E) | TA | GC | Soleus | Spleen
---|---|---|---|---
mdx | C57 | mdx | C57 | mdx | C57 | C57
---
F4/80 | ![Image](image39.png) | ![Image](image40.png) | ![Image](image41.png) | ![Image](image42.png) | 130Kda
β-actin | ![Image](image43.png) | ![Image](image44.png) | ![Image](image45.png) | ![Image](image46.png) | 42Kda

(F) ![Image](image47.png)
(G) ![Image](image48.png)
(H) ![Image](image49.png)
Figure 8.2.1. P2X7 expression and signalling in wild-type and mdx muscles at 4 months.
Representative immunoblots showing levels of dystrophin (Dp427), P2X7, phospho-ERK1/2 and ERK1/2, in specific muscle groups (TA, GC and soleus) of 4 month old mdx and C57BL10 (WT) mice (A) and P2X7 expression levels in diaphragm from the same animals (B) – Densitometric analysis of western blots analysed by ANOVA revealed significantly higher levels of P2X7 receptor expression in TA, GC and soleus and diaphragm muscles and basal ERK1/2 phosphorylation levels were also significantly higher in mdx TA and soleus muscles compared with age matched controls (B and C, respectively; *P<0.001). The pan macrophage marker F4/80 proved undetectable in immunoblots of protein extracts from the mdx/C57 muscle tissues used in A+B (E). F4/80 immunostaining was however detected in small localised regions of mononuclear infiltration in both C57 (F) and mdx (G) cryosectioned TA muscle (representative images), visualised by colorimetric peroxidase staining (dark pink/purple; F and G) with methyl green nuclear counter stain (blue). Primary antibody was omitted for negative staining control (H).

8.3 Discussion

This study has reported for the first time that the P2X7 receptor protein displays a pattern of significant and tissue-specific up-regulation in the dystrophic mdx muscles at 4 months of age where, in general mdx muscles in situ clearly have higher levels of P2X7 expression and show significantly enhanced ERK signalling, which corresponds well with this increased P2X7 receptor expression levels found.

The possibility that infiltrating cells of the immune system (which, like macrophages are known to express P2X7 receptor) might be responsible for the up-regulations of P2X7 expression seen here in myoblasts and muscle in-situ has been ruled out by probing the P2X7 immunoblots with anti-F4/80 antibody, a pan macrophage marker (Khazen et al., 2005). These experiments showed no discernable levels of F4/80 expression in any muscle groups tested compared with a very strong specific signal detected in C57BL10 spleen (Figure 8.2.1), which is in agreement with
other immunolocalisation data from our lab and from others. Indeed, at 4 months of age, the hind limb muscle groups of the *mdx* mouse contain very low numbers of infiltrating cells with few discernable areas of infiltration, compared with the earlier periods (3-8 weeks), when infiltrations of mononuclear cells are large and pronounced. Figure 8.2.1; F-G shows that macrophages are indeed present in both C57 and *mdx* TA muscle at 4 months, though limited to a small number of small localised areas. Hence, infiltrating macrophages can be considered insufficient to explain the P2X7 expression in these muscles. The levels of P2X7 receptor expression in the populations of macrophages that are present are however unknown, but for such large up-regulations in P2X7 receptor expression to be explained by the possible existence of such a small numbers of seemingly undetectable cells seems rather implausible. It is even less likely in light of the demonstration of significant up-regulations in P2X7 receptor expression in immortalised and primary cultures of dystrophic myoblasts (see Chapter 6), which would point to P2X7 elevation occurring directly in myoblasts and myotubes. Importantly, P2X7 up-regulation in dystrophic myotubes has recently been confirmed in studies by Rawat *et al.*, (2010).

The extrapolation of the previously discussed up-regulations in P2X7 receptor expression found in *mdx*-derived myoblast cultures to *mdx* muscle *in vivo* suggests that some significant physiological relevance could be linked to this phenomenon. The P2X7-mediated component of the purinergic response in myoblasts has been generally ignored until now; hence any predictions relating to functional consequences of P2X7 receptor expression on these cells are completely novel. The P2X7-mediated effects may potentially have been identified before, but mistakenly attributed to P2X5 receptor-mediated responses (as discussed in Chapter 6). Characterisation of the functional consequences of P2X7 receptor activation in muscle *in vivo* are ongoing, however recent studies have demonstrated P2X7-mediated inductions in myoblast proliferation and increased P2X7 mRNA transcript expression levels upon differentiation in C2C12 cells (Martinello *et al.*, 2011). These responses
are clearly multifactorial as pharmacological P2X7 receptor inhibition in vitro has been shown to inhibit myotube formation in C2C12 cells, but only when cultured in differentiation inducing low serum media (Araya et al., 2004). Therefore, the up-regulated P2X7 receptor expression levels described here in dystrophic myoblasts and muscles in situ may have a significant role to play in the pathology of dystrophic muscle, potentially dictating the proliferation/differentiation status of muscle resident stem cell populations. However, although the heightened sensitivity of dystrophic myoblasts appears to be an inherent attribute of these cells, the factors orchestrating cascade activation may be intricately complex and dictated by potentially quite localised changes in extracellular milieu. Whatever the functional consequence of the described phenomenon, the results presented here reinforce the notion that the elevated levels of P2X7 expression detected in mdx muscles are indeed of muscle-specific origin and not an artefact of small numbers of infiltrating cells.
Analysis of the effects of pharmacological blockade of P2X7 receptor in *mdx* mice *in vivo*

9.1 Introduction

ATP, present in cytosol at millimolar concentrations, was first examined as a potential mediator of pathological effects by (Burnstock, 1996) in relation to its role as a chemical mediator of nociception. The same group considered the potential consequences of release of millimoles of ATP from the dystrophic muscle, proposing roles for P2X5 and P2Y1 receptors in activated satellite cells during muscle fibre regeneration (Ryten *et al.*, 2004). Our group has extended this line of investigation with the findings that P2X7 receptor expression and activity are significantly increased in both myoblasts and adult tissues derived from the *mdx* mouse model of DMD, suggesting a possible role for this receptor in the dystrophic pathology. Apoptosis has long been speculated to potentiate the dystrophic pathology in skeletal muscle, largely associated with increases in intracellular calcium (Matsuda *et al.*, 1995, Tidball *et al.*, 1995, Spencer *et al.*, 1997). As previously discussed, P2X7 receptors have well documented roles in the initiation of apoptosis (Di Virgilio *et al.*, 1996) for a review, hence the possibility exists of similar pathways operating in skeletal muscle, where ATP concentrations released into extracellular space would be high and thus the effects of purinergic signalling even more significant. However, P2X7 receptors have also been shown to confer growth advantages in different circumstances or specific cell lines (see Di Virgilio *et al.*, (2009) for a review), leading to the current hypothesis that P2X7 receptors may have a bi-functionality;
conferring metabolic and proliferative advantages during sustained low level or tonic stimulation and changing into the initiation of apoptotic cascades in response to short bursts of high level stimulation. It is possible that both mechanisms may be operating in dystrophic muscles.

The discovery of an up-regulation in functional P2X7 receptor expression in dystrophic muscle indicates a possibility of therapeutic intervention through the pharmacological blockade of P2X7 signalling in vivo. CBBG is a commonly used and highly selective antagonist of the P2X7 receptor (Jiang et al., 2000), demonstrating low in vivo toxicity over sustained periods (Remy et al., 2008, Taylor and Thorp, 1959), and as such, was a good candidate for blocking P2X7 activation in dystrophic muscle. The parent compound, CBB, has been shown to be non-toxic in the mouse over extended periods when injected intraperitonealy (i.p.) up to 500mg/kg (Taylor and Thorp, 1959). More recently, specific in-vivo P2X7 receptor blockade using CBBG injected IP in rats at concentrations of 50mg/kg (Peng et al., 2009) and 100mg/kg (Gourine et al., 2005) has shown beneficial effects under pathological conditions of high concentrations of extracellular ATP release into the extracellular environment in damaged or necrotic spinal cord and systemic inflammation. However, due to the lack of an in-vivo study of the half-life or metabolism of this compound in mice, an initial LCMS analysis of CBBG levels in the plasma of injected animals has been performed (data not shown). Since most of the CBBG in plasma was found bound to proteins and due to species-specific differences in receptor sensitivities, a concentration of 125mg/kg body weight was adopted for this study. Two injection regimens were employed to study the short and long-term effects of receptor blockade short term (daily for 30 days, starting at 3 weeks of age), or long term (weeks 3-14, every third day). In both cases injections of CBBG or saline solution were given i.p., with subsequent histological analysis of the effect of in-vivo P2X7 receptor blockade on both the percentage central nucleation, as a marker of the previous occurrence of necrosis (Karpati et al., 1989), and also the percentage of
revertant fibres as a marker of myogenic precursor activity and previous pathologically-induced regenerative activity (Yokota et al., 2006, Hoffman et al., 1990, Wilton et al., 1997).

9.2 Results

9.2.1. Analysis of relative proportions of revertant and centrally nucleated fibres in mdx TA muscle following in vivo CBBG injection

In order to assess the efficacy of pharmacological P2X7 inhibition in vivo, the well established methodology of enumerating centrally nucleated and revertant fibre numbers was applied to cryosectioned TA muscle sections immunostained with anti-dystrophin antibody (DSHB). Manual counts of central nucleation, revertant fibre number and total fibre number were obtained using ‘ImageJ, cell counter plugin’ (Rasband, 1997-2005). Percentage counts were obtained for numbers of revertant and centrally nucleated fibres from total fibre counts per transverse muscle section and averages obtained from multiple sections taken from similar areas of each muscle. This data was analysed using two-way ANOVA with post-hoc Tukey’s test (Origin 7.0). In the long-term injection group ANOVA revealed no significant differences in average percentage central nucleation of CBBG compared with saline injected mdx muscles of either age group (P>0.05), although an expected significant increase in percentage central nucleation was observed in older animals compared with younger ones (Figure 9.2.1; top left panel; *P<0.0001). No significant differences were found in revertant fibre numbers between CBBG and saline-injected muscles at 6 weeks of age (P>0.05), but 2 way ANOVA revealed a significant decrease in revertant fibre number recorded in CBBG-injected compared with saline injected muscles at 16 weeks (Figure 9.2.1; top right panel; *P=0.028 and 0.0003 for treatment and age, respectively).
Figure 9.2.1. Pharmacological inhibition of P2X7 receptors in vivo - effect on mdx muscle morphology. Graphical representations depicting the effect of in vivo CBBG administration on mdx TA muscle. ANOVA revealed that differences in central nucleation did not differ significantly between CBBG and saline (control) injected animals (P>0.05), although a significantly higher percentage central nucleation was observed in 16 week old animals compared with those of 4 weeks of age (upper left panel; *P<0.0001). CBBG had no significant effect on the number of revertant fibres observed in 4 week old animals (P>0.05), yet a significantly higher percentage of revertant fires was observed in CBBG injected 16 week old animals compared with age matched saline injected controls (upper centre panel; *P<0.0001). No significant differences were found in average revertant fibre number per revertant colony (upper right panel; P>0.05). Lower panel micrographs illustrate central nucleation (lower left panel) and revertant fibre identification by dystrophin immunohistochemical staining (lower right panel).
9.3 Discussion

The finding that percentage revertant number is significantly lower in CBBG-injected 16 week old *mdx* mice compared with saline injected age matched controls could suggest that the inhibition of P2X7 mediated signalling effected a decline in proliferative potential of myogenic precursors in this muscle, indicating that P2X7 receptors may indeed provide a potential growth advantage in *mdx* muscle of this age. Alternatively, it could suggest that muscle fibres of CBBG injected animals from this group may have been subjected to fewer cycles of degeneration/regeneration than the saline injected controls due to a reduction in the P2X7-mediated arm of the apoptotic/necrotic process which may be active during the earlier degenerative/regenerative phases in these muscles. Moreover, the impact of treatment on myofibre- and macrophage-mediated responses cannot be distinguished here, with both mechanisms co-existing in dystrophic muscle. The reduction in revertant fibre number was restricted to the group of 16 week old animals, injected over a longer period, and not significant in the 4 week old group, which could suggest that the effects of inhibition may only be manifested slowly, as would be expected due to the very low levels of revertant fibres normally present in adult *mdx* muscle: 1-5% as derived from this study, which is in agreement with figures quoted by other investigators (<5%; Dr Susan Brown, personal communication). Hence, the small yet significant decrease in revertant fibre expression by ~1% in the CBBG injected 16 week old *mdx* muscle may be indicative of a much larger effect on total proliferative activity of the myogenic precursor cells in these muscles, or indeed of the extent of P2X7 receptor’s role in proliferation/differentiation responses. Another possibility may be that non-P2X7 mediated effects may have masked the specifically myogenic effect of CBBG inhibition. At 4 weeks of age these muscles would contain high levels of infiltrating mononuclear cells. Macrophage-mediated effects in dystrophic muscle have been widely studied, but, paradoxically, not all have been reported to be detrimental; 4 week *mdx* muscle has been reported to contain M1 macrophages
responsible for lysing myoblasts via NO-mediated mechanism, as well as M2 macrophages that reduce lysis through competition for arginase (Villalta et al., 2009). The P2X7 receptor expression levels in these two populations has not been assessed and it is not clear which population has the dominant effect in-vivo, so it remains impossible to predict the effect of CBBG-mediated P2X7 receptor blockade in-vivo on either population, or the macrophage-mediated component of the degenerative/regenerative process in general.

The significant increase in percentage central nucleation with increasing age was in agreement with the age related pattern of necrosis described by Karpati et al., (1989), which was shown to peak at around 6 weeks of age. Thus, the group of 16 week old animals would have more time in which regeneration could occur, hence the cumulative phenomena of central nucleation observed (Yokota et al., 2006). No significant differences in percentage central nucleation were observed between CBBG and saline injected muscles in either age group, suggesting that the mechanism by which satellite cells become and remain activated and centrally nucleated may differ from that by which they proliferate and therefore undergo somatic mutations that give rise to revertant fibres – this is due to the fact that a significant decrease in revertant fibre number was recorded in CBBG injected mdx muscle from those animals receiving the longer injection course. This could suggest that the myofibres of CBBG injected animals had undergone fewer degenerative/regenerative cycles and therefore displayed a reduction in revertant fibre number as a result of this (Yokota et al., 2006). Alternatively, it could also potentially mean that CBBG had an anti-proliferative effect on the satellite cell population of dystrophic muscle, without affecting their potential for becoming centrally nucleated. This would indeed be in agreement with the observation that myostatin is able to increase myofibre size without increasing satellite cell number (Amthor et al., 2009). Hence CBBG may have had a influence over the proliferative but not the differentiation potential of dystrophic satellite cells, a phenomenon recently observed through the
pharmacological inhibition of P2X7 receptor signalling *in vitro* (Martinello *et al.*, 2011). The demonstration by this study that a reduction in revertant fibre number in CBBG injected animals is not accompanied by any change in average revertant fibre number per revertant colony would be in agreement with the idea that proliferation and migration/differentiation are separately regulated in muscle stem cells, however, the extent to which the factors of proliferation and differentiation affect revertant fibre colony size has not been explored in the literature to date.

Support for the notion of ATP-mediated pathology progression, and pharmacological inhibition of P2X7 receptor signalling conferring beneficial effects in dystrophic muscle *in vivo*, can also be found in the demonstration that suramin injection (a less specific P2 receptor antagonist) reduces both muscle damage and fibrosis in the *mdx* mouse (Taniguti *et al.*, 2011, Iwata *et al.*, 2007). One final noteworthy point is that visual inspection following dissection consistently demonstrated an almost complete lack of evidence that CBBG was able to effectively penetrate the muscles of injected animals. All other major organs, peritoneal cavity, and even skin appeared very distinctly blue, whereas the skeletal muscles of the hind limbs remained virtually normal in appearance. This would suggest that rather than insufficient dosage, the low level of observable effect is more likely due to the inefficient permeation of the dye into skeletal muscle tissue. The obvious way forward in this instance would be to try intra-muscular injections. However, no obvious significant differences in pathology were observed upon preliminary histological examinations of diaphragm muscles from CBBG injected mice, where the degree of dye penetration following i.p. injection would be assumed to be higher. Moreover, the characterised expression of multiple functionally active P2X7 splice variants in *mdx* TA muscle may suggest that the use of any P2X7 inhibitor *in vivo* is delayed by the requirement for elucidation of the splice variant selectivity of such drugs.
10

General Discussion

10.1 Introduction

Mutations in the DMD gene encoding dystrophin, are responsible for the most common congenital muscular dystrophy (O'Brien and Kunkel, 2001) and are sufficient to confer often co-existing deficiencies in muscular ambulation, respiration and circulation as well as central nervous system defects leading to variable degrees of specific cognitive and vision impairment (Anderson et al., 2002, Emery and Walton, 1967, Fitzgerald et al., 1994, Costa et al., 2007). The dystrophin protein itself classically represents the primary point of anchoring for a large complex of proteins known as the dystrophin associated protein complex (DAPC) at the muscle sarcolemma. Mutations and subsequent functional loss of this protein generates a compound phenotype through loss of DAPC complex interactions, with a range of specific mutations generating phenotypic diversity (Ohlendieck et al., 1993, Ohlendieck et al., 1991). Loss of function mutations in the dystrophin gene are responsible for the commonest and severest of the muscular dystrophies in humans known as Duchenne Muscular Dystrophy (DMD) (Monaco et al., 1986, Hoffman et al., 1987). Less frequent in-frame mutations of the same gene result in partially functional truncated dystrophin protein products responsible for the milder Becker’s Muscular Dystrophy (BMD) (Hoffman and Kunkel, 1989). Precise elucidation of the specific molecular mechanisms underlying any given disorder is paramount to therapeutic development, and further insights into the cellular and molecular abnormalities in DMD pathology are essential to understanding the causative mechanistic events surrounding the phenotypic abnormalities of dystrophic muscle.
10.2 Role of ectoATPases in DMD

LGMD2D - attributed to mutations in the protein product of the \( \alpha \)-SG gene, presents a similar phenotype to that of DMD, though occurring without loss of the dystrophin-glycoprotein complex-F-actin link (Roberds \textit{et al}., 1994). Since \( \alpha \)-SG has been ascribed one known function, that of an ecto-ATPase enzyme (Sandona \textit{et al}., 2004, Betto \textit{et al}., 1999) it thus provides a potential insight into the extracellular nucleotide-mediated component of DMD pathology without the compounding factors arising from membrane instabilities due to dystrophin complex loss; Importantly, this ecto-ATPase activity is particularly pronounced at high levels of extracellular ATP \([\text{ATP}]_e\) leading to the hypothesis that abnormalities in purinergic signalling may operate in dystrophic muscle, through the reduction of extracellular nucleotide metabolism following loss of \( \alpha \)-SG from the sarcolemma. Such a phenomenon would occur in both the absence of dystrophin and directly from \( \alpha \)-SG mutations. Our laboratory has previously identified a heightened sensitivity to ATP\(_e\) in an immortalised dystrophic myoblast cell line, which was shown to be P2X receptor dependent – mediated through influxes of extracellular Ca\(^{2+}\). Yet a P2Y component was also present and despite the documented functional abnormality of a specific receptor subtype, no differences in receptor expression patterns had been found in normal and dystrophic myoblasts (Yeung \textit{et al}., 2006). Following vesicular or hemichannel-mediated release, the availability of ATP and other nucleotides is modulated through their rapid degradation by the ectonucleotidase family of membrane proteins (Burnstock, 2009) leading to the hypothesis that abnormalities in the routine hydrolysis mechanisms, which normally tightly regulate the levels of all extracellular nucleotides, may confer localised nucleotide over-sensitisation sufficient to explain the altered responses documented in dystrophic myoblasts. However, results of this study excluded the effect of differential extracellular nucleotide hydrolysing potential as explaining the heightened purinergic sensitivity of dystrophic.
myoblasts. Instead, this work serves to highlight the novel role of specific P2X7 receptor expression abnormalities in conferring heightened ATP sensitivity to dystrophic myoblast cells – where significant up-regulation in P2X7 receptor transcript expression and coexistent up-regulation in protein expression have been documented in mdx-derived primary myoblasts, immortalised myoblast lines and muscles in situ.

10.3 Role of P2X7 receptors in DMD

Although not unequivocally causative of any particular pathology, P2X7 receptors have been implicated in multiple pathological disorders (Khakh and Burnstock, 2009) and possess a diverse array of functions; coordinating fast excitatory transmission in the central and peripheral nervous systems, orchestrating controlled changes in circulation via smooth muscle/endothelial-mediated contraction, and perhaps principally in the induction of cellular responses in immune cells such as lymphocytes, macrophages and microglia (Burnstock, 2007, Burnstock, 2009). P2X7 receptor expression has been shown to be widespread in tissues and cells of the immune system where they regulate the processing and release of cytokines and inflammatory mediators (Di Virgilio, 2007, Di Virgilio et al., 1996), and their uniquely lower sensitivity to ATP has made them the focus of intense interest in arenas of pathological inflammation, tissue degradation and cytolysis. Unlike other P2X receptors, P2X7 responds only when high ATP levels are present. To that end P2X7 has become synonymously a ‘danger’ sensor, with well documented roles in the development and potentiation of the immune system’s inflammatory response. The cellular/functional consequences of P2X7 activation have been widely studied in lymphocytes and macrophages and include: morphological changes via actin filament rearrangement (Pfeiffer et al., 2004), membrane blebbing (Wilson et al., 2002), cytolysis/apoptosis (Di Virgilio, 2000), IL-1β processing and release (Ferrari et al.,
1997), lysosomal impairment and autophagolysosome release (Takenouchi et al., 2009) and the conferment of proliferative advantages through mitochondrial depolarisation (Di Virgilio et al., 2009). Specifically in terms of myofibre regeneration, an important feature of P2X7 receptor function may be the dual nature of P2X7 activation, where a low level, tonic ATP stimulation is conferring metabolic and proliferative cellular advantages, whereas elevated to high levels and/or sustained or chronic stimulation is able to trigger cytolysis, apoptosis or autophagic cell death. Two mediators of such a functional balance have been suggested: [Ca^{2+}], mobilisation and opening of a large P2X7-dependent membrane pore through some still uncharacterised mechanism(s) (Di Virgilio et al., 2009).

It is now widely recognised that ATP is not only released by dead or dying cells but also physiologically, by live cells including muscle (Becq, 2010). Indeed, high concentrations of ATP released during muscle contraction have been shown to modulate Ca^{2+} homeostasis and muscle physiology, acting through purinergic receptors (Buvinic et al., 2009). Several purinergic receptors have been found differentially expressed in myoblasts, myotubes and muscle fibres at different stages of development (see below). More recently, P2X7 has emerged as physiologically important skeletal muscle receptor. Its activation stimulates myoblast proliferation (Martinello 2011) while its inhibition in differentiating cells prevented myotube formation (Araya et al., 2004). As mentioned earlier, changes in functional P2X7 receptor expression have been documented in various pathological environments (Di Virgilio, 2007, Di Virgilio et al., 2009), also including the muscle of the dysferlin deficient mouse model of LGMD2B (SJL/J mouse). In this mouse up-regulated P2X7 receptor expression was linked to LPS/BzATP-induced IL-1β secretion from myoblasts, and inflammasome formation (Rawat et al., 2010). In the same study, Rawat’s group also documented the up-regulated expression of P2X7 receptor protein.
in cultured mdx myotubes, (surprisingly, mdx myoblasts were not examined although SJL/J myoblasts were analysed). The present study extends our understanding of the roles for ATPe in DMD disease pathology, specifically in the mdx mouse model, where up-regulation of P2X7 receptor expression in dystrophic myoblasts has been shown to confer heightened functional responses. These responses could explain several of the observed abnormalities in dystrophic muscles.

The well documented roles of P2X7 receptors in ATP-induced lytic cell death and in immune-modulators release are of particular relevance in muscle, where ATP and indeed NAD are essential facilitators of muscle metabolism. Even more so in dystrophic muscle, where degenerating fibres provide potential sources of extracellular ATP at concentrations exceeding that which could be achieved anywhere else in the body. Differing outcomes of P2X7 receptor responses are thought to result from low level or tonic stimulation, compared with sudden or prolonged high level or excitotoxic ATPe stimulation (Di Virgilio et al., 2009). The heightened P2X7 receptor responses in dystrophic myoblasts may therefore potentially influence the balance between positive and negative ATP-induced effects on the stem cell pool of dystrophic muscle; where induction of proliferation, differentiation and apoptosis could result (Martinello et al., 2011, Araya et al., 2004). Indeed, tonic P2X7 receptor stimulation has been shown to induce a slow, controlled influx of Ca\(^{2+}\), promoting healthy metabolism and growth while avoiding the catastrophic effects of unchecked Ca\(^{2+}\) entry. Specifically, beneficial effects have been proposed to include the Ca\(^{2+}\)-induced enhancement of mitochondrial energy production, ER Ca\(^{2+}\) accumulation and increased cytosolic [ATP]i, promoting serum independent growth following basal autocrine P2X7 receptor stimulation (Di Virgilio et al., 2009, Adinolfi et al., 2005); an effect proposed to occur via a calcineurin regulated induction of the ‘nuclear factor of activated T cells’ (NFATc1) nuclear translocation in muscle (Adinolfi et al., 2009, Sakuma et al., 2003). Calcineurin was identified in the mass spectrometry analysis.
conducted for this thesis as a protein showing significantly increased levels of phosphorylation in ATP treated dystrophic myoblasts. Calcineurin phosphorylation (and subsequent activation) has previously been demonstrated in an ERK dependent manner (Aramburu et al., 2004), suggesting a potential role for P2X7 in calcineurin-NFAT mediated hypertrophy in dystrophic myoblasts. Indeed, it has been suggested that the limited beneficial effects of glucocorticoid treatment in DMD are due to the activation of the calcineurin-NFAT pathway (St-Pierre et al., 2004). Interestingly, one of the issues raised by Di Virgilio’s group, who focussed primarily on transfected HEK-293 cells, was the unknown origins of the low [ATP]e which would be able to explain the existence of such autocrine mechanisms in vivo – yet active/passive ATP release from muscle cells is well documented (Becq, 2010, Buvinic et al., 2009) and would indeed be pronounced in dystrophic muscle due to contraction-induced ATP release via both secretory and necrotic pathways (Allen et al., 2010b, Allen et al., 2010a, Allen et al., 2005, Head, 2010).

ATP has long been known to be released by skeletal muscle both dependently and independently of contraction (Forrester and Lind, 1969) with specific release later demonstrated from neuromuscular junctions (Silinsky, 1975) and more recently, in cultured myoblasts and myotubes (Martinello et al., 2011). Although the potential existence of extracellular ATP receptors in skeletal muscle was first demonstrated over a quarter of a century ago (Kolb and Wakelam, 1983), only now are its intricacies beginning to be resolved, highlighting the depth of signalling complexity to be explored. The proposal that extracellular ATP may be an important mediator of satellite cell plasticity stems from the regulated changes in expression of P2X5 and P2X6 receptors in developing chick muscle (Meyer et al., 1999) and P2X2, P2X5 and P2X6 receptors in developing rat muscle (Ryten et al., 2001). The same group proposed the P2X5 mediated induction of differentiation in C2C12 cells (Ryten et al., 2002), and implicated P2Y1, P2Y4, P2X2 and P2X5 receptors in the regeneration of adult skeletal muscle, illustrating the sequential expression of P2X5, P2Y2 and P2X2.
receptors in regenerating mdx muscle in situ. However, somewhat surprisingly, the possibility of macrophage infiltrations i.e. cells expressing these receptors, in 5 week mdx muscle was never addressed (Ryten et al., 2004). P2X2 and P2Y1 and P2Y2 receptors have been implicated in neuromuscular junction formation, stabilisation and function (Choi et al., 2003, Voss, 2009, Ryten et al., 2007) and the expression of all P2X receptor subtypes has been documented in cultured myoblasts (Yeung et al., 2006), although specific roles for other sub-types are unresolved at this time.

The functional involvement of P2X receptors in the differentiation of C2C12 cells has been demonstrated via autocrine ATP$_e$ response – an effect that could be replicated by oxidised ATP (oATP) application; a non-specific P2X inhibitor (Araya et al., 2004). Moreover, it has recently been proposed that concentrations of ATP$_e$ are significantly higher in the extracellular environments of myoblasts in comparison to myotubes, as a consequence of a low ecto-ATPase activity in myoblasts (Martinello et al., 2011). This minimal ATP hydrolizing activity of myoblasts was also found here, illustrating the potential significance of up-regulated P2X7 receptor levels in dystrophic myoblasts as shown here, especially in proximity to damaged/degenerating myofibres, where the functional effects of such up-regulation would be accentuated by elevated levels of [ATP]$_e$. The observed effects of ATP in C2C12 cells do however, appear to be multifactorial, with ATP$_e$ displaying opposing functions in proliferating/differentiating cells. Martinello’s group have demonstrated the P2X7-dependent induction of proliferation in ATP/BzATP treated C2C12 myoblasts 24h post serum removal. This effect was reversed by P2X7 receptor blocker oATP. Araya’s group, also using oATP, demonstrated the inhibition of differentiation in C2C12 cells 4 days in differentiation medium, proposed but not confirmed to also be a P2X7-dependent effect (Martinello et al., 2011, Araya et al., 2004). The reason for these differentiation stage-dependent differences in P2X7 receptor function in C2C12 cells is unclear; no significant changes in P2X7 receptor expression upon differentiation were documented by either group; Martinello showed P2X4 mRNA
levels to be up-regulated upon differentiation, which could potentially explain the induction of differentiation in C2C12 cells exposed to ATP_e documented by Araya’s group, since oATP was the only antagonist used to inhibit the differentiation effect, and oATP also inhibits many other P2X and P2Y receptors (Friedle et al., 2010). Unfortunately, this group did not investigate the P2X4-mediated component of the proliferation response in these cells, which could easily have been achieved with ivermectin pre-treatment. Also interesting here is the fact that Martinello’s group showed that higher concentrations of ATP_e proved toxic to myoblasts, which would be in agreement with P2X7 receptor stimulation switching from tonically beneficial to excitotoxically catastrophic effects (Di Virgilio et al., 2009). Martinello also confirmed the importance of autocrine ATP_e signalling in myoblasts, showing a reduction in myoblast survival following apyrase treatment (triggering ATP degradation). Together, these observations confirm the significance of the findings of this study; that up-regulation in functional P2X7 receptor expression confers heightened nucleotide sensitivity to dystrophic myoblasts, with the potential for localised and differentiation-stage dependent induction of proliferation, fusion/differentiation and cell death. Whether such responses in DMD are singularly controlled by autocrine/paracrine ATP_e signalling seems doubtful considering the high levels of ATP released from degenerating myofibres, although the exact levels of ATP release from normal and dystrophic myoblasts have not been addressed.

Studies are ongoing in our laboratory to determine whether the P2X7-dependent stimulation of myoblasts falls into the proposed tonic versus excitotoxic mode. The role of individual P2X7 splice-variants, specific subunit interactions, or indeed, novel interacting signalling partners in such effects also remains to be seen - such has been proposed in relation to pannexin-1’s role in lytic pore formation (Shen et al., 2010), an effect found here to be absent in proliferating myoblasts and in differentiated myotubes, despite the expression of pannexin-1 (Figure Appendix 4).
The potential for P2X7 receptors contributing to the dystrophic pathology was assessed in this study via *in vivo* injection of the specific pharmacological inhibitor CBBG. The significant reduction in revertant fiber number in *mdx* TA muscle following CBBG injection could indicate that the number of degeneration/regeneration cycles in these animals was reduced (Winnard *et al.*, 1993, Klein *et al.*, 1992, Yokota *et al.*, 2006). It could however, also indicate a role for the P2X7 receptor in myoblast proliferation, as has been described above (Martinello *et al.*, 2011), thus reducing the regenerative potential of injected *mdx* muscles. This is being investigated *in vitro* by pre-incubation of primary myoblast cultures with CBBG prior to ATP stimulation.

**10.4 P2X7 splice variant expression and function in *mdx* muscle**

P2X7k is the only mouse P2X7 splice variant to be characterised in the literature - displaying a propensity for heightened agonist sensitivity and prolonged activation leading to constitutive large pore formation and cell death in transfected HEK-293 cells. Up-regulation of P2X7k variant expression has been documented here in dystrophic myoblasts and muscles *in situ*, however, the true functional relevance of P2X7k *in vivo* has never been investigated. Neither indeed has its antagonist selectivity been documented, which, as alluded to in the chapter-specific discussion, is surprising considering it’s potentially significant contribution to cellular responses *in vivo* (Nicke *et al.*, 2009). No functional N-terminal P2X7 splice variants have been characterised in humans to date – therefore no comparisons can be made. Of the functional human C-terminal variants that have been described, P2X7B and P2X7J have both been reported to confer proliferative gains, yet by distinctly opposing mechanisms of function: P2X7B acts in the potentiation of P2X7A agonist responses, promoting growth and proliferation (Adinolfi *et al.*, 2010), and P2X7J in the hetero-oligomeric inhibition of P2X7A receptor channel formation. This oddly
enough has been suggested to confer similar gains of function in terms of the growth and proliferation of tumour cells by inhibiting the apoptotic cascade induction of the wild-type P2X7A response (Feng et al., 2006). In fact, a patent has been filed relating to the application a monoclonal antibody raised against P2X7J in the treatment of P2X7J-positive tumours. Though capable of forming functional ion channels, both P2X7B and P2X7J are deficient in large pore formation. Their ability to heterooligomerise with P2X7A could form the basis of a possible explanation for the absence of large pore formation in the myoblasts described here. Indeed, if this is further extended to the pattern of splice variant expression observed in mdx diaphragm shown here - where mouse P2X7a was the only splice variant found to be up-regulated, compared with limb muscles where up-regulation of P2X7a, P2X7b and P2X7c was found to coexist. The possibility then exists for P2X7 splice variants to convey significant functional differences for diaphragm muscle and its myogenic progenitors. Although mdx limb muscle degeneration is progressive and associated with a decline in replicative potential (Mouisel et al., 2010), mdx limb muscles do not display the progressive fibrosis associated with the human pathology and mdx diaphragm is widely regarded to better represent the human condition (Stedman et al., 1991, Andreetta et al., 2006, Coirault et al., 2003). One could speculate that the increased severity of disease progression in mdx diaphragm could be related to the P2X7 splice variant profile observed in this muscle type: The lack of any potentially dominant negative effect of P2X7b or P2X7c variant expression on the activity of P2X7a receptors would leave unchecked the P2X7-triggered apoptotic cascade (Di Virgilio, 2000), which, has previously been implicated in dystrophic satellite cell death through an undefined mechanism (Merrick et al., 2009, Jejurikar and Kuzon, 2003). However, the validity of such functional extrapolations between human and mouse variants remains to be confirmed. Conversely, the up-regulation of P2X7k variant expression shown in this study could be involved in the mechanism by which fibrosis progressively and preferentially accumulates in the mdx diaphragm but not in
mdx limb muscles, which has previously been linked to the constitutive over-proliferation of connective tissues (Coirault et al., 2003, Andreetta et al., 2006).

10.5 P2X7 expression in myogenic precursors

Satellite cells comprise approximately 2-7% of the nuclei associated with a particular myofibre in an adult muscle (Peault et al., 2007). The proposal of Pax7 transcription factor expression being a unique requirement for adult satellite cell function originated in the observation that Pax7 knockout mice showed no significant abnormalities in foetal myogenesis, whereas their postnatal muscle growth was severely impaired (Seale et al., 2000). As such, Pax7 has become a seminal marker for adult satellite cell populations in vivo (Oustanina et al., 2004, Peault et al., 2007, Zammit et al., 2006), although in vivo confirmation of its function in adult muscles have been questioned by others (Lepper et al., 2009). More recently, other populations of myogenic precursor cells have been shown to inhabit distinct niches in adult skeletal muscle outside the normal satellite cell-associated region beneath the basal lamina. Thus, pericytes have been shown to possess the capacity to transverse from their normal endothelial locations within blood vessels, crossing the extracellular matrix to reach mature myofibres. They are able to participate in myofibre regeneration through the expression of myogenic regulatory factors such as Myf-5 and by concomitant fusion to form new myofibres (Dellavalle et al., 2007). PW1+/Pax7- interstitial cells (PICs) are the latest in this line of novel myogenic progenitor cell populations to be proposed. Residing solely in the interstitial space between mature myofibres and characterised by a lack of Pax7 expression, PICs are a muscle-resident stem cell population distinct from satellite lineage and able to participate in the fusion and regeneration of mature skeletal muscle (Mitchell et al., 2010). Without completely ignoring the rather attractive, yet unfounded notion that the P2X7+/Pax7- cell population that we have described could represent another novel
population of myogenic progenitors at similarly distinct location, perhaps a more logical hypothesis may be that the cells defined here could indeed be a population of activated PICs taking residence externally to the basement membrane in response to some internal stimulus. They are unlikely to be pericytes since they appear to express Myf-5 (Dellavalle et al., 2007). Transcription factor expression profiling of P2X7 receptor expressing cells on a single isolated living fibre and derived myoblast primary cultures may shed some light on whether the cells identified in this study represent a homogeneous population of myogenic precursor. PCR using laser dissected mononuclear cells attached to living myofibres ex vivo could also be used here. Perhaps a more elegant study could employ the single cell based oil-water emulsion PCR methodology gaining popularity in the high throughput analysis of complex microbial and cancer cell specialisations (Zeng et al., 2010, Novak et al., 2010).

10.6 Consequences of altered P2X7 expression and function in dystrophic muscle

Abnormalities in ERK signalling cascade described here in relation to dystrophic myoblasts are likely to have consequences for a host of cellular responses. These could be broadly categorised by bifold signalling cascade induction leading to increased survival/growth or apoptosis/autophagy/necrosis and cell death (Mebratu and Tesfaigzi, 2009). It has been shown that MAPK family members are responsible for determining the activation state of satellite cells in adult muscle, with quiescence suggested to involve the p38α/β phosphatase MKP-1 (Jones et al., 2005). Ablation of this enzyme has been shown to exacerbate the dystrophic phenotype in mdx muscle (Shi et al., 2010). However, the dependence of MKP-1 on P2X7 activation remains to be seen. The multi-faceted nature of MAPK signal cascade in satellite cells is illustrated by the fact that ERK1/2 activation induces both proliferation and
differentiation of cultured myoblasts (Gredinger et al., 1998, Jones et al., 2001, Milasincic et al., 1996). Yet these observations have been proposed to be cell type specific: ERK1/2 activation induces proliferation in C3H-derived myoblasts, and differentiation in 23A2-, L6A1- and C2C12-derived myoblasts (Jones et al., 2005). The demonstration of P2X7-dependent ERK1/2 activation in myoblasts in this study, combined with the more recent demonstration that ATPe can induce either proliferation or differentiation of C2C12 myoblasts under different conditions via a mechanism proposed to be P2X7-dependent (Araya et al., 2004, Martinello et al., 2011) suggest that the previously observed differences in MAPK-mediated response in myoblasts may indeed be due to differential expression/activation of P2X7 receptor levels/variants between different cell lines or differences in conditions of individual cultures.

Besides heightened P2X7 receptor sensitivity, this study has also highlighted the role of NAD-mediated P2X7 receptor activation in dystrophic myoblasts. NAD has previously been shown to induce ATP-independent Ca²⁺ influx via ADP-ribosylation of ATP binding sites of P2X7 receptors (Domenighini and Rappuoli, 1996). This mechanism is active in murine T cells, yet in macrophages, NAD does not serve to gate the channel, it only decreases the gating threshold in response to ATP binding, demonstrating synergistic interaction between ATP and NAD to induce P2X7 signalling cascades (Seman et al., 2003b). NAD-mediated cell death has been demonstrated in a variety of disorders (Divirgilio et al., 1989, Zhao et al., 2011, Seman et al., 2003b). Here dystrophic myoblasts have been shown to display heightened NAD sensitivity, the functional relevance of which is under investigation. As already covered in the chapter-specific discussion, ART1 is thought to be the predominant ADP-ribosyl transferase expressed in skeletal muscle, where its expression has been shown to be up-regulated upon differentiation of C2C12 and C3H-derived myoblast cultures (Zolkiwska et al., 1992, Friedrich et al., 2008).
Experiments are underway to determine ART1 expression levels in *mdx* muscle and derived primary myoblast cultures.

In an attempt to broaden the current knowledge and elucidate the immediate functional consequences downstream of P2X7 receptor activation in myoblasts, a mass spectrometry based approach was employed here in assessing protein phosphorylation states between normal and dystrophic myoblasts following ATP treatment. Several proteins were identified as novel participants in the ATP response in dystrophic muscle, notably the ERM actin binding proteins, annexin A6 and PKCδ. The ERM proteins have well documented roles in cell shape changes, motility and proliferation (Bretscher *et al*., 2002). They have received recent attention because of their potential roles in cancer biology due to similarities with another ERM family member, the tumour suppressor protein, merlin (McClatchey, 2003, Morales *et al*., 2010, Ren, 2010), although there is no current literature linking P2X7 receptors and ERM proteins directly.

P2X7-dependent PKCδ phosphorylation has previously been reported to accompany the activation of ERK1/2 proteins (Bradford and Soltoff, 2002) and more recently has been shown to induce the production of reactive oxygen species in murine macrophages in a P2X7-dependent manner (Martel-Gallegos *et al*., 2010). If extended to satellite cells, it may confer negative effects on mitochondrial energy production, a phenomenon well documented in relation to altered Ca²⁺ homeostasis in *mdx* muscle (Kuznetsov *et al*., 1998, Shkryl *et al*., 2009, Menazza *et al*., 2010). Recently, over-expression of the endoplasmic reticulum Ca²⁺ ATPase pump SERCA in both mdx and δ-sarcoglycan null mice has been shown to rescue the dystrophic phenotype and confer normal mitochondrial morphology by a mechanism proposed to be Ca²⁺ dependent (Goonasekera *et al*., 2011). Principally regarded as a pro-apoptotic stimulus, PKCδ activation has previously been shown to function in both pro- and anti-apoptotic manners, activated through a caspase-3-mediated cleavage event.
(Allen-Petersen et al., 2010) that may relate to p38 activation prior to P2X7-dependent large pore formation (Donnelly-Roberts et al., 2004). Whether a functional link between caspase-3 mediated PKCδ cleavage and P2X7 activation exists to mirror that of the P2X7-dependent caspase-1-mediated IL-1β cleavage and secretion associated with the inflammatory response in macrophages and also known to operate in myoblasts (Rawat et al., 2010) remains to be seen. Therefore, the up-regulated phosphorylation of PKCδ seen here in dystrophic myoblasts treated with ATP_e could suggest that this signalling event may be linked to P2X7-induced ERK1/2 activation in these cells. However, the functional implication of PKCδ activation in myoblasts is unclear.

Perhaps then, a fine balance in P2X7 activation in satellite cells exists (where large pore formation has been found to be absent despite the expression of pannexin-1) between the activation of different members of the MAPK family proteins. Indeed, activation of p38 has been shown to be sufficient to confer constitutive activation to the quiescent satellite cell population (Jones et al., 2005). Therefore, the constitutive, tonic level of P2X7 receptor activation observed in dystrophic myoblasts here may be sufficient to trigger ERK1/2 and p38-mediated satellite cell activation and proliferation via the JAK2 tyrosine kinase pathway, which itself is a mediator of growth and proliferation in myeloid progenitor cells (Kovanen et al., 2000) and myoblasts, acting via the STAT pathway (Wang et al., 2008). This constitutive P2X7 receptor activation may then extend to myofibres as well, owing to the retention of P2X7 receptor in myotubes (Figure Appendix 3). In turn, constitutive activation of the JAK-STAT pathway could inhibit MRF expression and terminal differentiation, resulting in the retention of centrally nucleated myofibres; a hallmark of the pathology in the mdx mouse (Yokota et al., 2006). However, the JAK1/STAT1/STAT3 and JAK2/STAT2/STAT3 pathways have been shown to have opposing functions in this
regard. The JAK1 pathway has been shown to repress MyoD and MEF2 expression and the JAK2 pathway to enhance their expression, via unresolved mechanisms (Wang et al., 2008).

Another partner in this cascade may be annexin A6, shown here to display significantly higher phosphorylation status in dystrophic myoblasts in response to ATP\textsubscript{e} stimulation. Its proposed role is as a negative effector of MAPK signalling and Ca\textsuperscript{2+} channel function through translocation to the plasma membrane (Grewal et al., 2005, Grewal et al., 2010) and it may be of significant relevance to the susceptibility of dystrophic myoblasts to MAPK cascade induction. As eluded to in the chapter-specific discussion, a particularly interesting property of the annexin A6 response in lymphocytes is its Ca\textsuperscript{2+} dependent translocation to membrane associated vesicles, proposed to play a functional role in inflammatory mediator release (Podszywalow-Bartnicka et al., 2007). It would be of significant relevance in relation to not only the proliferation of satellite cells, but also to the establishment of the immune response characteristic for the mdx muscle pathology. Indeed, Rawat’s group have recently highlighted the potential role of toll-like receptors (TLR-2 and TLR-4) in mediating P2X7-dependent IL-1\textbeta release in myoblasts derived from the dysferlin null mouse in response to stimulation with lipopolysaccharide (LPS) and BzATP (Rawat et al., 2010). This response is more typical for macrophages than skeletal muscle cells, a fact which poses two questions: What role can LPS play in the co-stimulation of P2X7 in dystrophic muscle; and what is the functional link bringing together P2X7 receptors, TLRs and dystrophin expression in skeletal muscle? On the first point, although sources of LPS are widespread, no significant roles have been documented for pathogen-mediated responses in dystrophic muscle. However, LPS is just one example of molecules that bind to TLRs and P2X7 receptors and activate the
inflammasome pathway. Amongst those are endogenous danger/alarm signals, eg. HMGB1, S100 and some other less characterised proteins. These molecules present in muscle may be the stimulus responsible for inflammatory cascade induction in dystrophic muscles.

10.7 Functional relevance of altered P2X7 expression in dystrophic muscle and the potential for therapeutic intervention

In addressing the origins of P2X7 receptor up-regulation in mdx myoblasts, one protein of particular interest is biglycan, a small leucine-rich proteoglycan (SLRP) protein found sequestered in the extracellular matrix (EM) and secreted in soluble form by activated macrophages (Schaefer et al., 2005). There it complexes with TLR2, TLR4 and P2X7/P2X4 receptors to form a larger multimeric mediator of inflammasome formation, driving caspase-1-dependent IL-1β processing and secretion (Babelova et al., 2009). Biglycan null mice display mild dystrophic phenotype, proposed to be a product of biglycan’s role in tethering selected components of the DAPC (Mercado et al., 2006, Brandt and Pedersen, 2010, Brandan et al., 2008). Moreover, biglycan expression has been shown to be up-regulated in mdx muscle (Bowe et al., 2000). Interestingly, it has been shown recently that components of the DAPC are rescued by biglycan’s recruitment of utrophin to the sarcolemma, following the demonstration that systemically delivered recombinant biglycan confers utrophin up-regulation and a reduction in pathology in the mdx mouse (Amenta et al., 2011). This may illustrate a functional distinction between soluble and membrane bound biglycan derivatives. If up-regulation of the membrane bound biglycan protein confers therapeutic gain in the mdx mouse through partial DAPC restoration, then perhaps the soluble protein secreted by macrophages has the opposite effect, mediating the P2X7-dependent biglycan inflammasome response.
Therefore, therapies aimed at inhibiting the soluble biglycan protein may also be of therapeutic merit here. In support of this theory is the observation that biglycan has recently been shown to be secreted by myoblasts and up-regulated in cells undergoing differentiation (Henningsen et al., 2010), suggesting the possibility for a positive feedback mechanism involving P2X7 receptor stimulation and biglycan secretion that would be constitutively active, based on the results presented here. Hence the secreted form of biglycan may have a detrimental function in mdx muscle, so far masked by the therapeutic benefits conferred through delivery of the membrane-bound form, and may be involved in potentiating the immune system response of dystrophic muscle. However, additional complexities can be envisaged, when considering the role of other cell types. For example, biopsied fibroblasts have been shown to be one of the main sources of biglycan in DMD muscle (Fadic et al., 2006). Indeed, TLR4 expression in myoblasts is induced by IL-6 via STAT3 (Kim et al., 2011), hence TLR4 expression and biglycan recruitment could be P2X7-dependent in myoblasts based on the ERK1/2 activation data presented here. Moreover, IL-6 secretion is induced by IL-1β in a dose dependent manner in myoblasts (Gallucci and Matzinger, 2001, Gallucci et al., 1998) and IL-1β is itself processed and released in a P2X7-dependent manner in both myoblasts and macrophages (Rawat et al., 2010, Babelova et al., 2009, Ferrari et al., 1997, Di Virgilio, 2007). Thus, heightened P2X7 receptor expression and activation, inducing apparently self perpetuating processing and release of IL-1β and IL-6 may serve to unify several functional and indeed morphological pathological hallmarks of mdx muscle. Moreover, IL-6 secretion can also be P2X7-dependent in fibroblasts (Solini et al., 1999), suggesting the potential for contributions from other cell types. Additionally, subpopulations of macrophages have been described to contribute different functional effects in regenerating dystrophic muscle. A CD68+ M1 population is involved in the pro-inflammatory tissue damaging response, and a CD163+/ CD206+ M2 population, further divided into M2a, M2b and M2c, dependent on their response to IL-4, IL-6 and IL-10
(Villalta et al., 2009, Tidball and Villalta, 2010). Therapeutic benefits have been documented using IL-10 mediated inhibition of M1 macrophage activation in mdx muscle (Villalta et al., 2010), suggesting the possibility for similar manipulation of IL-6 induced signaling cascades. However, the demonstration of heightened myoblast differentiation responses to IL-10 in the same study eludes to the intricacy of crosstalk existing between myoblasts, macrophages and probably fibroblasts, where elucidation of proper distinction between so called ‘myokine’ (Brandt and Pedersen, 2010, Pedersen, 2010) and cytokine signaling must first be addressed, so as to alleviate the possibility of inadvertently down-regulating potentially beneficial regenerative effects.

Finally, this study has shown P2X7 receptor expression to be retained following the differentiation of myoblasts to myotubes, suggesting a role for this receptor in both satellite cells and contractile myofibres. Myofibre damage in DMD muscles has been shown to result from increased [Ca^{2+}], levels not attributable to sarcolemmal damage (Millay et al., 2009). Additionally, abnormalities in Ca^{2+} influx via L-type, TRP and stretch activated channels have been documented in mdx myotubes (Millay et al., 2009, Vandebrouck et al., 2002, Kumar et al., 2004, Yeung et al., 2005, Friedrich et al., 2004), and Ca^{2+} channel blockers have proved beneficial in delaying the dystrophic pathology in limb muscles (Jorgensen et al., 2011). The data presented in this study suggests that inhibitors of ATP receptor-mediated Ca^{2+} influx may be beneficial in reducing the severity or slowing the onset of the DMD pathology. Indeed, a broad P2 receptor antagonist has previously been shown to confer therapeutic benefit in mdx mice (Iwata et al., 2007).
10.8 Conclusion

Current research into therapeutic intervention in DMD relate largely to vector borne gene therapy and stem cell based approaches, and although much has been learned, problems of low transgene expression, immune rejection, low proliferative and migratory potentials and difficulties in assessing expression/migration levels are continually addressed in the literature (Arechavala-Gomeza et al., 2010, Meng et al., 2011, Meng et al., 2010, Morgan et al., 2010, Mendell et al., 2010, Trollet et al., 2009). Complimentary to these ideas, this study has identified a specific ATP receptor abnormality in skeletal muscles of the adult mdx mouse. With up-regulated ATP receptor expression and high levels of immune cell invasion, the arena of dystrophic muscle may become ever more inviting to the purinergic investigator; offering a novel and in many ways unique in vivo opportunity to study the entire nucleotide receptor family in its full glory. P2X7 has been shown here to be integral to the phenotype of the mdx mouse, representing an extension of previously known mechanisms of purinergic signalling in myoblasts and highlighting a novel role for P2X7 in dystrophic muscle. It is proposed that this receptor may be a candidate for therapeutic evaluation, and indeed may offer some insight into possible ways in which myoblasts can be affected by the multifaceted Ca\textsuperscript{2+} abnormalities of dystrophic muscle.
Further studies

11.1 Analysis of P2X7 splice variant expression in mdx/P2X7 double knockout mouse models.

Complementary to the idea of pharmacological \textit{in vivo} P2X7 receptor blockade in the \textit{mdx} mouse, is the notion of generating \textit{mdx}/P2X7 double knockout (DKO) animals for analysis of the effect of complete P2X7 receptor expression ablation on the dystrophic pathology in these animals, thus subverting those problems associated with antagonist selectivities, dosages, injection courses and methodologies. To date, two P2X7 KO mouse models are available to the scientific community: one generated by GlaxoSmithkline Ltd. (GSK) and the other by Pfizer Ltd. Both have invested in the generation of P2X7 KO mouse models following the intense interest in the receptor over recent years. Both these knockouts are available to our laboratory. DKO animals were genotyped using PCR based analysis of genomic DNA extracted from ear clips taken at 2 weeks of age. Specific regions of DNA were amplified using primer sets able to differentiate the single nucleotide mutation in exon 23 of the \textit{mdx} strain from the WT sequence (Amalfitano and Chamberlain, 1996) as previously described (see Figure 3.2.1), which would be absent in homozygous \textit{mdx} animals. Other primer sets were designed that would be able to differentiate P2X7 KO from WT alleles based on overlapping sequences of the lacZ and neomycin gene cassettes inserted during the generation of the GSK and Pfizer P2X7 KO animals respectively (Solle \textit{et al.}, 2001, Sikora \textit{et al.}, 1999).
The recently described P2X7k variant (Nicke et al., 2009) was found to escape inactivation in both the GSK P2X7 KO and mdx/P2X7 DKO animals models (Figure 11.1; A), and indeed a similar situation was observed for the currently un-published P2X7c variant, which escaped inactivation in the Pfizer P2X7 KO animal through a similar mechanism (Figure 11.1; B) – although semi-quantitative PCR reproducibly demonstrated much lower levels of this variant in the Pfizer P2X7 KO than were consistently found for P2X7k variant expression in the Glaxo P2X7 KO animal.

Even the low level expression of P2X7 splice variants in DKO animals suggested here may be sufficient to mask any beneficial effects of silencing this receptor against the mdx genetic background, yet these animals may still prove useful - with the diversity of proposed conditions and pathways involving the P2X7 receptor, the existence of splice variant specific KO animals may allow variant specific functions to be more clearly defined. Data available to date concerning any derivation of function from P2X7 KO animals must be treated with some caution. The general consensus in the P2X7 field has been that the available P2X7 antibodies were unreliable based on their signal retention in the KO animal models (Sim et al., 2004), however, data collected by our lab and Murrell-Lagnado’s group (Cambridge), suggests that effects previously attributed to antibody-specificity are actually the result of novel splice variant expression patterns (manuscript in preparation). Although P2X7 pharmacological profiles have been classically documented using HEK cells or oocytes, due to the technical ease of transfection and patching, respectively, it would none the less be interesting to characterise the expression and function of certain variants in myoblasts derived from Glaxo- and Pfizer-derived KOs and DKO; the links this study has suggested between P2X7 and ERK1/2 activation consist of bi-fold responses, where ERK1/2 activation can lead to both survival and growth or apoptosis and cell death by as yet little understood mechanisms.
Solutions to the problem of splice variant expression in the current KOs might include: The creation of a true P2X7 KO using homologous recombination to excise the entire gene in embryonic stem cells prior to implantation and generation of a new KO line. Alternatively, other informative methodologies might involve the generation of a P2X7 over-expressing strain, possibly in an inducible and tissue-specific manor, or alternatively to biopsy normal C57 muscle, transfect the derived primary myoblasts with P2X7 under viral promoter control and transplant the cells back into the normal mouse to determine whether the increased purinergic sensitivity would be beneficial or detrimental to the overall muscle pathology.

Homozygous DKO s were obtained in the F3 and F4 generations of GSK and Pfizer derived litters respectively, as confirmed by PCR analysis using primer sets designed to amplify sequences of gDNA specific to WT or KO variants of \textit{mdx}, GSK P2X7 KO and Pfizer P2X7 KO animals. For P2X7 variants, PCR products could be visualised together due to differences in product size, whereas \textit{mdx} variants were visualised separately since the \textit{mdx} mutation comprises a single base pair mutation, which confers no difference in PCR product size. Amplification of KO specific sequences combined with absence of WT sequence amplification for both dystrophin and P2X7 was used as confirmation of homozygosity in the generation of \textit{mdx}/P2X7 DKO animals. Characterisation of both DKO animals is ongoing.
Figure 11.1. P2X7 splice variants escape inactivation in P2X7 KO animals. RT-PCR demonstrated the expression of P2X7k variant in tissues of the GlaxoSmithKline (GSK) P2X7 KO mouse and mdx/GSK P2X7 DKO mouse (A; lower panel). Expression of P2X7c variant was detected in tissues of the Pfizer P2X7 KO mouse (B; lower panel). Upper panels in A and B illustrate the design of KO constructs.


MILASINCIC, D. J., CALERA, M. R., FARMER, S. R. & PILCH, P. F. 1996. Stimulation of C2C12 myoblast growth by basic fibroblast growth factor and insulin-like growth factor 1 can occur via


Appendices

Work presented in these appendices relates to experiments carried out by the author himself, by a colleague, Dr. Wotjek Brutkowski, and by a collaborator, Mr Morten Ritso (University of Newcastle). That presented here (with permission) aims to complement and clarify certain statements that appear in the main thesis text.

Figure Appendix 1. Desmin staining of wild-type/mdx immortalised myoblast cultures. Immunofluorescence images showing time course of desmin (green) expression in differentiating H-2Kb-tsA58 and H-2Kb-tsA58/mdx-derived myoblast lines over 6 days (upper panels). 488c denotes secondary antibody staining only with primary omitted (lower panels; negative control). Nuclei are counterstained using DAPI (blue).
Figure Appendix 2. Annexin V staining of ATP$_e$ treated wild-type/mdx immortalised myoblasts. Flow cytometry-derived plots depicting percentage gating following ATP$_e$ treatment of H-2Kb-tsA58 (right panels) and H-2Kb-tsA58/mdx (left panels) immortalised myoblasts. Apoptosis (assayed for through Annexin V translocation to the outer membrane leaflet) was not observed in either genotype following treatment with ATP$_e$ (500μM; 10 mins) or Staurosporine (2μM; 4 hours).
Figure Appendix 3. **Up-regulations in P2X7 receptor expression in dystrophic myoblasts are retained following differentiation.** Representative immunoblot analysis of P2X7 receptor expression in H-2Kb-tsA58 and H-2Kb-tsA58/mdx-derived cultured myotubes following 8 days in differentiation media.

Figure Appendix 4. **Pannexin-1 is expressed in cultures of wild-type and mdx immortalised myoblasts and myotubes.** RT-PCR showing Pannexin-1 expression in H-2Kb-tsA58 and H-2Kb-tsA58/mdx-derived myoblasts (mb) and 8 day differentiated myotubes (mt). GAPDH represents a positive control.