P2X7 receptor knockout alleviates the pathology in the *mdx* mouse model of Duchenne muscular dystrophy

By

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Abstract

Duchenne muscular dystrophy (DMD) is a hereditary, X-linked, muscle wasting disease with no known cure. It is caused by altered mechanical stability of muscle cell membranes combined with altered cell signalling and inflammatory infiltrations due the absence of the cytoskeletal protein dystrophin, the product of the DMD gene. Progressive muscle fibre degeneration combined with chronic inflammation leads to a severe functional impairment, progressive disability, and ultimately to premature death. DMD presents predominantly in skeletal muscles but brain and bone are also affected.

Analyses in the \textit{mdx} mouse, the most commonly used model of DMD, has led to the identification of an increased expression and function of the P2X7 purinoceptor in dystrophic muscle cells and muscles \textit{in situ}. This ATP-gated receptor has been implicated in a number of human diseases that, combined with its well-known role in inflammatory cells, suggested that P2X7 upregulation might also be important for the pathogenesis of DMD.

To test the role of the P2X7 purinoceptor in DMD pathogenesis and its potential as a target for treatment, two \textit{mdx}/P2X7\textsuperscript{-/-} double mutant mouse strains were generated and compared to the \textit{mdx} mouse with respect to several critical disease parameters during an acute degenerative stage of disease. Histological, molecular, biochemical, and functional analyses revealed reductions in both muscle and non-muscle pathology in \textit{mdx}/P2X7\textsuperscript{-/-} mice, with significant improvements in key molecular and structural parameters. These included lower serum creatine kinase levels and decreased sarcolemma permeability to blood-born molecules, indicative of less sarcolemma damage. P2X7 ablation also resulted in increased minimum Feret’s diameter, a morphological indicator of muscle regeneration. While the fraction of muscle fibres with centralised nuclei was not significantly different, levels of myogenin, a protein indicator of muscle differentiation, were also higher in \textit{mdx}/P2X7\textsuperscript{-/-} mice. These changes were concomitant with an overall decreased inflammatory signature in \textit{mdx}/P2X7\textsuperscript{-/-} mice compared to \textit{mdx} and an increase in muscle strength, \textit{in vitro}. Examination of diaphragms (undergoing continuous degeneration/regeneration in the \textit{mdx} mouse) from 20 month old mice also showed the increase in minimum Feret’s diameter and continued reduced inflammation in the \textit{mdx}/P2X7\textsuperscript{-/-} group. Aged heart tissue from these \textit{mdx}/P2X7\textsuperscript{-/-} also presented less inflammatory infiltrate and reduced fibrosis compared to \textit{mdx}. Moreover, micro-CT analyses showed greatly reduced osteopenia in \textit{mdx}/P2X7\textsuperscript{-/-} bones when compared to 6 month \textit{mdx} mice. Amelioration of all symptoms was proportional to the extent of
receptor depletion, where single P2X7 isoform loss was less effective than the complete knockout.

These observations support involvement of the P2X7 purinoceptor in the dystrophic pathology in this model of DMD and are consistent with the well-known role of this receptor in other disorders. P2X7 purinoceptors are likely to act *via* several different molecular pathways, for example increased Ca$^{2+}$ influx that affects dystrophic cells directly as well as through reduced inflammation. Indeed, this study represents the first known analysis of an effect of P2X7 ablation on a chronic inflammatory phenotype localised to skeletal muscle.

These data from the most widely used model of DMD suggest that the P2X7 receptor can be also involved in human pathology and specific receptor antagonists could be considered for targeted pharmacological intervention to delay progression of this lethal disease.
Declaration

Whilst registered as a candidate for the above degree, I have not been registered for any other research award. The results and conclusions embodied in this thesis are the work of the named candidate and have not been submitted for any other academic award.

Anthony J. Sinadinos
1st September 2014

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I would like to offer most thanks to my supervisory team, Prof. Dariusz Górecki, Dr. Stephen Arkle, and Dr. Jerome Swinny, for their constant advice, generous assistance, and un-erring patience during these many years. Beyond their plentiful guidance, they are admirable professional role-models whom I can only hope to emulate, with reflection and practice, on my own journey through science.

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Lastly, thank-you friends and family for caring and supporting me for so long, and for whose assistance I would otherwise not be here.
Quotes

The indifference of nature:

“Nature is not cruel, only pitilessly indifferent. This is one of the hardest lessons for humans to learn. We cannot admit that things might be neither good nor evil, neither cruel nor kind, but simply callous - indifferent to all suffering, lacking all purpose.”

Richard Dawkins
River Out of Eden: A Darwinian View of Life

The unique potential of human labour to alleviate natural suffering:

“Men can be distinguished from animals by consciousness, by religion or anything else you like. They themselves begin to distinguish themselves from animals as soon as they begin to produce their means of subsistence, a step which is conditioned by their physical organisation.”

Karl Marx
The German Ideology
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Abbreviations

ACh  acetylcholine
ADP  adenosine diphosphate
AICAR  5-aminoimidazole-4-carboxamide ribonucleotide
AMP  adenosine monophosphate
AMPK  adenosine monophosphate activated-kinase
ASC  apoptosis-associated speck-like protein containing caspase activation and recruitment domain
ATP  adenosine triphosphate
ATPe  extracellular ATP
BCA  bicinchoninic acid
BMD  bone mineral density
BSA  bovine serum albumin
CBB  Coomassie brilliant blue
CCL  CC chemokine ligand
CD[*.*]  cluster of differentiation [sub-type]
CK  creatine kinase
CoV  coefficient of variation
COX2  cyclooxygenase-2
CXCL/R  CXC (motif) ligand/receptor
DAMP  damage associated molecular pattern
DAPC  dystrophin associated protein complex
DGC  dystroglycan complex
DMD  Duchenne muscular dystrophy
DRP2  dystrophin related protein-2
ECM  extracellular matrix
EDTA  ethylenediaminetetraacetic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>eMyHC</td>
<td>embryonic myosin heavy chain</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular-signal-regulated kinase</td>
</tr>
<tr>
<td>Foxo3</td>
<td>forkhead box O3</td>
</tr>
<tr>
<td>Foxp3</td>
<td>forkhead box P3</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GC</td>
<td>gastrocnemius muscle</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
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<td>Gr-1</td>
<td>granulocyte receptor-1</td>
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<td>Grb2</td>
<td>growth factor receptor-bound protein 2</td>
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<td>GTP</td>
<td>guanosine triphosphate</td>
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<td>GZMB</td>
<td>granzyme-B</td>
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<td>H&amp;E</td>
<td>haematoxylin and eosin</td>
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<td>HDAC</td>
<td>histone deacetylase</td>
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<td>HLA-G</td>
<td>histocompatibility antigen, class I, G</td>
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<td>HMGB1</td>
<td>high mobility group box 1</td>
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<td>ICE</td>
<td>interleukin-1β converting enzyme</td>
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<td>IDO1</td>
<td>indoleamine 2,3-dioxygenase 1</td>
</tr>
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<td>IFN-γ</td>
<td>interferon-gamma</td>
</tr>
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<td>insulin-like growth factor binding protein</td>
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<td>immunoglobulin G</td>
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<td>IPAF</td>
<td>ICE(interleukin-1β converting enzyme)-protease activating factor</td>
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<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
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<td>IL-[<em>.</em>]</td>
<td>interleukin-[sub-class]</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>Ly6G</td>
<td>lymphocyte antigen 6 complex [locus G]</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
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<td>μCT</td>
<td>micro computed tomography</td>
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<tr>
<td>MDSC</td>
<td>myeloid derived suppressor cell</td>
</tr>
<tr>
<td>MEF2</td>
<td>myocyte enhancer binding factor-2</td>
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<td>mTOR</td>
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<td>NAD(P)⁺</td>
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<td>natural killer cell p46-related protein</td>
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<td>NMD</td>
<td>neuromuscular disorder</td>
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<td>nNOS</td>
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<td>retinoic acid related orphan receptor-C (gene)</td>
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<td>STAT3</td>
<td>signal transducer and activator of transcription 3</td>
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<tr>
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<td>thymus (derived) regulatory lymphocyte</td>
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<td>tris-acetate-EDTA</td>
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<td>TGFβ</td>
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<td>uridine triphosphate</td>
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General Introduction

1.1 Duchenne muscular dystrophy: A multicomponent disorder

Duchenne muscular dystrophy (DMD) is the most common and one of the most severe of the muscular dystrophies (Emery, 2001; Kaplan, 2011). DMD was first described by Edward Meryon and Guillaume Benjamin Amand Duchenne in the 1850 and 60s (Meryon, 1852; Duchenne, 1867). They defined a degenerative neuromuscular disorder that was hereditary. It is now established that DMD is an X-linked congenital muscle wasting disease affecting approximately 1 in 3500 of all male births (Emery, 1991). Symptoms include progressive skeletal muscle degeneration leading to generalised muscle wasting and loss of function, particularly of the proximal muscle groups: First affected by wasting are legs and pelvis muscles followed by shoulder, neck and arm, and eventually respiratory muscles. The lost muscle is replaced by connective tissue and accompanying fibrosis hampers muscle regeneration further still. Cardiomyopathy is present but heart failure is not the leading cause of death (Mosqueira et al., 2013). DMD patients show the first symptoms in early childhood, typically lose ambulation by adolescence and the natural progression of this disease often leads to death in the second decade of life (Eagle et al., 2002). Death is most often caused by respiratory failure directly attributed to the progressive weakening of breathing muscle activity (Mosqueira et al., 2013). With improved treatment and care strategies patients can live even into their 40s and, subsequently, death by heart failure is becoming more common (Hermans et al., 2010).

In addition to lethal muscle symptoms, DMD is also associated with less pronounced and often less known brain and bone phenotypes. Specifically, reduced cognitive ability is observed in many DMD patients (Rapaport et al., 1991; Anderson et al,
2002; Taylor et al., 2010). For instance, in one of the largest single centre studies, Taylor et al. (2010) reported that reduced full-scale intelligence quotients positively correlated with DMD gene mutations resulting in the loss of CNS dystrophin isoforms (see next chapters) that are expressed in specific brain regions as well as muscle (Lidov et al., 1990; 1993; 1995; Man et al., 1991; Gorecki et al., 1992; Lidov, 1996; Austin et al., 2000; Aleman et al., 2001). It is now recognised that the cognitive impairment is a pleiotropic effect of the mutant DMD gene in non-muscle tissues with other molecular factors contributing to the widespread phenotype.

A second non-muscle abnormality associated with the DMD pathology is a reduced bone mineral density (BMD), evident even in childhood. Initially considered to be related to the known role of locomotion-induced mechanical stress contributing to normal bone development, low BMD is now considered another effect of the mutant DMD gene and not a result of reduced muscle loading alone (Hsu, 1982; Larson & Henderson, 2000).

**Molecular mechanisms of DMD**

The primary dysfunction in DMD is the loss of the cytoskeletal protein dystrophin (Hoffman et al., 1987a). Full length dystrophin is a large (427kDa) structural protein that couples the intracellular actin cytoskeleton of the muscle-fibre to extracellular matrix proteins. There are several isoforms of dystrophin, each derived from alternative transcription from one of at least seven promoters, many of which are tissue specific (for review see Blake et al., 2002). Further complexity arises due to alternative splicing of the primary transcripts, resulting in a plethora of structurally diverse dystrophin isoforms, which are expressed in a tissue-specific and developmentally regulated manner (Sironi et al., 2002). Full length dystrophin is comprised of four major domains, of which the N-terminus allows binding to actin via three main sites (Way et al., 1992; Norwood et al., 2000), and the cysteine rich C-terminus allows sarcolemmal localisation and stabilisation via binding a unique set of dystrophin-associated proteins (see below) (Arahata et al., 1991; Sweeney & Barton, 2000). The other two include central rod and cysteine-rich domains (Figures 1.1).
Although encoded for by the largest currently identified gene (~2.5 Mb) and encompassing over 14 kb, full-length dystrophin mRNA represents only around 0.01 to 0.001 percent of total muscle transcripts (Hoffman et al., 1987a; 1987b). Protein expression, despite its functional importance, is similarly sparse (Hoffman et al., 1987a). The subcellular location of dystrophin, rather than its load, is important for its function. Dystrophin helps to protect muscle from contraction-induced injury and is also important as an intracellular scaffold and binding partner, helping to regulate cell signalling via its interaction with a sarcolemmal protein complex termed the dystrophin-associated protein complex (DAPC; also referred to as the dystroglycan complex [DGC]) (Rando, 2001; 2002) (Figure 1.2). The core DAPC of skeletal muscle consists of α and β-dystroglycans, α–ε sarcoglycans and the sarcospan situated in and on the sarcolemma and of the intracellular α-dystrobrevin and syntrophins. Dystroglycans form a receptor for extracellular matrix proteins such as laminin, agrin, and perlecan, while syntrophin and dystrobrevin have additional downstream interacting partners (see below).

Muscle dystrophin interacts with the actin cytoskeleton via a binding site in the 30 kDa N-terminal domain (Way et al., 1992; Norwood et al., 2000), while the C-
terminal domain interacts with DAPC members, thus linking the sub-sarcolemmal cytoskeleton to the cell membrane and further to the extracellular matrix (**Figure 1.2**) (Campbell & Kahl, 1989; Ervasti *et al*., 1990; Ervasti & Campbell, 1991; Ibragimov-Beskrovnaya *et al*., 1992). This link between the cytoskeleton, cell membrane, and extracellular matrix provides structural stability to the sarcolemma during physiological muscle contraction and stretch. The loss of mechanical stability of sarcolemma in the absence of dystrophin has been considered the key mechanism of DMD pathology and explains the elevated serum creatine kinase (CK) levels.

**Figure 1.2.** Schematic representation of the dystrophin associated protein complex (DAPC) (from Rando, 2001). Grb2: growth factor receptor-bound protein 2.
The DAPC is also found in the central and peripheral nervous systems, in secretory tissues such as the pituitary gland (Pocsai et al., 2010), and at restricted access boundaries like the blood-brain and blood-testis barrier (Vajda et al., 2002; Nico et al., 2005; Lien et al., 2007; 2012), the choroid plexus (Haenggi et al., 2004), and in kidneys (Loh et al., 2001). Its composition in different tissues may vary from the skeletal muscle complex and it is anchored there by specific dystrophin isoforms.

Therefore, loss of dystrophin leads to the destabilisation of the DAPC, which may directly contribute to muscle as well as non-muscle symptoms including the cognitive deficits observed in at least a subset of DMD patients (Taylor et al., 2010; Nardes et al., 2012). At the molecular level, the heterogeneous penetration of cognitive dysfunctions might be due to various mutations causing the loss of specific dystrophin isoforms whilst sparing other variants. The severity of mental retardation has been correlated with the loss or modification of C-terminal dystrophin regions, indicating the importance of distally located DAPC binding domains for the function of brain dystrophins (Moizard et al., 1998; Taylor et al., 2010).

The loss or alteration of many of the proteins that make up the DAPC due to mutations of their encoding genes also result in a variety of specific muscle disorders of diverse severity. For instance, several of the limb girdle muscular dystrophies are caused by mutations in the genes for sarcoglycan and dystroglycan (Kaplan, 2011). This points to the importance of the complex. However, while the impaired sarcolemmal integrity and homeostatic function are recognised consequences of the loss of dystrophin and the DAPC, the downstream cellular and molecular events leading to specific DMD abnormalities are still not understood fully and some are subject of a long-lasting debate (Carpenter & Karpati, 1979; Fong et al., 1990; Turner et al., 1991; Yoshida et al., 1997).

The lack of dystrophin reducing membrane resistance to mechanical stress can explain some but not all abnormalities in skeletal muscles and offers no explanation for altered cell signalling or pathology occurring in non-contractile tissues, for example the cognitive deficit associated with loss of specific dystrophin isoforms in the brain.
1.2 The *mdx* mouse as an animal model of DMD

The use of animal models in the study of human diseases relies on the conservation of biology between related species.

Several models of DMD are non-mammalian, such as zebrafish (Chambers *et al.*, 2001; Rubinstein, 2003) and *C. elegans* (Baumeister & Ge, 2002). Mammalian models include dystrophic dog (Cooper *et al.*, 1988; Sharp *et al.*, 1992), cat (Winand *et al.*, 1994) and, the recently developed, pig (Klymiuk *et al.*, 2013). The most widely used model for pre-clinical studies of DMD ([http://treat-nmd.eu/research/preclinical/dmd-sops/](http://treat-nmd.eu/research/preclinical/dmd-sops/)) is the *mdx* dystrophic mouse (Bulfield *et al.*, 1984).

The *mdx* mouse was discovered as a spontaneous dystrophin mutant in C57BL/10ScSn line. It lacks full length dystrophin as a result of a cytosine to thymine missense mutation in exon 23 (Sicinski *et al.*, 1989). The mRNA transcript with a premature stop codon is unstable, which results in its degradation and no protein translation. Therefore, both DMD patients and the *mdx* mouse share a common aetiology, which is the lack of a functional dystrophin protein.

Despite a shared causal mechanism, the *mdx* mouse is not an exact model for human DMD. Progression of muscle degeneration follows a different time course in *mdx* mice to that seen in humans. The main difference is that *mdx* mice present a sudden onset of skeletal muscle necrosis, which peaks at 28 days and decreases to low-level degeneration-regeneration until the cumulative effects become more apparent again at later ages. The exception is *mdx* diaphragm, which shows progressive damage throughout life yet does not lead to significantly premature death. Several explanations have been offered for the overall less severe presentation of the disease in *mdx* mice. From a biophysical perspective, it has been noted that body-size dependent properties such as muscle force generation, tendon mass, and biomolecule diffusion do not scale linearly, relative to total mass, between mice and humans (Partridge, 2013). Also, the pattern of growth differs between humans and mice, where mice gradually and uniformly accrue weight over their juvenile-to-adolescent-to-adult development, whilst humans undergo saltatory bouts of growth during early juvenile development and again during adolescence. Beyond these factors, there are species-specific features of a cellular and molecular nature. For instance, one of the
molecular differences used to explain, albeit partially, differences between the *mdx* mouse and human DMD patients is the compensatory expression of the dystrophin analogue, utrophin. This protein is found in adult *mdx* and DMD patients but considered to provide more protection in mice (Kleopa *et al*., 2006) where *mdx*/utrophin double mutants present with more severe phenotype (Grady *et al*., 1997). Another example is a greater regenerative potential of satellite cells in mice relative to humans, which might depend on longer telomeres found in mice (Sacco *et al*., 2010) and, in general, DMD patients express fewer regeneration-associated genes than *mdx* mice (Turk *et al*. 2005).

Nevertheless, the acute phase of dystrophic pathology provides an appropriate model for testing the pathological mechanisms as well as therapeutic approaches for DMD (Shelton & Engvall, 2005). Any delay in the onset of damage or improvement in disease parameters indicating alleviation of the disease progression in this acute phase can be easily detected. The mouse model of DMD is therefore accepted as the most suitable and affordable for pre-clinical studies of disease biology and the testing of new treatment modalities. The other mammalian models of DMD such as dog and pig are used for therapeutic studies in the more advanced stages as it is thought treatment strategies proven in these large animal models may be more applicable to humans (Partridge, 2013). Work with these larger mammals is considerably more expensive and it is less acceptable to perform terminal or invasive procedures for early-stage hypothesis testing in these more sentient species.

In short, analyses and comparisons of pathological features and dystrophic processes in DMD patients and in animal models led to a much better understanding of disease mechanisms than would be afforded by studies using clinical material only.

### 1.3 Downstream effects of the absence of dystrophin

Major secondary systemic and cellular level abnormalities that are faithfully replicated in animal models of this disease include:

- Chronic inflammation of dystrophic muscles.
- Chronically elevated intracellular Ca\(^{2+}\) levels in skeletal muscle fibres.
• A regeneration defect, such that damaged muscle is increasingly less able to be repaired over time.
• Cell signalling dysregulation.
• A metabolic crisis, such that muscle becomes less efficient at utilising and managing nutrients.
• Elevated levels of oxidative stress mediators.

Many of these features of DMD are directly associated with the biology of the main affected tissue, namely skeletal muscles. In vertebrates, skeletal muscle accounts for up to half of the total body mass and thus represents the single largest organ. The primary function of skeletal muscle is voluntary force production, allowing body movement and stabilizing body position. To fulfil its role, skeletal muscle has a unique combination of properties including excitability, conductivity, contractility, extensibility, and elasticity, which allow the tissue to respond to and propagate signals, shorten when generating force and then return to its original shape. The main muscle cell, the myofibre, is a multi-nucleated and post-mitotic highly specialised and metabolically active cell containing finely tuned assemblies of contractile force-generating proteins. Its growth and repair are maintained by a resident stem cell population termed satellite cells giving rise to myoblasts, which can fuse with existing fibres or with each other to form new myofibres, thus re-enacting early muscle development. However, developmental pathways within individual muscles are different with significant changes in gene expression between head and trunk and between slow and fast myofibres (Mok & Sweetman, 2011; Porter et al., 2001), with further complexity imposed by genetic modifiers active in specific muscle groups (Swaggart et al., 2011). This diversity, albeit not fully understood, appears responsible for specific muscle diseases affecting specific muscle groups or distinct muscle fibres only.

Moreover, skeletal muscle is one of the major sites of glucose (Benito, 2011) and fatty acid (Phillips et al., 1996; Pan et al., 1997; Furler et al., 2001) homeostasis and also helps to control total body pH (Robergs et al., 2004; Kemp, 2005). These functions highlight the metabolic influence skeletal muscle imposes on the organism and can explain the widespread impact of muscle wasting.
Furthermore, in response to acute damage or in pathological states, muscle contains various infiltrating cells releasing molecules, which significantly influence the local microenvironment and contribute to its growth and repair but may also, when released into the bloodstream, affect distant organs (Pedersen & Febbraio, 2012).

Muscle loading is a key catalyst for osteogenesis and increased bone mineral density, especially during adolescence (Slemenda et al., 1994; Wang et al., 2007). Reduced muscle loading contributes to the lower bone mineral densities of DMD patients (Bianchi et al., 2003; Soderpalm et al., 2007; Rufo et al., 2011; Mayo et al., 2012).

The multicomponent nature of the DMD phenotype has been uncovered using global expression profiling strategies like microarray analysis. Chen et al. (2000) found multiple cellular pathways to be systematically altered in human DMD patients compared to healthy individuals. These included the overexpression of genes encoding for embryonic myosin heavy chain, inflammatory mediators, as well as those indicative of a general metabolic breakdown. All of these disease pathways have been later confirmed as differentially activated between dystrophic and non-dystrophic muscles in both humans and mdx mice (Haslett & Kunkel, 2002; Haslett et al., 2002; Porter et al., 2002). Generally, it was found that more genes are upregulated than downregulated in dystrophic compared to healthy muscle, consistent with the degeneration-regeneration profile of a tissue containing increased numbers of activated endogenous and infiltrating cell types (Porter et al., 2002). Some disease-related genes have been found expressed with a pathological weighting in DMD patients even at pre-symptomatic ages (Pescatori et al., 2007).

Inflammation

As many as 30% of all genes that are differentially expressed in DMD muscle are related to inflammation (Porter et al., 2002). Even before acute periods of degeneration, mdx mouse muscle is infiltrated by a variety of leukocytes predominately comprised of neutrophils, macrophages, and activated T-cells (Spencer et al., 2001; Wehling et al., 2001; Wehling-Henricks et al., 2008).

Both the innate and the adaptive system responses are important in DMD. The form of inflammation present in dystrophic muscle is termed ‘sterile' as no pathogens are
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involved and it may also be found in chronic inflammatory conditions including arthritis and atherosclerosis. The role of immunity in the pathology of DMD is consistent with the prevailing view that the immune system responds not only to ‘non-self’, but also to autologous ‘danger’ signals (danger-associated molecular patterns; DAMPs). DAMPs can contain molecular patterns shared by microorganisms and cytoplasmic components but can also be self-molecules, such as adenosine triphosphate (ATP), heat shock proteins, reactive oxygen species (ROS), uric acid or high-mobility group box 1 (HMGB1), that are usually shielded from the immune system, or normally expressed at only marginal levels. Recognition of DAMPS is therefore believed to act as an alert to non-physiologic, necrotic cell death (Rock et al., 2010; Lukens et al., 2012).

Components of the innate immune system are recruited in response to DAMPS. Therefore, macrophages make up the bulk of inflammatory cells in DMD muscles (Spencer et al., 2001; Wehling et al., 2001; Evans et al., 2009). Along with neutrophils, this cell type is responsible for cytolysis, which may further exacerbate the inflammatory condition. However, macrophages infiltrating muscles are heterogeneous and the phenotype of these infiltrating macrophages changes upon acute muscle injury and over the course of disease progression. As found in the mdx mouse, at an acute degenerative period around 4 weeks of age, muscle is mainly infiltrated by type M1 macrophages (Villalta et al., 2009). This cell-type is largely responsible for the cytolysis of muscle fibres and is linked with the Th1 T-cell activation profile. Later in time, M2a and M2c macrophages increase in number (Tidball & Villalta, 2010). These macrophages have been found to reduce inflammation (Gordon, 2003; Martinez et al., 2008; Villalta et al., 2009) and promote regeneration of injured muscle, although they may also promote the extracellular matrix (ECM) build-up that is a well-known disease feature of dystrophic muscle (Vidal et al., 2008).

The inflammatory response to muscle injury is different in DMD compared to that found in acute muscle injury, as the pro-inflammatory component dominates over the pro-regenerative one. The reason for this might be that DMD inflammation may also be coupled to a breakdown in peripheral tolerance to self-molecules (Tidball & Villalta, 2010), which appears to occur in chronic myopathic conditions. Dystrophin is
also immunogenic under certain circumstances. Moreover, other proteins that are normally excluded from muscle are found in the dystrophic tissue. For instance, the fibrin matrix precursor, fibrinogen, is found in human and mouse dystrophic muscle whereas it is restricted inside blood vessels in healthy subjects (Vidal et al., 2008; 2012). Fibrinogen induces the classical inflammatory cascade, resulting in IL-1β, TNFα, and IL-6 release and thus exacerbates the problem.

Therefore, therapies reducing inflammation have the potential to reduce DMD damage, providing that they would not result in reduced muscle regeneration.

**Altered muscle regeneration**

Protecting and enhancing dystrophic muscle regeneration is particularly important as dystrophic muscle precursor (satellite) cells develop a reduced potential for muscle regeneration caused by a process of replicative senescence (Sacco et al., 2010). Satellite cells are able to respond to acute muscle injury but in the chronic disease state they become exhausted. Muscle tissue in DMD patients, and to a lesser extent the mdx mouse (see above), progressively lose ability to regenerate properly. Myoblasts derived from patients with DMD have been shown to have a reduced differentiation potential *in vitro* (Melone et al., 1999). In this sense, muscular dystrophy mimics many other muscle wasting conditions such as cancer cachexia, diabetes, renal failure, heart failure, and age-related sarcopenia.

**Elevated intracellular Ca^{2+} and DMD**

Abnormal ion homeostasis is yet another secondary feature of DMD pathology. This includes an increase in intracellular Ca^{2+} (Bodensteiner & Engel, 1978; Bertorini et al., 1982; Dunn & Radda, 1991) and Na^{+} (Dunn & Radda, 1991; Dunn et al., 1993), although the rise in Ca^{2+} is considered more important for disease pathogenesis or progression and it is indeed one of cellular hallmarks of DMD.

Experimentally introduced intracellular Ca^{2+} is sufficient to cause a dystrophic phenotype in mice, independent of sarcolemmal membrane fragility (Millay et al., 2009) while drugs that reduce intracellular Ca^{2+} levels significantly reduced muscle
degeneration in mdx mice in vivo (Iwata et al., 2005). Furthermore, the majority of proteolytic activity in damaged muscle is considered the result of an increased level of cytosolic Ca\(^{2+}\), subsequent calpain activation (Spencer et al., 1995; Spencer & Mellgren, 2002), and thiol protease activity also independent of lysosomal degradation pathways (Zeman et al., 1985; Furuno & Goldberg, 1986; Goldberg et al., 1986). Additionally, a chronic increase in intracellular Ca\(^{2+}\) has been implicated in altered developmental program in adult myofibres associated with aforementioned reduced differentiation potential (Chen et al., 2000).

Despite endogenous differences in the basal levels of cytosolic Ca\(^{2+}\) between mice (~82nM) and humans (~55nM), the loss of the full-length dystrophin was shown to result in an attenuated Ca\(^{2+}\) regulation in single fibre in vitro assays in both models (Fong et al., 1990). This reduction in Ca\(^{2+}\) regulation was deemed more pronounced when there was a higher extracellular Ca\(^{2+}\) level, indicating the involvement of a calcium leak channel (Turner et al. 1991; for review see Whitehead et al., 2006). Ca\(^{2+}\) sequestration impairment in dystrophic muscle also has the potential to contribute to this ionergic disease pathway (Culligan et al., 2002; Goonasekera et al., 2011).

**Cell signalling abnormalities resulting from the absence of dystrophin**

Lack of dystrophin leads to disruption of the DAPC and the aforementioned decrease in sarcolemmal stability. In addition to these structural abnormalities, lack of dystrophin also results in the loss of anchoring for cell signalling molecules. The best known example is the neuronal nitric oxide (NO) synthase (nNOS). nNOS is a constitutive protein present within muscle cells and is localised to the intracellular side of cell membrane via an attachment to syntrophins within the DAPC (Miyagoe-Suzuki & Takeda, 2001) (Figure 1.2). Metabolic instability and muscle sarcolemma breakdown has been partly ascribed to the delocalisation of nNOS in the absence of dystrophin in DMD (Li et al., 2011b). At the cell membrane, NO donated by nNOS is thought essential for promoting satellite cell activation and fusion but when delocalised it may contribute to overall nitrosative stress leading to nitrosylative protein modifications and the loss of muscle force (Eu et al., 2003; Bellinger et al., 2008a; 2008b; 2009).
A less understood abnormality resulting from the absence of dystrophin involves extracellular ATP (ATPe) breakdown. Signalling by ATPe (see below) is regulated by specific mechanisms such as direct receptor desensitization and degradation of ATP by ecto-ATPases present in the extracellular space (Robson et al., 2006). The latter both eliminates the ATP and generates new signalling molecules (ADP, adenosine) with actions often opposing those of ATP. In addition to nucleoside triphosphate diphosphohydrolases (NTPDases) and ectonucleotide pyrophosphatase/ phosphodiesterases found expressed in muscles (Yegutkin, 2008), the $\alpha$-sarcoglycan of the DAPC (Figure 1.2) has been found to be an atypical ATP-hydrolase activated by very high ATP concentrations (Betto et al., 1999; Sandona et al., 2004). In myotubes in vitro, about 25% of the overall ATP-hydrolyzing activity has been ascribed to $\alpha$-sarcoglycan. In dystrophic muscles, sarcoglycan is depleted or missing, which affects the ectoATPase-mediated regulatory circuit and tips the balance towards ATP release over degradation. Moreover, mutations in the sarcoglycan gene result in limb-girdle muscular dystrophy despite the presence of dystrophin and generally intact links between intracellular cytoskeleton, the cell membrane, and extracellular matrix proteins (Lim & Campbell, 1998). This points to the importance of this DAPC member in the dystrophic pathology.

1.4 ATP signalling and the dystrophic pathology

Several of the secondary abnormalities in dystrophin-deficient muscles, such as decreased ATPe degradation due to loss of ectonucleotidase activity of $\alpha$-sarcoglycan, the chronic inflammation, and increased intracellular $\text{Ca}^{2+}$ levels in dystrophic fibres, may suggest ATP signalling alterations in dystrophic tissues. ATP is a nucleotide best known for its role in cellular metabolic processes, providing the final hydrolysable energy source for numerous ATPase controlled mechanisms. In skeletal muscle, ATP is normally present in cell cytoplasm at levels reaching $\sim$5-9mM (Hochachka & Matheson, 1992; Kushmerick et al., 1992; Hetherington et al., 2001), where it is regularly hydrolysed to drive the actin-myosin cross-bridge cycle, leading to muscle contraction (Drury & Szent-Gyorgyi, 1929; Lymn & Taylor, 1971; Ma & Taylor, 1994; Gordon et al., 2000; Barclay, 2003). Since the pioneering work of Burnstock in the 1970s (Burnstock, 1971; 1972), it has been confirmed that under physiological
conditions small quantities of ATP are released and this ATPe acts as autocrine and paracrine signalling molecule (Burnstock, 2006). For example, at the neuromuscular junction ATP is released with acetylcholine (ACh) to potentiate skeletal muscle fibre contraction (Buchthal & Folkow, 1948).

While small amounts are released under physiological conditions, very high levels of ATPe are released from injured muscles and, acting as a DAMP, can promote inflammatory cell infiltrations (see below). Therefore, ATPe in skeletal muscle can be involved in a range of processes, from physiologically beneficial to pathologically catastrophic. ATP exerts these actions via activation of specific purinoceptors.

**P2 receptors**

The physiological and pathological actions of ATP are mediated by specific transmembrane receptors (Burnstock & Kennedy, 1985). These purinergic receptors consist of two families of nucleotide gated transmembrane proteins. One set, the metabotropic P2Y, are G-protein coupled receptors (GPCRs) linked to a variety of intracellular signalling cascades that are driven by primary effector proteins including phospholipase C, adenylate cyclase, and the small soluble GTPase RhoA. Eight P2Y (1, 2, 4, 6, 11, 12, 13, and 14) receptor subtypes responding to purines have been identified so far in man (for review see Erb et al., 2006). Although several P2Y receptors have been identified in skeletal muscles they are not the subject of this work and therefore will not be discussed further.

The other family is ionotropic P2X receptors, ATP gated ion channels with seven identified subtypes (P2X1-7) having various affinities for ATPe. The P2X receptors are transmembrane proteins between 379 (P2X6) and 595 (P2X7) amino acids in length that display 40-50% protein sequence homology. Each P2X receptor consists of two transmembrane domains separating a large extracellular loop from cytoplasmic C- and N-termini (North, 2002; Kawate et al., 2009) (Figure 1.3).
Figure 1.3. Schematic representation of a P2X receptor. This receptor has a single ~280aa extracellular-loop and two trans-membrane domains (M1 and M2) separating intracellular N- and C-termini. The extracellular loop is shown with 10 conserved cysteine residues that may allow disulphide bridge formation. (adopted from Dunn et al., 2001). The ATP binding site is located within the extracellular loop of the protein. The most up-to-date models for the receptor class are derived from zebrafish P2X4 crystallography study (Kawate et al., 2009; Hattori & Gouaux, 2012).

P2X receptor subunits co-assemble into homo- or hetero-trimers to form a functional receptor channel (Nicke et al., 1998; Barrera et al., 2005; Gonzales et al., 2009; Kawate et al., 2009; Li et al., 2010b).

These receptors, when activated, form a non-selective pore permeable to small cations, including Ca\(^{2+}\). When the channel is in its open state, cations are allowed to travel along an electrochemical gradient. P2X2, 2/3, 2/5, 4, and 7 receptors can also exhibit multiple open states in response to ATP, characterized by a still ill-understood permeability to larger (molecular weights of several hundred Da) molecules such as N-methyl-D-glucamine (NMDG) or ethidium bromide (Khakh et al., 1999; Virginio et al., 1999; Compan et al., 2012; Browne et al., 2013). The P2X receptors are variously sensitive to their primary ligand ATP, with P2X7 displaying the lowest sensitivity to ATP coupled with a slow desensitisation (Gever et al., 2006). The P2X7 receptor is structurally notable amongst the P2X receptors for possessing a long C-
terminal tail, accounting for the majority of the increased amino acids in the polypeptide sequence (Figure 1.4).

During development and regeneration in adults, muscle fibres in species ranging from chick to rodents to humans have been shown to express P2Y1 and 2 (Tung et al., 2004), and P2X1, 2, 4, 5, 6, and 7 receptors (Ryten et al., 2001; 2004; Jiang et al., 2005; Yeung et al., 2006). Our understanding of the specific physiological roles of these receptors is, however, still incomplete. They have been implicated in numerous cellular processes, including, but probably not restricted to, the proliferation of myoblasts (Martinello et al., 2011), the differentiation of myotubes (Ryten et al., 2002), vasoconstriction, ion homeostasis, and apoptosis.

For example, secondary myotube generation and ACh expression was related to P2X2, P2X5, and P2X6, expression in developing rat skeletal muscles (Ryten et al., 2001; 2002). It was also shown that P2X7 receptors may have a role in proliferation and differentiation in C2C12 mouse skeletal muscle cells (Araya et al., 2004; Martinello et al., 2011).

P2X1 receptors have been found to mediate vasoconstriction and blood flow is integral to maintaining the metabolic integrity of skeletal muscle (Buckwalter et al., 2003; 2004; Kluess et al., 2005a; 2005b). However, this effect has been linked with P2X1 receptor expression in smooth muscles (Ralevic & Burnstock, 1996; Lewis & Evans, 2000a; 2000b; Jiang et al., 2005).

In addition, several P2X receptors have been implicated in paracrine/autocrine signalling especially related to inflammation. It is in this second role, signalling in pathological conditions, where specific P2X receptors have been found of particular importance for the pathogenesis of DMD.
Figure 1.4. P2X7 receptor amino acid sequence comparison across species. Amino acid sequences corresponding to both transmembrane domains (TM1 and TM2) are highlighted. Noticeable is the conserved length across mammalian P2X7 receptors of different species despite the pronounced polymorphisms (blue colour indicates less conserved residues and red indicates highly conserved ones). Query submitted to NCBI: BLAST.
**Purinergic receptor involvement in skeletal muscle pathology**

It has been predicted that at ATPe concentrations in excess of 100μM, ectoATPases cannot cope with ATPe overload (Bodin & Burnstock, 1996). Taking into account that skeletal muscle has the largest ATP reserves of all tissues, it seems unlikely that ecto-ATPases would abate the effects of cellular ATP released from damaged cells. When combined with decreased ectoATPase activity in the absence of sarcoglycan, ATPe levels would become high, forming an environment that would clearly favour over-activation of P2 purinoceptors. This led our laboratory to hypothesise that the dystrophic phenotype may involve abnormalities in purinergic signalling. DNA microarray analysis of dystrophic muscle from *mdx* mice revealed upregulated expression of P2X4 purinoceptor transcripts (Porter *et al.*, 2002) that may be caused by infiltrating macrophages. This finding is consistent with a very strong inflammatory component associated with the DMD pathology (Ichim *et al.*, 2010). It is also the case that tissue macrophages express P2X4 in addition to the P2X7 purinoceptor (Yeung *et al.*, 2004).

Intriguingly, further studies showed that *mdx* myoblasts expressed significantly higher levels of P2X7 purinoceptor proteins than wild type controls (Young *et al.*, 2012). This up-regulated P2X7 purinoceptor expression has been shown to be retained following the differentiation of dystrophic mouse myoblasts to myotubes. Moreover, elevated expression and function of P2X7 purinoceptor in dystrophic skeletal muscle groups have been demonstrated in situ (Yeung *et al.*, 2004; 2006; Young *et al.*, 2012). These observations correlated with an earlier discovery of an altered P2X7 purinoceptor function in DMD patient lymphoblasts (Ferrari *et al.*, 1994).

### 1.5 P2X7 and DMD

Among the P2X receptors, P2X7 has many properties that make it well suited to potentiate DMD disease characteristics. P2X7 purinoceptors are activated by a much higher [ATP]e concentration than other P2X purinoceptors. Thus, P2X7 has been dubbed a “danger receptor” responding to excessive, non-physiologic ATPe levels (“danger signal”) released in cases of necrotic cell death. The P2X7 receptor is also
known as the canonical death receptor, due to an association with both apoptotic and necrotic pathways in numerous cell types (Schulze-Lohoff et al., 1998; Wang et al., 2004; Kong et al., 2005; Sugiyama et al., 2005; 2010; Haanes et al., 2012).

The up-regulated expression and function of P2X7 purinoceptors in dystrophic cells suggests that it may there play roles that were recognized in several other disorders. The well-documented involvement of P2X7 purinoceptors in ATP-induced cell death and in release of immune/inflammatory modulators (see below) is of particular relevance in dystrophic muscles, where degenerating fibres provide potential sources of ATPe at concentrations exceeding those that could be reached anywhere else in the body. Death of myofibres would exacerbate the disease while death of myoblasts would further prevent their regeneration.

In humans, P2X7 cDNA has been detected under physiological conditions in a variety of tissue types, including glia (Pannicke et al., 2000; Wang et al., 2002; Chen et al., 2008; Murphy et al., 2012), airway epithelia (Groschel-Stewart et al., 1999), salivary glands (Soltoff et al., 1990; Nakamoto et al., 2009), pancreas (McMillian et al., 1993; Christoffersen et al., 1998; Novak et al., 2010), pituitary gland (Kimm-Brinson et al., 2001), kidney (Rassendren et al., 1997; Harada et al., 2000; Hillman et al., 2002), skin fibroblasts (Solini et al., 1999), smooth muscle (Cario-Toumaniantz et al., 1998), cells of haematopoietic origin including osteoblasts, osteoclasts and most innate and adaptive immune cells (Collo et al., 1997), as well as in skeletal muscle (Rassendren et al., 1997).

It is now recognised that, in certain cellular contexts, basal activation of P2X7 may be pro-survival and growth promoting (Adinolfi et al., 2005a; 2005b; Di Virgilio et al., 2009). The relative significance of either cell death or growth-promoting effects depend on the expressing cell type but data from our laboratory suggests that in myoblasts P2X7 stimulation is able to switch between tonically beneficial (e.g. growth), and excitotoxically catastrophic effects depending on the receptor expression and ATPe levels. Enhanced P2X7 receptor activation in dystrophic muscle cells leads to a whole range of effects including increased intracellular Ca$^{2+}$ levels, extracellular-signal-regulated kinase (ERK) phosphorylation, altered muscle regeneration potential (Yeung et al., 2006; Young et al., 2012) and autophagic cell death (Young et al., 2015).
Based on pharmacological experiments, Ca$^{2+}$ imaging studies, and protein expression comparisons, it is reasonable to suppose that P2X7 receptors could contribute to the significant increase in intracellular Ca$^{2+}$ in dystrophic muscle (Ryten et al., 2004; Yeung et al., 2006; Young et al., 2012). The myofibre damage in DMD has been shown to result from increased cytosolic Ca$^{2+}$ levels that is not attributable to sarcolemmal damage alone (Millay et al., 2009). While abnormal increases in Ca$^{2+}$ influx via L-type, transient-receptor-potential, and stretch-activated channels into mdx myotubes have been documented (Vandebrouck et al., 2002; Millay et al., 2009; Kumar et al., 2004; Friedrich et al., 2004; 2008) further increases could be caused by the elevated expression and activity of P2X7 ion channels.

Additionally, upon prolonged activation, the P2X7 receptor has the capacity to form or to promote formation of a transmembrane pore capable of passing large molecules into the cell (Ferrari et al., 1996; Browne et al., 2013). This pore formation allows massive influx of ions and release of the intracellular content (DAMPs), therefore facilitating a sterile inflammatory response that can ultimately lead to cell death.

Therefore, P2X7 stimulation has the potential to mediate muscle cell lysis also via the activation or modification of a sterile inflammatory response. In this regard, P2X7 receptor overexpression has been linked to numerous chronic inflammatory conditions including rheumatoid arthritis (Baroja-Mazo & Pelegrin, 2012), atherosclerosis (Solini et al., 1999), glomerulonephritis (Turner et al., 2007), and Crohn’s disease (Kurashima et al., 2012). Again related to the relative expression of P2X7 receptors, it has been shown that 1) the receptor responds to ATP as an autocrine/paracrine danger sensor and 2) the receptor may be modulating the immune cell phenotype to promote prolonged pro-inflammatory infiltrations. In one recent example, P2X7 receptors were implicated in the deterioration of immune self-tolerance known to perpetuate chronic inflammatory psoriasis (Mathers et al., 2012). In another example study, pharmacological inhibition of P2X7 receptors was used to improve post cardiac transplant survival rates in a mouse model (Vergani et al., 2013).

Considering that inflammation is a hallmark of DMD, the abnormal function of P2X7 purinoceptors appears to close the loop whereby damaged dystrophic muscle would release large quantities of ATP but also other autologous DAMPs, which initiate and perpetuate immunity in response to tissue damage. Infiltrating macrophages, as
described earlier, have been shown to contribute to both skeletal muscle degeneration and regeneration. This intricacy of crosstalk between myoblasts, macrophages, and probably other cell types in dystrophic muscles, as well as the balance between tonically beneficial (e.g. growth) and toxic, catastrophic, effects of P2X7 purinoceptor activation on myoblasts and muscle fibres, requires elucidation. This signalling network has a potential fundamental influence over disease progression and thus presents a good candidate target for pharmacological interventions, providing that P2X7 blockade could alleviate muscle damage without down-regulation of potentially beneficial regenerative processes.

One of the fundamental strategies used in functional investigations of P2X7 receptors has been the generation and analyses of mice in which the P2X7 gene has been disrupted.

1.6 P2X7 knock-out mice and their relevance to this study

There are currently two P2X7 KO mice available for use. These were created by researchers working for Glaxo Smith Kline (Chessell et al., 2005) and Pfizer (Solle et al., 2001). The Glaxo P2X7r KO mouse contains a lacZ insertion in exon 1 (of 13) of the P2X7 gene, whilst the Pfizer KO mouse has a neomycin cassette replacing a region of exon 13 (also see Methods 2.1 and Results 3.3.1). Both mice are viable and superficially indistinguishable from wild-type controls. Analyses of P2X7 KO mice, from either Glaxo or Pfizer, have supported hypotheses indicating the involvement of the purinergic receptor in numerous biological pathways. For instance, the P2X7 receptor has been linked with IL-1β maturation and release from macrophages (Solle et al., 2001) and adjuvant-induced inflammation and neuropathic pain (Chessell et al., 2005). These observations reflect the aforementioned associations between P2X7 and chronic inflammatory conditions. In other studies the P2X7 KO mice were used to test associations between the receptor and diseases. In one example, the Glaxo P2X7 KO mouse showed significant renoprotection in an established model of glomerulonephritis (Taylor et al., 2009b) and P2X7 KO reduced collagen deposition and TGFβ levels (Goncalves et al., 2006). In another study, Pfizer P2X7 KO mice were protected from experimentally induced carotid artery thrombosis (Furlan-Freguia et al., 2011). The P2X7 KO mice have, therefore, an established role in
testing the involvement of this receptor in disease and there are clear similarities in pathobiology between several of the abovementioned disorders and DMD, for instance associated with immune and inflammatory responses.

The two P2X7 receptor KO animals differ in important ways, precluding their use as interchangeable KO models. Indeed, our laboratory has been involved in identification of the molecular differences in both available P2X7 KO mouse strains (Nicke et al., 2009; Masin et al., 2012). The Glaxo P2X7 KO mouse is a hypomorph where one of the receptor isoforms, the P2X7 ‘K’ variant, resulting from the use of an alternative promoter, escapes inactivation (Nicke et al., 2009). Although expressed at very low levels, P2X7 ‘K’ is highly active when compared to the predominant full-length ‘A’ variant and can explain the residual responses observed in some tissues from this KO. The biological significance of this P2X7 variant is unclear as it is not found in man but, in terms of this study, the Glaxo P2X7 KO mouse has been considered against the full-functional Pfizer P2X7 KO for effects of dose-dependency, to compare cellular and biochemical features of mdx dystrophinopathy between the two models. To establish the impact of the P2X7 receptor on the dystrophic pathology and to assess its suitability as a specific therapeutic target, we have used selective breeding to develop two lines of mdx mice with the P2X7 gene disrupted (see 1.8 Aims and hypothesis for a more detailed breakdown of subsequent work-flow).

1.7 Summary: a recapitulation

DMD is a complex disease involving cell-structural and metabolic abnormalities, for which there are currently palliative therapies only. Skeletal muscle is an important organ, ordinarily helping to regulate total body metabolism, blood pH, and osteogenesis, in addition to being the primary effector unit of voluntary force production. Downstream of dystrophin absence, secondary pathological features of DMD include the loss of the DAPC and the accompanying mechanical tether between the intracellular actin cytoskeleton and extracellular matrix proteins; an increase in muscle intracellular Ca$^{2+}$; a reduction in muscle regeneration potential over increasing cycles of degeneration and regeneration; chronic inflammation; delocalised cellular nNOS leading to increased protein nitrosylation and reduced potential for muscle force generation; a neurological deficit; and an osteopenic phenotype with increased
risk of bone fracture. Although it is likely that DMD pathology includes many other salient cellular and molecular features, all of the above mentioned pathways are shared, to a greater or lesser extent, between mice and humans.

The P2X7 receptor shows increased expression in dystrophic mouse muscle cells compared to healthy controls, in vitro, and at both acute degenerative and regenerative phases of disease progression in vivo. Furthermore, dystrophic muscle cells are more sensitive to ATP induced permeabilisation and calcium build-up, as well as phospho-ERK1/2 activation.

Given the known role of the receptor in cell death and growth processes, and related to the potential of the receptor to mediate chronic inflammatory conditions, it was decided to further explore the role of P2X7 in the pathogenesis of DMD, using a well-established model of this disease, the mdx mouse. By comparing the mdx mouse to two dystrophic P2X7 KO (dKO) mice according to several key cellular and molecular disease parameters, it was hoped to derive particular biological functions of the P2X7 receptor in DMD disease progression. It was expected that, by removing P2X7 function, it would be possible to alleviate mdx disease pathology, also providing data relevant for the evaluation of the potential for P2X7 pharmacological interventions with recourse for clinical therapeutic purpose in human DMD patients (Figure 1.5).
Figure 1.5. Schematic depiction of hypothetical involvements of the P2X7 receptor in *mdx* and DMD pathogenesis. **a** Loss of dystrophin leads to multiple secondary molecular, cellular, and system level disease features. Each of these may be exacerbated by P2X7 hyper-activation. **b** The P2X7R is depicted as mainly coinciding and slightly preceding pathological progression over non-linear time. Pathology is here defined as the sum of the compounding and interacting disease features depicted in ‘a’. In *mdx* hindlimb skeletal muscle, the phase of acute degeneration begins after ~3 weeks and is waning from ~6 weeks (top panel). Natural progression of the disease also results in late-term atrophy and disease pathology, including exaggerated fibrosis, that we have provisionally ascribed to one published time-point, at 62 weeks (Pastoret & Sebille, 1995). Our hypothesis is that P2X7R ablation may be capable of reducing or delaying pathology over disease development (Purple trajectory). Onset and disease severity may also be modified in an activity dependent manner, such that exercised *mdx* mice would present increased pathology, but this has not been purposefully integrated into this model. In *mdx* diaphragm, disease progression is considered progressive, similar in principle to human DMD, in general (lower panel).
1.8 Hypothesis, aims, and objectives

The main aim of this study is to evaluate the effects of P2X7 receptor knock-out on histological, biochemical, molecular, and functional parameters of mdx pathology. Given the known roles of P2X7 in cell death, chronic inflammatory diseases, and its functional upregulation in dystrophic mouse muscle, this receptor has the potential to exacerbate multiple aspects of mdx disease progression through triggering muscle cell death, abnormal regeneration and Ca\(^{2+}\) homeostasis, as well as maintaining chronic inflammation. **It is hypothesised that inactivation of the P2X7 receptor will ameliorate disease in the mdx mouse model of muscular dystrophy.**

Specific objectives are as follows:

- To characterise mdx/P2X7\(^{-/-}\) double mutant mice generated by crossing mdx with two different strains of P2X7 KO mouse and to compare morphological and molecular parameters at the acute phase of muscle disease progression in 4 week old mice.

- To profile P2X7 receptor expression and co-localisation with immune cell sub-populations in mdx skeletal muscle and to compare infiltrating cells between 4 week old mdx and mdx/P2X7\(^{-/-}\) muscles.

- To measure and compare functional aspects of disease such as sarcolemmal breakdown and muscle force generation *ex vivo* and *in vivo*.

- To test the influence of P2X7 depletion on secondary features of dystrophic pathology by comparing bone mineral density and in behavioural tests *in vivo* in mdx and mdx/P2X7\(^{-/-}\) mice.

- To perform preliminary pharmacological experiments *in vivo* on the effects of administration of P2X7 antagonist, CBB, on serum CK levels at the acute phase of muscle disease progression in 4 week old mice.

- Histologically assess aged diaphragm and heart tissue, to see if mdx disease pathology continues to be alleviated or is worsened over a longer-term by P2X7 ablation.
2

Materials and methods

2.1 Animals

Mice were bred and maintained in a controlled environment (12 hour light/dark cycle, 19-23°C ambient temperature, 45-65% humidity). They were fed a standard pellet diet (Economy rodent breeder diet, Special Diet Services, Witham, UK) and allowed tap water ad libitum.

To limit heterogeneity in outcome measures, only male mice were used for experimentation.

Mice were killed by CO₂ asphyxiation followed by cervical dislocation.

Wild-type control

The C57BL/10ScSnJ wild-type (WT) mouse strain was used as a healthy control (Jackson laboratory s/n: 000476; from here abbreviated to C57 BL/10). This mouse was derived from the C57BL/10J sub-strain.

DMD mouse model

The dystrophic mouse used in this study was the C57BL/10ScSn-Dmdmdx/J (Jackson lab. s/n: 001801; abbreviated to mdx). This mouse was discovered in 1976 as a spontaneous mutant in C57BL/10ScSn mice at the Agricultural Research Centre, U.K (http://jaxmice.jax.org/strain/001801.html).

P2X7 knock-out mice

Two P2X7 receptor KO mice were used in this project.
One P2X7−/− KO mouse (Jackson lab s/n: 005576) was created in the C57BL/6 strain by introduction of a neomycin resistance cassette into exon 13 of the P2X7 gene. This mouse was created by Christopher A. Gabel of Pfizer Pharmaceuticals (Solle et al., 2001). In this study we refer to this animal as Pfizer P2X7−/− KO.

The other P2X7−/− KO mouse was also created in the C57BL/6 mouse, this time by insertion of a LacZ gene into exon 1 of the mouse P2X7 gene. This mouse was created by researchers working for Glaxo Smith Kline (Chessell et al., 2005). In this study we refer to this animal as Glaxo P2X7−/− KO.

**mdx/P2X7 double-KO mice**

Two mdx/P2X7−/− dKO mice were generated by selective breeding of mdx with either a Pfizer or Glaxo dKO (Figure 2.1). A female mdx mouse was crossed with a male homozygous KO mouse from one of two P2X7 KO mouse strains, developed by Pfizer and Glaxo. From the subsequent litter, all male F1 mice were dystrophic and P2X7+/- heterozygous. Back-crossing these males with parental P2X7+/+ mdx females led to a heterogeneous litter composed of 100% dystrophic males, of which 50% were P2X7+/+ and 50% were P2X7+/−. Genotyping was used to select P2X7+/− heterozygous males and females. These P2X7+/− mdx males and females were crossed. Mating individuals from this litter together resulted in a litter of 100% dystrophic animals of which 25% were also P2X7−/−. Double mutant individuals from this cross were identified via genotyping to establish the mdx/P2X7−/− colony. The characterisation of the resultant litters are defined in Chapter 3.
2.2 Ethical considerations

The major ethical consideration in this study related to the use of an experimental mouse model for biomedical research. University local ethical approval and Home Office licenses have been granted for the use of animals in this research. All mice were processed in accordance with recommended UK Home Office animal welfare guidelines governing the treatment of experimental animals


Mouse husbandry, including day-to-day cleaning and monitoring of animal living conditions, was performed by specialist senior animal technicians under the
supervision of a veterinary surgeon. This team have been trained and have experience to identify symptoms of disease and stress in the mice, and can react appropriately to minimise suffering or to aid recovery. This can also include removing specific animals from certain test regimes if conditions change or humanely killing animals whose health deteriorates without signs of improvement.

Various levels of animal discomfort are to be expected in many experimental procedures involving animals. All procedures used in this study, including breeding of harmful mutants (knockout mice), general handling, occasional collecting of ear punches for genotyping, and the use of intraperitoneal injections for drug delivery, are classified as “Mild” or below (unclassified) according to the Home Office directives. Mdx or P2X7 receptor knockouts are known to be mild conditions not associated with pain or decreased viability. Adopting the 3R principles, every attempt was made to minimise any discomfort associated with experimental procedures. All the preliminary testing of the effects of P2X7 receptor inhibition that could be performed in vitro has been completed for previous published studies (Jiang et al., 2005; Yeung et al., 2004; 2006; Young et al., 2012; Masin et al., 2012). There is no reasonable non-animal alternative to test the effects of receptor ablation on this complex disease involving interplay between muscle degeneration and regeneration, inflammation, as well as bone and CNS abnormalities.

The numbers of animals used in each experiment were based on statistical power calculations (e.g. appendix 9.10) and previous experience with specific methods. To minimise the numbers of animals euthanised, as many individual tissues were collected from each animal as was possible. Samples not immediately used were stored.

### 2.3 List of reagents

<table>
<thead>
<tr>
<th>Product</th>
<th>Manufacturer</th>
<th>Associated assay(s)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Methylbutane</td>
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<td>Cryosectioning</td>
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<td>Sigma Aldrich</td>
<td>Masson’s Trichrome</td>
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<td>Life Technologies</td>
<td>Nucleotide electrophoresis</td>
</tr>
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<td>Agarose gel loading buffer</td>
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</tr>
<tr>
<td>Ammonium chloride</td>
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<td>Immunolocalisation</td>
</tr>
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<td>Material/Reagent</td>
<td>Supplier</td>
<td>Application</td>
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<tr>
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<td>-------------</td>
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<tr>
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<td>Masson’s Trichrome</td>
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<td>Sigma Aldrich</td>
<td>Western blotting</td>
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<td>Sigma Aldrich</td>
<td>Protein quantification</td>
</tr>
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<td>Protein quantification</td>
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<td>Complete Mini Protease Inhibitor Cocktail Tablets</td>
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<td>P2X7 inhibitor study</td>
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<td>Copper (II) sulphate (4% w/v)</td>
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<td>Histology</td>
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<td>ImmPACT NovaRed</td>
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<tr>
<td>ImmPRESS polymerised reporter</td>
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<td>IHC</td>
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### Table 2.1. List of reagents. *main assays associated with products in the current study; not exhaustive.*

2.4 Buffers and solutions

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<th>Composition</th>
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<td><strong>TAE buffer (x1)</strong></td>
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Chapter 2: Materials and methods

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<tr>
<td>Poly-L-lysine (x50)</td>
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<td>4% PFA</td>
<td>Paraformaldehyde (2-4% w/v), in TBSt</td>
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<td>TBS(t)</td>
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<td>Autofluorescence quenching solution</td>
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<td>Tris-HCl (resolving)</td>
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<td>NaCl (118mM), KCl (4.7mM), NaHCO3 (24.88mM), KH2PO4 (1.18mM), Glucose (11.1mM), MgSO4 (0.82mM), CaCl2 (2.52mM)</td>
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<td>Aniline blue diammonium salt (31.77mM, w/v), Glacial acetic acid (2% w/v), DDH2O</td>
</tr>
</tbody>
</table>

Table 2.2. List of buffers and solutions.

2.5 List of antibodies

<table>
<thead>
<tr>
<th>Protein</th>
<th>Manufacturer</th>
<th>Host</th>
<th>Antigen Spec.</th>
<th>Size on WB</th>
<th>Rec. Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>Sigma</td>
<td>Rabbit</td>
<td>Many</td>
<td>42 kDa</td>
<td>1/1000</td>
</tr>
<tr>
<td>β-tubulin</td>
<td>Imgenex</td>
<td>Rabbit</td>
<td>Many</td>
<td>~40-55 kDa</td>
<td>1/2000 WB</td>
</tr>
<tr>
<td>CD11b</td>
<td>BD Biosciences</td>
<td>Rat</td>
<td>M</td>
<td>~170 kDa</td>
<td>1/500 WB, 1/300 IF</td>
</tr>
<tr>
<td>CD163</td>
<td>Santa Cruz</td>
<td>Rabbit</td>
<td>M</td>
<td>130 kDa</td>
<td>1/500 WB, 1/500 IF</td>
</tr>
<tr>
<td>CD4</td>
<td>eBiosciences</td>
<td>Rat</td>
<td>M</td>
<td>55 kDa</td>
<td>1/250 WB, 1/200 IHC</td>
</tr>
<tr>
<td>CD68</td>
<td>AbD Serotec</td>
<td>Rat</td>
<td>M</td>
<td>87-115 kDa</td>
<td>1/750 WB, 1/500 IF</td>
</tr>
<tr>
<td>CD8</td>
<td>eBiosciences</td>
<td>Rat</td>
<td>M</td>
<td>32-34 kDa</td>
<td>1/250 WB, 1/200 IHC</td>
</tr>
<tr>
<td>Collagen-Ia</td>
<td>Abcam</td>
<td>Rabbit</td>
<td>H, M, Rat, B, G</td>
<td>Not tested</td>
<td>1/500 IF</td>
</tr>
<tr>
<td>Collagen-IV</td>
<td>Chemicon (Millipore)</td>
<td>Goat</td>
<td>M</td>
<td>Not tested</td>
<td>1/500 IF</td>
</tr>
<tr>
<td>Antibody</td>
<td>Manufacturer</td>
<td>Host</td>
<td>Antigen Spec.</td>
<td>Source</td>
<td>Conjugate</td>
</tr>
<tr>
<td>-------------------</td>
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<td>---------------</td>
<td>--------</td>
<td>---------------------</td>
</tr>
<tr>
<td><strong>Dystrophin</strong></td>
<td>Gift from Derek Blake</td>
<td>Rabbit</td>
<td>M</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DSHB</td>
<td>Mouse</td>
<td>M</td>
<td></td>
<td>Full length</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
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<tr>
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</tr>
<tr>
<td><strong>EyMHC</strong></td>
<td>Santa Cruz, DSHB</td>
<td>Mouse</td>
<td>M</td>
<td></td>
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<tr>
<td></td>
<td></td>
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<td></td>
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<tr>
<td><strong>F4/80</strong></td>
<td>Abcam</td>
<td>Rabbit</td>
<td>M</td>
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<tr>
<td><strong>Foxp3</strong></td>
<td>eBioscience</td>
<td>Rat</td>
<td>M</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>GAPDH</strong></td>
<td>Sigma</td>
<td>Rabbit</td>
<td>Many</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IL-6</strong></td>
<td>R&amp;D systems</td>
<td>Goat</td>
<td>M</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td><strong>Ly6G (Gr-1)</strong></td>
<td>eBioscience</td>
<td>Rat</td>
<td>M</td>
<td></td>
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</tr>
<tr>
<td><strong>Myogenin</strong></td>
<td>Santa Cruz</td>
<td>Mouse</td>
<td>M</td>
<td></td>
<td></td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td><strong>Osteopontin</strong></td>
<td>R&amp;D systems, DSHB</td>
<td>Goat</td>
<td>M</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mouse</td>
<td>Rat H, Rb, M</td>
<td></td>
<td></td>
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<tr>
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</tr>
<tr>
<td><strong>P2X4</strong></td>
<td>Alomone</td>
<td>Rabbit</td>
<td>M</td>
<td></td>
<td></td>
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<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>P2X7</strong></td>
<td>Synaptic Systems</td>
<td>Rabbit</td>
<td>M, Rat</td>
<td></td>
<td></td>
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</tr>
<tr>
<td><strong>Pax7</strong></td>
<td>Abcam, DSHB</td>
<td>Rabbit</td>
<td>M, (+many)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td><strong>TGF-β</strong></td>
<td>Abcam</td>
<td>Rabbit</td>
<td>H, M, Rat</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Utrophin (MANCHO 8A4)</strong></td>
<td>DSHB</td>
<td>Mouse</td>
<td>H M</td>
<td></td>
<td>400 kDa</td>
</tr>
</tbody>
</table>

Table 2.3. Primary antibodies used for Western blotting and immunolocalisation analysis. M = mouse, H = human, C = chicken, Rb = rabbit, B=bovine.

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Host</th>
<th>Antigen Spec.</th>
<th>Conjugate</th>
<th>Rec. Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sigma</td>
<td>Goat</td>
<td>Rabbit</td>
<td>HRP</td>
<td>&lt;1/5000 WB</td>
</tr>
<tr>
<td>Sigma</td>
<td>Goat</td>
<td>Mouse</td>
<td>HRP</td>
<td>&lt;1/5000 WB</td>
</tr>
<tr>
<td>Life Technologies</td>
<td>Rabbit</td>
<td>Goat</td>
<td>HRP</td>
<td>&lt;1/5000 WB</td>
</tr>
<tr>
<td>Life Technologies</td>
<td>Goat</td>
<td>Rat</td>
<td>HRP</td>
<td>&lt;1/5000 WB</td>
</tr>
<tr>
<td>Life Technologies</td>
<td>Goat</td>
<td>Rabbit</td>
<td>Alexa Fluor 488</td>
<td>1/1000 IF</td>
</tr>
<tr>
<td>Life Technologies</td>
<td>Chicken</td>
<td>Rat</td>
<td>Alexa Fluor 647</td>
<td>1/1000 IF</td>
</tr>
<tr>
<td>Life Technologies</td>
<td>Chicken</td>
<td>Rabbit</td>
<td>Alexa Fluor 488</td>
<td>1/1000 IF</td>
</tr>
<tr>
<td>Life Technologies</td>
<td>Chicken</td>
<td>Mouse</td>
<td>Alexa Fluor 488</td>
<td>1/1000 IF</td>
</tr>
<tr>
<td>Life Technologies</td>
<td>Chicken</td>
<td>Goat</td>
<td>Alexa Fluor 594</td>
<td>1/1000 IF</td>
</tr>
<tr>
<td>Life Technologies</td>
<td>Donkey</td>
<td>Rabbit</td>
<td>Alexa Fluor 555</td>
<td>1/1000 IF</td>
</tr>
<tr>
<td>Life Technologies</td>
<td>Donkey</td>
<td>Rabbit</td>
<td>Alexa Fluor 594</td>
<td>1/1000 IF</td>
</tr>
<tr>
<td>Life Technologies</td>
<td>Donkey</td>
<td>Mouse</td>
<td>Cy3</td>
<td>1/1000 IF</td>
</tr>
<tr>
<td>Life Technologies</td>
<td>Donkey</td>
<td>Rat</td>
<td>Alexa Fluor 594</td>
<td>1/1000 IF</td>
</tr>
<tr>
<td>Life Technologies</td>
<td>Donkey</td>
<td>Goat</td>
<td>Alexa Fluor 546</td>
<td>1/1000 IF</td>
</tr>
</tbody>
</table>

Table 2.4. Secondary antibodies used for Western blotting and immunolocalisation analyses.
2.6 Nucleic acid analyses

2.6.1 Genomic DNA extraction

Mouse tail clip samples were removed from recently killed mice and ear punches were collected from living animals. Samples were incubated in proteinase K (40mAU/mg of tissue) for 6-8 hours or overnight in a shaking incubator at 55°C, to hydrolyse proteins and to inactive RNases and DNases. Next, a commercial DNA extraction spin kit (Qiagen DNeasy™ blood and tissue DNA extraction kit) was used to trap, purify, and then elute total genomic DNA, as follows: Digested sample containing intact DNA was mixed with a binding solution and centrifuged at 6,000 x g for 1 minute. DNA, trapped in the silica column, was then washed by centrifuging two volumes of ethanol solution through the column at 6,000 and then 20,000 x g, for 1 minute and 3 minutes, respectively. Finally, DNA was eluted in a Tris EDTA buffer (10mM Tris.Cl, 0.5mM EDTA, pH 9.0) ready for immediate use or long term storage at -80°C.

2.6.2 RNA extraction

RNA was extracted from tissue that had been crushed under liquid nitrogen using a pestle and mortar. Half of the tissue powder was set aside and used for protein extraction (see below). 1mL of TRIzol reagent was added per 100mg of muscle tissue. Following homogenisation by lavage through a 23 gauge needle and syringe, the sample-TRIzol solution was centrifuged at 12,000 x g for 10 minutes at 4°C. A fatty layer was then removed. Supernatant was incubated at RT for 5 minutes. 0.2mL of chloroform was then added per 1mL of TRIzol used for homogenisation. Following a short vigorous mixing for 15 seconds and 2-3 minutes of incubation at RT, samples were centrifuged at 12,000 x g for 15 minutes at 4°C. The aqueous phase was removed and 0.5mL of 100% isopropanol was added to this aqueous phase per 1mL of TRIzol used for initial homogenisation. Samples were incubated for 10 minutes and then centrifuged at 12,000 x g for 10 minutes at 4°C to form an RNA pellet. The supernatant was discarded and the pellet was washed with 75% RNase-free ethanol. Following a final centrifugation at 7,500 x g for 5 minutes at 4°C, the pellet
was resuspended in RNase free water for storage at -80°C or immediate downstream applications.

2.6.3 Nucleic acid quantification by spectrophotometry

2μl of neat nucleic-acid solution was placed on the measuring pedestal of a NanoDrop™ 1000 (Thermo Fisher Scientific) spectrophotometer, after zero calibration with Tris EDTA buffer. Nucleotide concentration was determined by measuring the peak absorbance of the solution at the optical wavelength of 260nm and protein contamination was quantified by calculating the ratio of the absorbance at 280 compared to 260nm. DNA and RNA have a known peak absorbance of light with a wavelength of 260nm, where an undiluted sample of DNA with an optical density of 1 will contain 50μg/mL of DNA and 40μg/mL for RNA. Samples with a 260/280 absorbance ratio equal to or greater than 1.8 were considered free from protein contamination and suitable to be used for PCR.

2.6.4 Polymerase Chain Reaction (PCR)

PCR was used to genotype experimental animals. 1-2μl of template DNA (<250ng) was added to a reaction tube containing 0.125μl of Taq DNA polymerase (NEB, 0.625 units/25μl reaction), 5μl DNA polymerase buffer (x5 stock), 1.5μl MgCl₂ (25mM), 0.5μl dNTPs (10mM), 0.5μl forward primers (20pmol/μl), 0.5μl reverse primers (20pmol/μl), made up to 25μl with double distilled H₂O. For convenience, a pre-prepared commercial master-mix (Promega™ GoTaq® 2xGreen Master Mix) was also sometimes used, to which forward primers, reverse primers, and DNA template were added. The following PCR amplification cycle profile was then applied using a thermal cycler (Primus 96 Plus®, Peqlab Biotechnologie GmbH, Erlangen, Germany):

Denaturing at 94°C for 3 minutes, followed by 35-40 cycles of 94°C for 30-40 seconds, 50-62°C annealing for 30-40 seconds, and elongation at 72°C for 1 min per kb of expected PCR product length. This was followed by a final 10 minute elongation step at 72°C.
Specific primer pairs were used to discriminate between the WT, *mdx*, and P2X7\(^{-/-}\) KO alleles. See Table 2.5

PCR product were stored at 4°C overnight or <-20°C indefinitely.

<table>
<thead>
<tr>
<th>Primer pair sequences</th>
<th>Product size</th>
<th>Annealing temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dystrophin WT</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fv = p9427 (5'-AACTCATCAAATATGCCTGT)</td>
<td>105 bp</td>
<td>55-56°C</td>
</tr>
<tr>
<td>Rv = p260E (5'-GTCACCTAGATGTTGAAGCCATTTAG)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Dystrophin mdx</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fv = p9427 (5'-AACTCATCAAATATGCCTGT)</td>
<td>105 bp</td>
<td>55-56°C</td>
</tr>
<tr>
<td>Rv = p259E (5'-GTCACCTAGATGTTGAAGCCATTTAA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>P2X7 WT (Pfizer)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fv = (5'-TGGACTTCTCGGACCTGTCT)</td>
<td>363 bp</td>
<td>59°C</td>
</tr>
<tr>
<td>Rv = (5'-TGGCATAGCAGCTTAGCA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pfizer KO (neomycin)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fv = (5'-CTTGGGTTGGAGGGACTTATTC)</td>
<td>280 bp</td>
<td>59°C</td>
</tr>
<tr>
<td>Rv = (5'-AGGGTGGAGTACAGGAGATC)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>P2X7 WT (Glaxo)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fv = ‘A’ (5'-TGCCCATCTTCTGAACAC)</td>
<td>565 bp</td>
<td>59°C</td>
</tr>
<tr>
<td>Rv = ‘C’ (5'-CTTCCTCTTCTTAAGTTCC)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Glaxo KO (lacZ)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fv = ‘A’ (5'-TGCCCATCTTCTGAACAC)</td>
<td>393 bp</td>
<td>59°C</td>
</tr>
<tr>
<td>Rv = ‘E’ (5'-GCAAAGCCATTAAGTTGGG)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.5. Sequences of PCR primers used for genotyping.

### 2.6.5 Agarose gel electrophoresis of PCR products

PCR product was mixed 4:1 with a loading buffer (10% \(^{v/v}\) glycerol, 0.05% \(^{v/v}\) bromophenol blue), prior to loading onto a 2% \(^{w/v}\) agarose gel, unless the Promega\textsuperscript{TM} GoTag\textsuperscript{®} 2xGreen Master Mix was used, in which case the PCR product could be loaded immediately following PCR. Agarose gels were prepared by mixing 2g of electrophoresis grade agarose per 100ml TAE buffer. The TAE buffer-agarose mixture was then heated in a microwave until agarose dissolved completely, stopping heating periodically to mix the solution. 2-5µl of Web Scientific web-green DNA stain was added to per 100mL of cooled agarose gel before it solidified. The samples were loaded and the gel was then run at 3-5V/cm in 1xTAE buffer for time required to resolve bands of interest, against a 100 bp DNA ladder (Life Technologies) used for determination of the PCR product size.
2.6.6 RNA quality control by agarose gel electrophoresis

An aliquot of neat total RNA was loaded onto a 1% \( \text{w/v} \) agarose gel prepared as described above and run under non-denaturing conditions to assess sample quality. Good quality mouse RNA should present two crisp bands at \(~5\text{kb}\) and \(2\text{kb}\) corresponding to the 28S and 18S subunits of the rRNA, with band intensity ratio of \(~2:1\) (see Appendix 9.6).

2.6.7 RNA hybridisation to RNAstable™ matrix

Room temperature storage and delivery of isolated RNA was achieved with the use of a thermo-stable dissolvable RNA stabilization method. RNA of known concentration was added into and dried overnight within individual wells of a 96-well plate coated with Biomatrica® RNAstable. The plate was then sealed in a foil bag containing a desiccant.

2.7 Histological and immunostaining procedures

2.7.1 Muscle collection and storage

For histology and immunolocalisation, hind limb muscles including the tibialis anterior (TA) and gastrocnemius (GC) were used because of their well-established pathological expression profiles. Analysis of both of these muscle groups has been recommended in a recent standard operating procedure for the use of \(mdx\) mice in DMD pre-clinical research (Willmann et al., 2012). Other muscles collected included the diaphragm and the heart.

Using a clean dissection kit, skin layers were peeled away and skeletal muscles were excised from tendon to tendon. Section sampling was always performed within the middle third of muscle samples, away from manipulation sites and in the mid-belly section of the muscle.

Following collection, samples were placed onto ice-cold tin foil to minimise protease sample degradation or they were flash frozen in a 1.5mL Eppendorf tube placed in
liquid nitrogen if the downstream application was Western blotting. Dissected muscle intended for cryosectioning was arranged on a cork disk in a small amount of OCT medium in a longitudinal orientation, so as to eventually achieve transverse sections, and frozen in liquid nitrogen chilled 2-methylbutane and stored at -80ºC.

### 2.7.2 Muscle tissue cryosectioning

Frozen muscle was transferred to a cryostat chamber and allowed to equilibrate to -20ºC. Using OCT cryo-resin, the cork disk upon which the tissue sat, was affixed to a chuck. 5-10μm thick cryosections were then cut from the middle third of the sample and collected on poly-L-lysine coated glass slides. These sections were allowed to air dry for several hours before long term storage at -80ºC.

### 2.7.3 Haematoxylin and eosin staining

Haematoxylin and eosin (H&E) staining was performed to assess sample quality and tissue pathology following cryosectioning. Frozen muscle sections were collected onto a glass slide, allowed to reach room temperature and to dry. Sections were then covered in Vector® Haematoxylin QS nuclear stain solution for 45 seconds and rinsed in running tap water before 1 minute incubation in 95% v/v ethanol. Alcoholic eosin Y solution was applied to sections for 1 minute. Sections were then dehydrated in an ascending alcohol series (ethanol 95% v/v x2, 100% v/v x2) placed in xylene for 1 minute and finally mounted in DPX synthetic mounting medium with a glass coverslip. Slides were thoroughly dried before microscopic analysis.

### 2.7.4 Masson trichrome staining

The trichrome method employed here is the TREAT-NMD-recommended protocol ([http://treat-nmd.eu/research/preclinical/dmd-sops/](http://treat-nmd.eu/research/preclinical/dmd-sops/)). Briefly, 10μm thick heart cryosections were fixed in 4% w/v PFA, 0.1M PBS followed by Bouin’s fixative, stained with Biebrich Scarlet-Acid Fuchsin solution (Sigma HT151) for 20 minutes, washed in water and incubated in a phosphotungstic/phosphomolybdic acid solution (5% w/v phosphotungstic acid; 5% w/v phosphomolybdic acid; DDH2O) for 4x 3 minutes, directly before incubation in Aniline Blue solution (2.5% w/v; 2% v/v acetic
acid; \( \text{DDH}_2\text{O} \) for 15 minutes. Subsequently, sections were washed in water and incubated in glacial acetic acid solution (1% \( \text{v/v} \); \( \text{DDH}_2\text{O} \)) for 1 minute. Finally, sections were washed, dehydrated, mounted and visualized using bright-field Axiozoom V.16 microscope (Zeiss).

### 2.7.5 Immunohistochemical staining

Frozen muscle sections on glass slides were allowed to reach room temperature and to dry. Individual sections to be incubated with primary antibody and the negative controls were delineated using a hydrophobic wax marker pen (Vector Labs). Samples were then fixed in a 2-4% \( \text{w/v} \) paraformaldehyde solution in TBSt for 15 minutes at 4°C, followed by two washes in TBSt. Sections were blocked for 20-30 minutes in a 10% \( \text{v/v} \) serum (in TBSt) from the species in which the secondary antibody was derived. The primary antibody solution in TBSt, containing 10% \( \text{v/v} \) serum, was then applied to sections for 2 hours at room-temperature or overnight at 4°C. Following incubation, antibody solution was removed, sections were washed by three 5 minute incubations in TBSt, and endogenous peroxidases were quenched by applying 0.3% \( \text{v/v} \) \( \text{H}_2\text{O}_2 \) (in TBSt) directly to the sections for 5 minutes. Following another three 5 minute washes, Vector® labs ImmPRESS secondary antibody complex was added and incubation carried out for 30 minutes. Following two 5 minutes TBSt washes, sections were incubated with the Vector® labs colorimetric ImmPACT NovaRed substrate for 10 minutes, to visualise the signal. Sections were next rinsed with two 5 minute washes in distilled water, followed by a nuclear counterstaining with a diluted Haematoxylin QS (30 second incubation). Finally, sections were passed through an ascending alcohol series (95% ethanol x2, 100% ethanol x2) and xylene - 1 minute incubation each, and mounted with DPX.

### 2.7.6 Immunofluorescence staining

Frozen muscle sections were allowed to reach room temperature and to dry. Individual sections were delineated using a hydrophobic wax marker pen. Samples were then fixed in a 2-4% \( \text{w/v} \) paraformaldehyde solution in TBSt for 15 minutes at 4°C, followed by two washes in TBSt. At this stage, a 100mM \text{NaBH}_4, 50mM \text{NH}_4\text{Cl},
incubation was applied to the sections for 30 minutes to help subdue autofluorescence that may be present in a sample by reducing aldehyde to hydroxyl groups. Following three 5 minute washes in TBSt, sections were blocked for 20-30 minutes in a 10% v/v serum in TBSt from the species in which the secondary antibody was derived. The primary antibody solution in TBSt, containing 10% v/v serum, was then applied to sections for 2 hours at room-temperature or overnight at 4°C. Three 5 minute TBSt washes were then performed before a 1 hour room temperature incubation with secondary antibody diluted in 2% v/v serum containing Hoechst nuclear counterstain.. Sections were finally washed three times for 10 minutes before mounting in Merk Millipore FluorSave™ fluorescence mounting media.

Fluorescence images were captured using a Zeiss LSM 710 confocal microscope. For montage composition, multiple adjacent optical windows were captured with some overlap to aid joining.

2.8 Muscle morphometric analysis

From skeletal muscle tissue sections that have had individual muscle fibres delineated using an anti-collagen type IV fluorescent staining, an image processing sequence was used to measure morphometric variables in a semi-automated manner following the logical image processing steps, as described by Briguet et al. (2004).

Individual x20 fields of view were captured on a Zeiss LSM 7.10 confocal microscope and montaged together using Fiji (Schindelin et al., 2012), a packaged version of American National Institutes of Health (NIH) open source image processing software ImageJ (Rasband, 1997-2012). A macro was created to facilitate the montage process (see Appendix 9.3). Image processing steps undertaken across whole montaged muscle cross-sections included i) subtracting background components to minimise background noise that could interfere with further analysis, ii) applying band-pass thresholds to differing colour components, iii) dilating borders to close inconsistent gaps, iv) skeletonising these borders, v) applying a convolution filter to translate pixels uniformly for border detection. The ‘analyse particles’ function was used to generate a mask of the muscle fibre borders, simultaneously eliminating stray ‘non-border’ signals. An image of the threshold delimited nuclei was
then overlaid on the border mask, before another ‘analyse particles’ command was used to measure morphometric variables including ‘area’ and ‘minimum Feret’s diameter’. A macro was created to perform all image processing operations with minimal user input.

Central nucleation was also quantified across these analysed cross-sections by co-opting a ‘holes’ measure; the ‘holes’ were introduced to delineated muscle fibres by overlaying the threshold defined nuclei onto the border mask.

Internet videos describing our implementation of the above procedures are available from the following private links: http://youtu.be/GZVaRQYgGQU - macro defined muscle morphometric analysis using Fiji. http://youtu.be/oxyM7r7VYp0 - using the morphometric output to measure central nucleation frequency.

2.9 Sarcolemmal integrity assay

2.9.1 Mouse IgG staining

When muscle sarcolemma is compromised, blood borne molecules that are normally excluded from the cell may be allowed to enter the cytoplasm. Immunoglobulins are one such example. Using a secondary antibody raised against mouse IgG, an immunofluorescence approach was used to delineate compromised sarcolemma as a percentage of IgG positive to total fibres in 4 week GC samples from C57, mdx, and Pfizer dKO mice. The immunofluorescence procedure was as described earlier.

Quantification of IgG stained area was achieved using Fiji and this value was expressed as % of total cross-sectional area.

2.9.2 Serum creatine kinase assay

A common clinical diagnostic marker for DMD is the level of serum CK. CK is an important metabolic enzyme present within muscle cells (Wallimann et al., 2011). As it is normally restricted to the intracellular compartment, CK found in the serum is a measure of sarcolemmal break-down.
Whole blood was collected from recently killed mice by cardiac puncture using a 25 gauge needle and syringe, then stored at 4°C. Clotted blood was then heated to 37°C in a water bath for 10 minutes just prior to centrifugation. To minimise the chance of induced haemolysis, two low-speed centrifugations were adopted over one longer spin. Firstly, blood was centrifuged at 2,300 x g_{av} for 5 minutes. Serum supernatant was then removed to a new Eppendorf tube for a second 5 minute spin at 2,300 x g_{av}. The serum fraction was again transferred to a new tube, this time for storage at -80°C or immediate testing.

5µl of each sample was mixed with 250µl of CK enzymatic assay reagent (BIOO Scientific), containing excess ADP, glucose, and NAD(P)^+, as well as hexokinase and glucose-6-phosphate dehydrogenase (quantities unspecified by kit manufacturer) in single wells of a 96-well optical plate (Nunc-96). A standard series, containing a set quantity of NADH serially diluted with a standard dilution buffer (equivalent standard CK concentrations of 800, 400, 200, 100, and 50 IU/L) and the CK reagent was included in another 5 wells. One well was reserved for a negative control, containing only CK reagent and standard dilution buffer (equivalent standard CK concentration of 0 IU/L). Light absorbance at 340nm was measured in all wells immediately following CK reagent introduction and after 5 minutes of incubation, using a microplate spectrometer (POLARstar OPTIMA, BMG LABTECH, GmbH, Germany). NAD(P)H, the final product of the coupled enzymatic reactions has a peak absorbance of light at 340nm wavelength. Absorbance value increase over 5 minutes was multiplied by the assay-defined constant 2,186 (conversion factor), to obtain CK activity in International Units per litre (IU/L).

2.10 Protein isolation and analyses

2.10.1 Protein extraction

Skeletal muscles obtained from both left and right leg of the mouse were crushed under liquid nitrogen to generate a well-mixed powder that could be split for protein and RNA extractions. On ice, 500µl of Complete Lysis-M reagent (Roche Applied Science) was added per 100mg of crushed tissue. The Complete Lysis-M reagent contained an additional 1 Roche Mini Protease Inhibitor Cocktail tablet and 2 Roche PhosSTOP phosphatase inhibitor cocktail tablets per 10mL of reagent. The sample
was then titration mixed using a large bore pipette tip before further thorough mixing by syringe titration through 23 gauge and then 25 gauge needles. The resultant homogenate was then centrifuged for 20 seconds at 800 x g to pellet debris. The protein-containing supernatant was kept at -80°C for long-term storage or used immediately.

2.10.2 Protein quantification by BCA assay

Protein reduces Cu\(^{2+}\) proportional to BCA chelation of Cu\(^{+}\), resulting in a colorimetric reaction that is the basis for the BCA protein assay (Nagaoka et al., 2006; Abou-Khalil et al., 2013). The light absorbance at 550nm of BCA reagent containing protein extract at three dilutions was compared to a linear range of bovine serum albumin (BSA) standard concentrations between 1µg and 10µg.

1 to 10µl of BSA (Sigma Aldrich, 1mg/ml) was added, in duplicate, to a 96 well optical plate (Nunc-96) as a standard. Test samples were added to separate wells at 1, 3, and 5 µl, in duplicate. To each standard and test sample-containing well, 200µL of BCA working reagent (50 parts BCA, 1 part of 4% w/v CuSO\(_4\)) was added. The plate was then placed in a shaking incubator at 55°C for 15 minutes. Absorbance at the 550nm was measured using a microplate reader spectrometer (POLARstar OPTIMA, BMG LABTECH, GmbH, Germany). Protein concentration was determined by comparing standards of known concentrations to test samples of a similar volume.

2.10.3 SDS polyacrylamide gel electrophoresis (PAGE)

Equal quantities of protein extracts (20-40µg) were mixed 1:1 with 2 x Laemmli sample buffer (65.8mM Tris-HCl, pH6.8, 2.1% w/v SDS, 26.3% v/v glycerol, 0.1% v/v bromophenol blue), containing 5% v/v \(\beta\)-mecaptoethanol and heated at 95°C for 5 minutes. The resultant samples were then added to individual lanes of a two-phase PAGE gel before electrophoresis.

PAGE gels were made with a lower percentage stacking gel (4% w/v) overlaying a resolving gel (6-12% w/v). The resolving gel was composed of DDH\(_2\)O, 30% v/v acrylamide/bis, 1% v/v of 10% w/v SDS, and 25% v/v 1.5M Tris-HCl (pH 8.8). The stacking gel comprised of 61% v/v DDH\(_2\)O, 13% v/v of 30% acrylamide/bis, 1% v/v
10% w/v SDS, and 25% v/v 0.5M Tris-HCl (pH 6.8). Stacking and resolving gel components were mixed in separate 15 ml Falcon tubes and polymerising agents: 10% w/v APS (0.005% [stacking]-0.01% [resolving] v/v) and TEMED (0.002% v/v) were added just prior to pouring the gels. Alternatively, PROTEAN® TGX™ (Bio-Rad Laboratories Ltd) pre-cast polyacrylamide gels were used. Gels were assembled in a Mini-PROTEAN 3 Gel Cassette and Electrophoresis Module (Bio-Rad Laboratories Ltd) and immersed in SDS-PAGE running buffer. Electrophoresis voltage was set to 100V until the bromophenol blue had reached the resolving gel component. Proteins were separated through the resolving gel at 120V until proteins of interest had reached the middle of the gel, as determined against a 3-7 µl visible protein marker ladder (PageRuler™ Plus, Thermo Scientific) inserted into either or both flanking lanes.

2.10.4 Western blotting

Gels with separated proteins were placed within a Western sandwich set up (BioRad) containing a PVDF membrane. Proteins were then transferred using an electrophoresis rig containing a Western blot transfer buffer. Transfers were run at 100V for ~2 hours or at 40V overnight.

Following transfer, PVDF membranes were removed and washed prior to a 1 hour blocking with 5% w/v non-fat milk in TBSt. Membranes were then cut into strips according to expected molecular weights of target proteins and using protein ladder as a guide to allow multiple antibodies raised in the same species to be used with one blot. The primary antibody incubation was carried out for ~2 hours at room temperature or overnight at 4°C, in 5% w/v milk in TBSt. Membranes were then washed three times for 5 minutes in TBSt before secondary antibody incubation in 5% w/v milk in TBSt for ~1 hour at room temperature. After three final washes, membranes were incubated with chemiluminescent substrates (Millipore) and protein bands were visualised using a Syngene G:Box equipped with GeneSnap image capture and processing software. Subsequently, membranes were washed and dried for storage or re-probing.
For protein band quantification and comparison, relative band densitometry was employed using Fiji image analysis and Microsoft Excel spreadsheet software. Protein bands of specific size were assigned percentage values proportional to their relative densities and this was divided by similarly appropriated percentage values for sample-specific actin or β-tubulin bands, to give a comparative level of target protein relative to a loading control (as described in: http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/).

Two strategies were employed to re-probe PVDF membranes. The first involved placing the membrane(s) in a Western stripping buffer (see Table 2.2) for 2 hours at 50°C, with constant agitation. Membranes were then washed three times for 5 minutes in TBSt, before methanol reactivation, another wash series, and the standard antibody incubation procedure (as described above). The second approach involved quenching the horseradish peroxidase enzyme coupled to the secondary antibody on the membrane by incubating it in H₂O₂ (30% v/v) for 15-20 minutes at 37°C. Membranes were then washed three times for 5 minutes, before methanol reactivation and antibody incubations. The second approach can only work if the primary antibody is raised in different species, but this method is useful if expected protein bands are of very similar sizes.

### 2.11 *In vitro* diaphragm tissue bath tetanic force measurements

Whole diaphragms with ribs attached were excised from 4 week and 4 month C57 BL/10, *mdx*, Glaxo, and Pfizer *mdx/P2X7−/−* mice and placed into a Krebs Ringer solution (composition in table 2.2), taking care not to handle the muscle, wherever possible. Sutures were then tied to two points on either side of a segment of attached rib, which were then connected to an immobile plastic clamp. A centrally derived triangular section of the diaphragm was used for testing. Contractile force was translated along another suture, tied to the central tendon apex of the approximately equilateral triangular section of muscle. This suture was, in turn, attached to a mechanical force transducer (ADInstruments), amplifier, and data acquisition setup. Excitation of muscle was achieved *via* local field potentials through platinum electrodes in an oxygenated (95% O₂; 5% CO₂) Krebs Ringer solution, at a constantly maintained temperature of 37°C.
Chapter 2: Materials and methods

Each diaphragm was stretched in very small increments from an initial resting state to establish the optimal excitation-to-force generation length \((L_o)\). Diagnostic testing also confirmed that the voltage twitch stimulus of 140V (2msec width) was adequate to elicit a maximal twitch response \((P_t)\). To achieve a maximal isometric tetanic force response \((P_o)\), diaphragm sections were subject to a 140V (2msec) stimulus train at 100Hz frequency for 0.5-1 seconds. The test regime involved collecting 6 twitch responses, followed by 6 tetanic trains, with 2 minutes rest period between each. Maximal twitch and tetanic response was taken from the tallest of the respective force traces. All forces were normalised to muscle wet weight and expressed as Newtons per mg of tissue \((N/mg)\). The utilised testing strategy was a close match for a method used by Gosselin et al., (2003), and followed recommendations presented within the TREAT-NMD standard operating procedures for the use of experimental animals: [http://www.treat-nmd.eu/downloads/file/sops/dmd/MDVDMD_M.1.2.002.pdf](http://www.treat-nmd.eu/downloads/file/sops/dmd/MDVDMD_M.1.2.002.pdf)

### 2.12 P2X7 inhibitor study

A preliminary P2X7 inhibitor study was undertaken using Coomassie Brilliant Blue G (CBB). CBB (0.22\(\mu\)m in PBS, filter sterilised) was injected intraperitoneally \((i.p.)\) daily at 125mg per kg of mouse body weight, from post-natal day 2 until collection at 4 weeks of age. PBS injection regime run in parallel was used as a negative control. Blood was collected from all experimental animals for subsequent serum CK analysis.

### 2.13 Tissue preparation for bone histomorphometry

6 month old male \(mdx\) (n=4), C57 (n=4), Glaxo and Pfizer single (n=2, 5) and double-mutant (n=3, 4) mice were killed and then quickly had their skin and internal organs removed before being placed into a 4% \(^w/v\) paraformaldehyde fixative overnight. This bone and skeletal muscle specimen was then placed into 70% \(^v/v\) ethanol (in 0.1M PBS) for 2 hours before a final incubation and storage in 80% \(^v/v\) ethanol, to complete fixation and to preserve bone architecture for morphometric analysis.

Micro-computed tomography \((\mu CT)\) was undertaken by Dr. Andrea Del Fattore and Prof. Anna Teti of the University of L’Aquila, Italy. To determine bone mineral density and thickness the following parameters were analysed: Bone volume/total volume \((BV/TV, \%)\), trabecular thickness \((Tb.Th, \mu m)\), trabecular space \((Tb.Sp, \mu m)\),
and the trabecular number per mm$^2$ (Tb.N, n.mm$^{-2}$). This analysis was analogous to their previously published work (Rufo et al., 2011).

### 2.14 Statistics

Statistical tests were performed using Minitab™16 statistical analysis software. For comparison of one response variable from 2 independent groups, a Student T-test was preferred when sample values could be assumed, or determined, as normally distributed. The parametric equivalent for more than 2 groups was an ANOVA analysis, with Tukey-Kramer post-hoc test to determine individual pairs of differences. The non-parametric equivalent was the Kruskal-Wallis test, with no subsequent option for post-hoc analysis. For all analyses, an $\alpha$-level of 0.05 was chosen. Vertical bars in graphical representations of data represent SEM, where significance is indicated by asterisks.

A note on sample numbers per group

For statistical comparisons, a minimum number of three biological replicates, i.e. each sample from a different mouse, were required. In many cases we have been able to include more than three samples per group to increase the power of comparisons. As the data spread from each group has been used to determine variance to be applied to an ideal distribution, it is not a requirement that there be equal N number per group for parametric comparison. It was attempted to keep N numbers similar within tests.

Between tests, the N number can vary considerably due to inherent differences in the spread of data determined by one method over another. An example of this is serum CK comparisons (p. 173, Figure 5.1), which required higher N number per group than other studies before power was achieved to determine differences between groups. Serum CK level is a useful diagnostic marker in human and animal dystrophinopathy studies but as a more global measure of muscle membrane destabilisation, it is well acknowledged to present a high degree of data-spread variance over disease threshold level (http://treat-nmd.eu/research/preclinical/dmd-sops/).
2.15 Contributions by others

Not all data presented within this thesis are from studies undertaken solely by the main author. The following table details contributions by specific collaborators (Table 2.6)

<table>
<thead>
<tr>
<th>Method</th>
<th>Contributor(s)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal husbandry and euthanasia</td>
<td>Specialist technicians</td>
<td></td>
</tr>
<tr>
<td>Nucleic acid procedures</td>
<td>Thesis author</td>
<td></td>
</tr>
<tr>
<td>Genotyping by PCR</td>
<td>Thesis author</td>
<td></td>
</tr>
<tr>
<td>Quantitative PCR</td>
<td>Thesis author and Prof. Pawel Kalinski and Dr. Ravikumar Muthuswamy, University of Pittsburgh, USA</td>
<td>Collaborators prepared cDNA from RNA sent to them and performed qPCR. Data analysis undertaken by thesis author</td>
</tr>
<tr>
<td>Histology and immunolocalisation studies</td>
<td>Thesis author</td>
<td></td>
</tr>
<tr>
<td>Muscle morphometry – method design, development and implementation</td>
<td>Thesis author</td>
<td>Based on Briguet et al., 2004</td>
</tr>
<tr>
<td>Sarcolemmal integrity assays</td>
<td>Thesis author</td>
<td></td>
</tr>
<tr>
<td>Protein isolation and analyses by Western blotting</td>
<td>Thesis author</td>
<td></td>
</tr>
<tr>
<td><em>In vitro</em> diaphragm tissue bath force measurements</td>
<td>Thesis author</td>
<td></td>
</tr>
<tr>
<td>P2X7 inhibitor study</td>
<td>Thesis author and specialist technicians</td>
<td>Injections performed by animal technicians</td>
</tr>
<tr>
<td>Bone histomorphometry</td>
<td>Thesis author and Prof. Anna Teti and Dr. Andrea Del Fattore, University of L’Aquila, Italy</td>
<td>Animal bones prepared by thesis author and sent to Italy for micro-CT. Data analysed by the author.</td>
</tr>
</tbody>
</table>

Table 2.6. Table of collaborator contributions for data presented in the thesis.
3

Effect of P2X7 receptor ablation on muscle structure and growth at the acute and post-acute phases of *mdx* disease

3.1 Introduction

3.1.1 Histological profile of *mdx* mouse muscle

Human DMD patients undergo chronic muscle degeneration and tissue remodelling that results in reduced muscle force generation, collapse of tissue ultrastructure necessary to efficiently translate that force, and the replacement of muscle with fat and fibrotic scarring (Mann *et al.*, 2011; Klingler *et al.*, 2012). Satellite cells that would normally activate to repair damaged muscle, or that merge to form new fibres, are thought to exhaust in response to significantly increased replication, in a process termed replicative senescence (Schultz & Lipton, 1982). This process is thought to be partly dependent on the reduction in telomere length over the repeated cycles of satellite cell activation and proliferation associated with chronically diseased muscle (Decary *et al.*, 2000; Sacco *et al.*, 2010). Mice possess longer telomeres than humans, and this has been suggested as one reason for the less severe form of the disease in the *mdx* mouse model compared to human DMD (Sacco *et al.*, 2010). Satellite cells and myoblasts may also be more susceptible to cell death due to the increased presence of ATP released from damaged fibres combined with an increased expression and activity of ionotropic purinoceptors, such as P2X7, which were found in dystrophic myoblasts and myotubes (Yeung *et al.*, 2006; Martinello *et al.*, 2011; Young *et al.*, 2012).
The majority of muscles in the dystrophic \textit{mdx} mouse do not undergo chronic and continuous degeneration, instead going through cycles of degeneration and regeneration. The first degeneration period occurs at around 3-6 weeks in the \textit{mdx} mouse (Dangain & Vrbova, 1984; Tanabe \textit{et al}., 1986; Coulton \textit{et al}., 1988; Roig \textit{et al}., 2004; Yeung \textit{et al}., 2004; Willmann \textit{et al}., 2012). Within this period, muscle fibres undergo pronounced necrosis and apoptosis, also leading to enhanced clearance of cellular debris involving mononuclear cell infiltrations and sterile inflammation mechanisms. Another histological marker is an increase in the incidence of central nucleation as a product of enhanced muscle repair and myogenic precursor cell fusion events.

Muscle regeneration is ultimately regulated by a set of myogenic regulatory factors (MRFs) that are differentially expressed in response to micro-environmental stimuli including mechanical stress, autocrine and paracrine chemical signalling, and extracellular matrix interactions. The major MRFs expressed during secondary muscle regeneration include c-met, myoD, myf5, myogenin, MRF4, and myocyte enhancer binding factor-2 (MEF2) (Grounds \textit{et al}., 1992; Yun & Wold, 1996; Cornelison & Wold, 1997) (Figure 3.1). These factors have a sequential and contingent expression profile in a continuum from quiescent to activated, inducing proliferation and then differentiation of myogenic precursor cells. A graded expression of several of these factors means that individual myogenic precursor cells can express multiple MRFs at any time, dependent on their progression through the myogenic program (Figure 3.1: see Boldrin \textit{et al}., 2010; Dilworth & Blais, 2011).
Figure 3.1. A map of myogenic regulatory factors (MRF) involved in myofibre regeneration. Satellite cells that are present between the sarcolemma and the basal lamina of muscle fibres are activated upon tissue injury, which leads to their proliferation, differentiation, and then fusion to repair or to form new myofibres. Each step of the regeneration process is marked by the expression of multiple MRFs, as indicated (from Boldrin et al., 2010). MPC (myogenic precursor cell).

Another morphological parameter that is modified in the disease state is muscle fibre size. In DMD, following a greater than average growth up to five years of age, muscles progressively degenerate, regenerate, and split resulting in smaller than average fibres observed in adolescence and adulthood (Watkins & Cullen, 1982). In the \( \text{mdx} \) mouse, a well characterised pattern of muscle fibre size changes that differs from that of the human pathology is evident (Dangain & Vrbova, 1984; Tanabe et al., 1986; Coulton et al., 1988; Roig et al., 2004). Within the acute period of muscle fibre degeneration-regeneration, fibres are on average smaller in the \( \text{mdx} \) mouse, mostly due to the increased presence of smaller, regenerating, centrally nucleated, fibres. After this degenerative period, \( \text{mdx} \) muscle partially recovers, a subset of muscle fibres hypertrophy to compensate for an inherent weakness, whereas specific muscle force (normalised to muscle fibre cross-sectional area), remains lower. Muscle necrosis persists, albeit at low levels (Lynch et al., 2001). Dystrophic muscle
therefore presents a greater variation in muscle fibre sizes than is present in non-
diseased control tissue. Finally, there is a late-phase atrophy, from around 62 weeks of 
age, whereby the persistent degeneration of muscle fibres is no-longer compensated 
for by regeneration (Pastoret & Sebille, 1995).

Although DMD muscles generally have no dystrophin, in many DMD patients a small 
proportion of muscle fibres show strong dystrophin staining. These "revertant fibres"
are thought to arise due to somatic mutations in individual cell nuclei that correct and 
restore the dystrophin reading frame. The frequency of such somatic mutations 
increases with each satellite cell division and therefore the number of revertant fibres 
increases with the number of degeneration-regeneration cycles. Therefore, the 
revertant fibre count has been used as a discrete disease progression marker (Yokota 
et al., 2006; Young et al., 2012) in addition to the central nucleation index or levels of 
expression of various myogenic proteins. Revertant fibres are a good indicator of total 
regenerative fibre turnover since they represent a cumulative record of all regenerative 
events, whereas levels of myogenic proteins have a transient expression profile and 
are restricted to particular phases of regeneration.

Specific muscle fibres are differentially susceptible to disease progression. Naturally 
smaller muscle fibres, tend to be less susceptible to DMD-related damage (Karpati & 
Carpenter, 1986; Karpati et al., 1988a), perhaps related to the cellular mechanics of 
Ca^{2+} handling in smaller compared to larger cells (Khurana et al., 1995). Despite this 
observation, an entire branch of treatment strategies in the DMD field involves 
attempting to exogenously increase muscle fibre size, since it has been shown that 
artificially increased fibre size increases force production and reduces susceptibility to 
contraction-induced damage (Wagner et al., 2002; Peter et al., 2009; Gehrig et al., 
2010; Kim et al., 2011; Selsby et al., 2012; von Maltzahn et al., 2012).

In the human disease, certain muscle groups are more severely affected than others 
with those of the limbs, the diaphragm, and the heart being more affected. Within 
affected muscle groups fast-type, glycolytic, fibres are preferentially affected over 
slow-type oxidative fibres (Webster et al., 1988). In the mdx mouse, this is also the 
case (Moens et al., 1993; Ljubicic et al., 2011) and there is even a fibre type 
switching from fast-to-slow type over the progression of the disease (Carnwath & 
Shotton, 1987). Based on this observation, different mouse muscles have been
preferred for pre-clinical testing (Willmann et al., 2012) with the TA and GC hindlimb muscles classified as ‘appropriate’ and ‘particularly appropriate’ for downstream morphological or biochemical analyses (Figure 3.1).

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Type</th>
<th>Morphology</th>
<th>Biochemistry</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diaphragm\textsuperscript{a}</td>
<td>Fast</td>
<td>✓✓</td>
<td>✓✓</td>
<td>✓✓</td>
</tr>
<tr>
<td>Extensor digitorum longus (EDL)\textsuperscript{b}</td>
<td>Fast</td>
<td>✓</td>
<td>✓</td>
<td>✓✓</td>
</tr>
<tr>
<td>Gastrocnemius (GC)\textsuperscript{c}</td>
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<td>✓✓</td>
<td>✓✓</td>
<td>✓*</td>
</tr>
<tr>
<td>Quadriceps\textsuperscript{d}</td>
<td>Mixed</td>
<td>✓</td>
<td>✓✓</td>
<td>X</td>
</tr>
<tr>
<td>Biceps/triceps\textsuperscript{e}</td>
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<td>✓</td>
<td>✓</td>
<td>X</td>
</tr>
<tr>
<td>Tibialis anterior (TA)\textsuperscript{f}</td>
<td>Fast</td>
<td>✓✓**</td>
<td>✓</td>
<td>✓*</td>
</tr>
<tr>
<td>Soleus (SOL)\textsuperscript{g}</td>
<td>Slow</td>
<td>✓✓</td>
<td>✓</td>
<td>✓✓</td>
</tr>
</tbody>
</table>

Table 3.1. Mouse muscles used for pre-clinical morphological, biochemical, and functional studies. ✓✓ = particularly appropriate; ✓ = appropriate; X = not suitable. \textsuperscript{a} Severe phenotype, should be used as much as possible; \textsuperscript{b} Small size, more suited for functional assays; \textsuperscript{c} Large size, heterogeneous fibre composition, affected by exercise; \textsuperscript{d} Large size, affected by exercise; \textsuperscript{e} As back-up if GC or quadriceps not sufficient; \textsuperscript{f} Large size, accessible; \textsuperscript{g} Small size. As a purely slow muscle, soleus is more preserved from dystrophic pathology.

* Only in \textit{in situ}. ** Less affected by standard exercise regimes that induce muscle damage and contraction-induced pathological injury but badly affected during onset of necrosis (Figure and legend from Willmann et al., 2012).

3.1.2 \textit{The P2X7 receptor and skeletal muscle regeneration}

The P2X7 receptor is expressed in a large number of cell types. Along with an upregulation in the disease state, our laboratory has previously shown P2X7 to be expressed on myoblasts and myotubes (Yeung et al., 2006; Young et al., 2012). Work by us and others has also confirmed its expression in an \textit{in vitro} model of muscle resident satellite cells (Banachewicz et al., 2005), at the neuromuscular junction (Deuchars et al., 2001), and in inflammatory cells infiltrating \textit{mdx} muscles (Yeung et al., 2004).

One of the best characterised cell lineages in which P2X7 receptors are known to be expressed is the haematopoietic, including most cells of the innate and adaptive immune systems (Collo et al., 1997; Lister et al., 2007) and also osteoblasts and osteoclasts (Gartland et al., 2003; Ke et al., 2003; Panupinthus et al., 2007; 2008;
An important component of \textit{mdx} and DMD disease progression is chronic muscle inflammation (Tidball, 2009). Given the association of P2X7 with diseases with an inflammatory component (Chu et al., 2012; Kimbler et al., 2012; Murphy et al., 2012; Cieslak et al., 2011; Baroja-Mazo & Pelegrin, 2012; Chatterjee et al., 2012) and the aforementioned involvement of inflammatory cells and mediators (Table 3.2) in muscle regeneration (Spencer et al., 1997; Spencer & Tidball, 2001; Nguyen & Tidball, 2003a) it is pertinent to consider its effect on regenerative processes in dystrophic muscles. It is particularly important as P2X7 receptor inhibition or ablation has been shown to reduce the release of several of these inflammatory markers including active IL-1$\beta$ (Solle et al., 2001; Labasi et al., 2002) and TNF$\alpha$ (Chessell et al., 2005), while the level of the anti-inflammatory IL-10 has been shown to be reduced by P2X7 receptor activation (Rizzo et al., 2009).

Moreover, there is data suggesting that P2X7 receptors, in addition to influencing immune cell functions, may regulate muscle regeneration directly. The P2X7 receptor has been shown to promote myoblast proliferation (Martinello et al., 2011) as well as myoblast-to-myotube differentiation (Araya et al., 2004).
<table>
<thead>
<tr>
<th>Molecule</th>
<th>Expressing cell</th>
<th>Effect on muscle</th>
<th>References</th>
</tr>
</thead>
</table>
| IL-4     | • Regenerating myotubes  
        • M2 macrophages  
        • Th2 lymphocytes | • IL-4 KO mice had reduced muscle diameter due to reduced NFATc2 dependent myoblast to myotube fusion | (Horsley et al., 2003) |
| IL-10    | • Myoblasts  
        • Myotubes  
        • M2 macrophages  
        • T-cells, mainly Th2 and T-reg | • Prevents IGF-1 resistance by inhibiting the IL-1β induced JNK pathway, resulting in myogenin expression in myoblasts | (Strle et al., 2008) |
| IL-6     | • Satellite cells  
        • Myotubes  
        • M1 macrophages  
        • T-cells  
        • Neutrophils | • Applied to rat myoblasts in vitro leads to enhanced differentiation  
        • An IL-6 Ab reduced muscle atrophy in mice overexpressing IL-6  
        • Muscle disuse atrophy was extended in IL-6 knockout mice coinciding with delayed differentiation markers  
        • IL-6 induces myoblast proliferation via a STAT3 dependent pathway  
        • IL-6 mAb did not improve mdx regeneration profile | (Okazaki et al., 1996)  
        (Tsujinaka et al., 1996)  
        (Washington et al., 2011)  
        (Serrano et al., 2008)  
        (Kostek et al., 2012) |
| IL-1β    | • Myoblasts  
        • Myotubes  
        • M1 macrophages  
        • NK cells  
        • Neutrophils | • Physiological concentrations of IL-1β block IGF-1 induced protein synthesis and myogenin expression | (Broussard et al., 2004) |
| TNFα     | • Myotubes  
        • M1 macrophages  
        • Th1 lymphocytes  
        • Neutrophils | • Physiological concentrations of TNFα inhibits IGF-1 induced protein synthesis and myogenin expression in both mice and pigs  
        • TNFα promotes the proteasome-led destabilisation of MyoD leading to delayed myoblast cell-cycle exit  
        • TNFα inhibits myogenic differentiation via NF-κβ pathway  
        • Prolonged recombinant TNFα application decreases total muscle proteins | (Miller et al., 1988; Layne & Farmer, 1999; Broussard et al., 2003)  
        (Langen et al., 2004)  
        (Langen et al., 2001)  
        (Fong et al., 1989) |
| Osteopontin | • Osteoblasts  
        • Myoblasts  
        • Macrophages  
        • T-cells | • Immobile osteopontin promoted the fusion of myoblasts whilst soluble osteopontin promoted myoblasts proliferation and resulted in smaller, rounder, myotubes | (Uaesoontrachoon et al., 2008) |

Table 3.2. Selected inflammatory cytokines and their influence on skeletal muscle regeneration. Expressing cells listed are limited to those populations that can be present within skeletal muscle. The colour coding represents the general impact of the effect on muscle regeneration, from pro-regenerative (blue), mixed (purple), to deleterious (red).
3.1.3 Chapter summary

The P2X7 receptor has the potential to affect various aspects of DMD disease progression due to its expression on muscle precursor cells as well as infiltrating immune cells. The effect of P2X7 ablation in the \textit{mdx} mouse muscles were analysed using morphological and biochemical methods in two \textit{mdx} P2X7\textsuperscript{+/−} dKO mouse strains developed specifically for this study. Morphometric analysis was performed to compare muscle fibre size variation, fibre size distribution and the central nucleation index between \textit{mdx} and \textit{mdx}/P2X7\textsuperscript{+/−} muscles. The choice of mouse muscles for these analyses was based on published guidelines (Willmann \textit{et al.}, 2012). To account for potential involvement of P2X7 in myoblast proliferation and differentiation, satellite cell quantification was undertaken to compare \textit{mdx} with \textit{mdx}/P2X7\textsuperscript{+/−}.

Western blotting was used to compare the expressions of myogenic and regenerative markers: myogenin and eMyHC. These analyses were supported with a revertant dystrophin-expressing fibre count comparison in \textit{mdx} compared to \textit{mdx}/P2X7\textsuperscript{+/−} muscles.

The muscle expression of the dystrophin analogue, utrophin, was compared between \textit{mdx} and Pfizer \textit{mdx}/P2X7\textsuperscript{+/−} mice to evaluate possible changes in compensatory upregulation (Rafael \textit{et al.}, 1998) and as it is also linked with a shift to a slow-oxidative myogenic program (Gramolini \textit{et al.}, 2001; Selsby \textit{et al.}, 2012).

Finally, diaphragms at the age of \(\sim\)20 months was chosen to observe changes in minimum Feret’s diameter and revertant fibre numbers in Pfizer \textit{mdx}/P2X7\textsuperscript{+/−} compared to \textit{mdx}, as at this age pathology has progressed to a very advanced stage in one of the most severely affected muscles in this mouse model of DMD.
3.2 Results

3.2.1 Characterisation of the mdx/P2X7\textsuperscript{+/−} double-mutant mouse strains

The mdx mouse has been identified by Bulfield et al. (1984) in C57 BL/10 strain and found to be a spontaneous point mutation in exon 23 of the mouse dystrophin gene (Figure 3.2; Sicinski et al., 1989).

Using primers sets specific for each allele (Figure 3.2), regular genotyping was undertaken to identify the genetic status (Figure 3.3a). The status was also confirmed at the protein level by Western blotting method showing that Pfizer and Glaxo mdx/P2X7\textsuperscript{+/−} animals lacked both dystrophin (Figure 3.3b and d) and P2X7 receptor (Figure 3.3b and c). Additionally, it was found that mdx/P2X7\textsuperscript{+/−} heterozygous male mice had the P2X7 expression level qualitatively intermediate between the mdx and C57 mouse (Figure 3.3d).

Immunofluorescence analysis of P2X7 protein expression was undertaken using the most specific and best characterised antibody (177 003 Synaptic Systems). Using this antibody, P2X7 expression was found to localise to regions of mononuclear cell infiltrations in and around areas of skeletal muscle fibre degeneration in the mdx mouse at 4 weeks of age (Figure 3.4a). P2X7 receptor mRNA and protein is expressed in normal and dystrophic myoblasts and myotubes in vitro (Yeung et al., 2006) and here P2X7 receptor localisation in the muscle fibre cytoplasm and at the sarcolemmal border of muscles in the mdx mouse has been found (Figure 3.4b). In the 4 week old mdx/P2X7\textsuperscript{+/−} mouse, no such P2X7 fluorescence signal was detected (Figure 3.4a) thus confirming its specificity in mdx samples.
Figure 3.2. DNA sequences of P2X7 receptor and DMD loci, along with diagnostic PCR primer alignment regions (blue). The Glaxo P2X7 KO mouse has a LacZ insertion following the ATG codon in exon 1 (The top section). WT primer pairs flank exon 1 and a KO specific reverse primer was complementary to LacZ (sequence not shown but insert location is indicated by the LacZ arrowhead). The Pfizer P2X7 KO mouse has a neomycin resistance cassette insertion replacing a sequence within exon 13, normally encoding Cys$^{506}$ to Pro$^{532}$ (The middle section). A wild type (WT) primer pair flanks the insertion region, and the KO primer pair is neomycin specific (sequence not shown). (The bottom section) The $mdx$ dystrophin mutation involves a nucleotide substitution from a cytosine (WT: larger font) to a thymine ($mdx$: Black, below the wild-type sequence) within exon 23. The reverse primer allows for discrimination between WT and $mdx$ sequences, and the forward primer is shared (sequence data from blast.ncbi.nlm.nih.gov/).
Figure 3.3. PCR and Western blot characterisation of Glaxo and Pfizer \textit{mdx}/P2X7\textsuperscript{+/−} compared to C57 BL/10 and \textit{mdx} mice. \textbf{a}) Example PCR product analysis to confirm KO status of Glaxo \textit{mdx}/P2X7\textsuperscript{+/−} (dKO), Pfizer \textit{mdx}/P2X7\textsuperscript{+/−} (dKO), and \textit{mdx} compared to C57. Specific dystrophin WT bands (105 bp) were present in C57, with faint read through bands in the KO genotypes likely due to the primer poorly discriminating between one nucleotide substitution at the 3’-end. Dystrophin \textit{mdx} mutant bands (also 105 bp) are shown only for Glaxo dKO, Pfizer dKO, and \textit{mdx}. Pfizer P2X7 KO status was determined with multiplex PCR containing primers for the P2X7 WT sequence spanning the beginning of exon 13 and a neomycin resistance cassette. Neomycin bands (280 bp) were present in both P2X7 KO mouse lines as neomycin was used as a selective marker in both. Specific Pfizer P2X7 WT bands (363 bp) were present, as expected, for C57, Glaxo dKO and \textit{mdx}. Glaxo P2X7 WT primers span exon 1, and specific bands (566 bp) were observed for C57, Pfizer dKO, and \textit{mdx}. KO bands (393 bp), representing amplification between the shared forward primer to a reverse primer within the LacZ motif, were present in Glaxo dKO only. \textbf{b}) Western blotting, showing increased P2X7\textsubscript{r} expression in \textit{mdx} compared to C57, and its absence in Pfizer P2X7\textsuperscript{−/−} and \textit{mdx}/P2X7\textsuperscript{−/−} TA muscles. Also note, dystrophin (427kDa) is present in C57 and Pfizer P2X7\textsuperscript{−/−}, but not in \textit{mdx} and \textit{mdx}/P2X7\textsuperscript{−/−} tissues. \textbf{c}) Western blots showing P2X7 receptor subunit bands (78 kDa) in \textit{mdx} that are absent in both Pfizer and Glaxo dKO skeletal muscle. \textbf{d}) Western blot (top) showing a lack of full length dystrophin (427 kDa) in both \textit{mdx} and Pfizer P2X7\textsuperscript{+/−} F1 heterozygous dystrophic mice compared to C57 WT controls. Another blot (bottom) indicating the increased expression of P2X7 in dystrophic skeletal muscle compared to C57, and also highlighting an intermediate expression of P2X7 in a \textit{mdx}/P2X7\textsuperscript{+/−} heterozygous mouse compared to \textit{mdx} mice. Right, a graphical representation of the Western densitometry data showing a significant decrease in P2X7 receptor protein in the F1 P2X7\textsuperscript{+/−} heterozygous \textit{mdx} mouse compared to \textit{mdx} (T-test, T=−3.65, df=1, P=0.022, assuming equal variance). All western-blotting bands at expected weights (\textit{Table 2.3}).
Figure 3.4. The P2X7 receptor is upregulated in *mdx* compared to C57 TA muscle, localised to regions of mononuclear infiltration and at the periphery of centrally nucleated muscle fibres, and is absent from the *mdx*/P2X7<sup>−/−</sup> (dKO) mouse. Immunolocalisation of P2X7 receptors in 4 week *mdx* TA compared to Pfizer *mdx*/P2X7<sup>−/−</sup> samples. a) Note the predominant endomysial localisation of P2X7 (green signal) particularly in the areas of pathological infiltrations of inflammatory cells (identifiable by concentrations of cell nuclei - blue) in *mdx* skeletal muscle and its near complete absence in Pfizer dKO tissue. In Pfizer dKO tissue, P2X7 signal may be found in a small subset of mononuclear cells infiltrating the muscle (e.g. arrows; and bottom inset). P2X7 signal is absent in the negative control (secondary antibody only: not shown). b) In 4 week *mdx*, P2X7 may also localise to the muscle sarcolemmal borders of regenerating fibres (arrows). Left images show magnified insert region from the total cross-section on the right. Also note, P2X7 is expressed in a nerve trunk innervating muscle (arrowhead, at same location in both channels and characteristic morphology in collagen IV staining (Hagg *et al*., 2001) and orange signal when co-localised with P2X7).
3.2.2 Histological examination of mdx and mdx/P2X7\(^{-/-}\) muscle at 4 weeks and 4 months

To monitor for the skeletal muscle damage present at the acute degenerative and post-degenerative phases of the mouse disease, 4 week and 4-5 month C57 wild-type, mdx, and Pfizer mdx/P2X7\(^{-/-}\) TA muscle sections were analysed histologically. Nuclei and cytoplasm were delineated using standard H&E staining.

At 4 weeks, coinciding with an acute period of muscle degeneration-regeneration in the mdx mouse (Dangain & Vrbova, 1984; Tanabe et al., 1986; Coulton et al., 1988; Roig et al., 2004; Yeung et al., 2004; Willmann et al., 2012), mdx and Pfizer mdx/P2X7\(^{-/-}\) TA presented clear regions of mononuclear cell infiltrations, fibre size variation, and instances of hyalinated or necrotic fibres that were absent in C57 controls (Figure 3.5). These regions of pathology were distributed sporadically across the whole spectrum of mid-belly muscle cross-sections. The heterogeneous distribution of pathological changes within a single muscle cross-section between contralateral muscles (Kobayashi et al., 2012), and between individual animals of the same genotype has previously been noted. Therefore, it was difficult to identify significant differences in histopathology between H&E stained 4 week mdx and Pfizer mdx/P2X7\(^{-/-}\) TA muscles by sight.

At 4 months, regions of inflammation were less pronounced in dystrophic samples than at 4 weeks. Fibre size variation was still evident and the incidence of central nucleation was greatly increased, indicative of the degenerative-regenerative history of these muscles (Figure 3.5).
**Figure 3.5.** H&E staining, showing comparable histological features in dystrophic 4 week (left) and 4 month (right) TA muscle from *mdx* (middle) and Pfizer *mdx/P2X7*−/− (dKO - bottom). Note, the highly characteristic increased muscle fibre size variation in dystrophic *mdx* and dKO groups compared to C57 (top). Also note, the increased central nucleation and numbers of interstitially located nuclei in dystrophic tissue compared to C57 controls.
3.2.3 **Quantification of satellite cell markers in skeletal muscles of mdx and mdx/P2X7−/− mice**

Considering the previously identified P2X7 receptor expression in muscle precursor cells, myoblasts and myotubes *in vitro* and *in vivo* (Araya *et al.*, 2004; Banachewicz *et al.*, 2005; Martinello *et al.*, 2011; Young *et al.*, 2012), an immunological analysis was undertaken to determine if P2X7 ablation would have any effect on satellite cell number.

Antibodies against collagen type-IV and Pax7 were used to identify quiescent or activated satellite cells in relation to extracellular matrix components representing the periphery of muscle fibres, in 4 month C57 and *mdx* TA tissue. As has been published before (Seale *et al.*, 2000), Pax7 (considered the satellite cell marker) localised to extracellular regions of mononuclear cell aggregation and to central nuclei inside regenerating fibres, in addition to a typical localisation beneath the basal lamina on the myofibre periphery (**Figure 3.6a**).

Pax7 immunostaining was then used in a cross comparison of C57 (wild type), *mdx*, and *mdx/P2X7−/−* TA muscles at 4 weeks and 4 months, ages coinciding with acute degenerative and post-degenerative phases of disease progression. At 4 weeks, Pax7 marker expression was greatly increased in dystrophic muscles compared to C57 (**Figure 3.6b** left panels). Pax7 was also more often found in extracellular regions of nuclear aggregation or coinciding with central nuclei in dystrophic tissue compared to C57 wild-type controls (**Figure 3.6a**). At both 4 weeks and 4/5 months, *mdx* Pax7 immunofluorescence in *mdx* was similar in terms of expression levels and patterning compared to *mdx/P2X7−/−* samples.

A Western blotting study was undertaken to compare total Pax7 expression levels in 4 week old C57, *mdx*, Glaxo and Pfizer *mdx/P2X7−/−* TA and GC muscle lysates (**Figure 3.7**). Total Pax7 expression levels were found not to differ significantly between dystrophic strains harbouring the wild-type or the mutated P2X7 genes.
Figure 3.6. Pax7 signal is visible in satellite cells (including activated), it is more pronounced in dystrophic muscle relative to control but there are no differences between mdx and Pfizer mdx/P2X7<sup>−/−</sup> tissues at 4 weeks or 4/5 months. **a)** Example immunofluorescence images showing Pax7 (green), within nuclei in cellular interstitial regions of active myoblast recruitment (left, arrow), in typical sub-sarcolemmal positions (right, arrowhead) in mdx and C57 skeletal muscle sections, respectively. Also note Pax7 expression within centrally placed nuclei of mdx. **b)** Pax7 signal across C57 (top), mdx (middle), and Pfizer mdx/P2X<sup>−/−</sup> (DKO: bottom) 4 week (left) and 4-5-month (right) tibialis anterior (TA) muscles. Images of 4/5 month samples also show collagen signal (red) over half of image capture area. Note the increased Pax7 signal in mdx and Pfizer mdx/P2X<sup>−/−</sup> tissues at 4 weeks compared to C57. Also, typical satellite cell placement on the periphery of myofibres is more evident in C57 than in dystrophic tissue.
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Figure 3.7. Western blotting of Pax7 satellite cell marker levels across genotypes, showing no significant differences in Pax7 expression in TA or GC at 4 weeks between mdx, Glaxo mdx/P2X7⁻/⁻, or Pfizer mdx/P2X7⁻/⁻. a) Example Western blots showing the expression of Pax7 across all four genotypes in 4 week gastrocnemius (GC) muscles. b) Graphical representation of TA (left) and GC (right) muscle Western blot densitometry data, showing no significant difference between any of the experimental groups at this time point (TA: ANOVA, F=2.34, df=2, P=0.136, N=3, 7, 6; GC: ANOVA, F=0.58, df=2, P=0.58, N=4, 4, 4). All western-blotting bands at expected weights (Table 2.3).
3.2.4 Morphometric comparisons between mdx and mdx/P2X7⁻/⁻ skeletal muscles

A systematic study was undertaken to determine the effect of P2X7 ablation on the morphology of dystrophic muscle fibres in vivo. To accomplish this, a semi-automated form of morphometric image analysis was developed using Fiji. Previous logical operations necessary to complete such an analysis have been described (Briguet et al., 2004). Using this study as a methodological template, macros were written that sequentially translated an immuno-labelled image into a binary 8-bit format, dilated borders to close illogical border gaps and which measured threshold-delimited areas, finally reporting their spatial properties (see Figure 3.8a and Appendix 9.3). Using this method, thousands of individual muscle fibres comprising a whole skeletal muscle cross-section, were analysed simultaneously. Results obtained from the whole cross-sectional analysis therefore negate the issue of the heterogeneity generated by random field sampling, which has the potential to unrepresentatively skew data. The accuracy of the semi-automated method was further confirmed qualitatively by overlaying a mask-output of the ‘analyse particles’ function of Fiji-ImageJ upon the original immunofluorescence image (Figure 3.9a). Furthermore, a more systematic manual versus semi-automatic count was employed to compare analyses of the same image (Figure 3.9b). Using an additional ImageJ plugin (Ferreira, 2009-2012), a fibre cross-section was colour-coded according to morphometric variables. Using this tool, fibre size heterogeneity was evident across muscle cross sections (Figure 3.8b). In line with previous observations, fibre size heterogeneity was more pronounced in mdx than in C57 skeletal muscles in these 4 month example animals (Briguet et al., 2004).

Using this validated semi-automated method, several morphometric variables were compared in 4 week and 4 month old mdx and Pfizer mdx/P2X7⁻/⁻ TA mid-belly cross sections. Indicative of many factors influencing muscle re-modelling processes, the level of muscle fibre size heterogeneity can be quantified and compared using a measure of the ‘coefficient of variation’ (CoV). The coefficient of variation is defined as the fibre size standard deviation divided by the mean, then multiplied by a constant x1000 (Briguet et al., 2004). Fibre size inconsistencies can be introduced due to the cryosectioning of tissue at variable oblique angles. To compensate for this, the fibre size unit of ‘minimum Feret’s diameter’ was adopted, as recommended by Briguet et al. (2004). Using the measure of fibre size variability across whole muscle cross-
sections, an average CoV of 370 was determined for 4 week mdx compared to 400 for Pfizer mdx/P2X7−/−. At 4 months, mdx TA had an average CoV of 535 compared to 548 for Pfizer mdx/P2X7−/−. This qualitative increase in the CoV for Pfizer dKO compared to mdx at both monitored ages was not statistically significant (Figure 3.10b).

Paracrine signalling cues and myogenic fusion events influence myofibre size following muscle tissue damage or mechanical stretch. Over the progression of the disease in the mdx mouse, myofibre sizes change according to the influence of these multiple factors leading to well-characterised differences between wild-type and dystrophic tissues (Roig et al., 2004). Comparing average fibre size using the semi-automated morphometric method, Pfizer mdx/P2X7−/− muscle had a significantly greater minimum Feret’s diameter than mdx at 4 weeks of age (Figure 3.11a). At 4 months, there was no significant difference in fibre size between Pfizer mdx/P2X7−/− and mdx TA muscle.

To separate fibres that had undergone active regeneration from those that had not, a sub-level analysis of the morphometry data was run to determine the difference in minimum Feret’s diameter (fibre size) between centrally nucleated and non-centrally nucleated mdx and Pfizer mdx/P2X7−/− TA muscle at 4 weeks. This analysis showed that the difference in muscle fibre size found between these genotypes was mainly an effect of an increase in the size of centrally nucleated Pfizer mdx/P2X7−/− fibres. Across a fibre size distribution histogram, this increase in centrally nucleated minimum Feret’s diameter is represented by the right shift of the data plot for Pfizer mdx/P2X7−/− relative to mdx (Figure 3.11b). This translates to an increase in the maximal modal myofibre size grouping from 18.75-21.25µm for centrally nucleated 4 week mdx TA fibres to 28.75-31.25µm for Pfizer mdx/P2X7−/−. The shallower peak in the percentage distribution of fibres, coupled to an extended upper range in overall distribution of fibre size, also helps to explain the slight qualitative increase in the coefficient of variation data for Pfizer dKO compared to mdx at this age (Figure 3.10b).

In accordance with Briguet et al. (2004), an image analysis method was integrated into the current Fiji macro to allow the quantification of centrally nucleated muscle fibres. Specifically, a mask of the filtered blue-Hoechst-nuclei channel was overlaid
onto an ‘analyse particles’ output, with the subsequent centrally nucleated fibres being recognised according to the presence of ‘holes’. The presence of central nuclei in post-mitotic muscle fibres indicate previous regeneration events and is used as a measure of pathological severity (Spurney et al., 2009). Loss of P2X7 did not result in a significantly different level of central nucleation in dystrophic muscle at either 4 weeks or 4 months (Figure 3.10a).
Figure 3.8. Summary of image processing steps used to perform a semi-automated morphometric image analysis and confirmation of global differences between disease and control tissue. a) Example images showing an original Collagen type-IV immunostained muscle section (top-left), followed by conversion to 8-bit grayscale (top-right), binary band-pass filtration before several pixel dilation and erosion steps (bottom-left) and software plugin particle analysis with a labelled mask output (bottom-right). b) Example fibre-size colour coded TA cross-sections from a C57 and an mdx mouse, derived from applying a ‘ROI colour coding’ plugin (Ferreira, 2009-2012) to a mask output from the semi-automated Fiji morphometric analysis of merged muscle cross-sections.
Figure 3.9. Comparison of semi-automatic versus manual morphometry, showing similar muscle fibre de-lineation and morphometric value outputs. **a)** An image of a collagen-IV (red) immunolabelled *mdx* skeletal muscle cross-section overlaid with the Fiji-ImageJ “analyse particles” mask, demonstrating a very high degree of muscle-boundary overlap. Nuclei (blue). **b)** Box plots and a histogram of morphometry data from 3 separate analyses, also showing a high degree of similarity between manual and automatic approaches.
Figure 3.10. The percentage of central nucleation and the coefficient of variation for minimum Feret’s diameter is not different between TA muscle fibres from *mdx* and Pfizer *mdx/P2X7−/−* at 4 weeks or 4 months. a) Average percentage of centrally nucleated muscle fibres in *mdx* and Pfizer *mdx/P2X7−/−* showed no significant difference in central nucleation (2-way ANOVA, \( F=1.18, \) df=1, \( P=0.299 \)). b) Average coefficient of variation showed no significant difference between *mdx* and Pfizer *mdx/P2X7−/−* at 4 weeks or 4 months (2-way ANOVA, \( F=2.93, \) df=1, \( P=0.113 \)).
Figure 3.11. Minimum Feret’s diameter is increased in 4 week TA from Pfizer mdx/P2X7−/− compared to mdx, and is not significantly so at 4 months. a) Graphical representation of average minimum Feret’s diameter of TA muscle fibres, showing a significantly greater fibre size in Pfizer mdx/P2X7−/− (dKO) compared to mdx at 4 weeks (T-test, T=-3.55, df=5, P=0.016) and no statistically significant difference at 4 months (T-test, T=-1.5, df=5, P=0.193). b) Histogram of minimum Feret’s diameter of centrally nucleated 4 week TA muscle fibres showing a right shift in the fibre size distribution of Pfizer mdx/P2X7−/− regenerated fibres compared to mdx.
3.2.5 Analysis of myogenic and regenerative markers in mdx and mdx/P2X7−/− skeletal muscles

To assess the effect of P2X7-receptor ablation on candidate MRFs, myogenin and embryonic myosin heavy-chain (eMyHC) expressions were analysed in TA muscle extracts from 4 week old mice by Western blotting. Both of these markers have been used to describe changes in muscle fibre turnover correlating with dystrophic severity (Chen et al., 2000). The late differentiation-associated myofibre transcription factor, myogenin, was found to be significantly up-regulated in Pfizer and Glaxo P2X7−/− samples compared with mdx, with no difference in the expression between the two mdx/P2X7−/− strains (Figure 3.12). As expected, C57 showed little myogenin expression (data not shown). At 4 weeks, levels of eMyHC were reduced in Pfizer mdx/P2X7−/− TA muscles compared to mdx and similar between both mdx/P2X7−/− mouse strains (Figure 3.13). C57 TA muscle showed no detectable eMyHC expression (not included in statistical comparison due to low N number).
Figure 3.12. Western blotting, showing increased myogenin protein expression in Glaxo and Pfizer mdx/P2X7/− muscle compared to mdx at 4 weeks. a) Representative Western blots from 3 different animals each for myogenin (~34 kDa), in mdx, Glaxo mdx/P2X7/−, and Pfizer mdx/P2X7/− (dKO), compared to β-tubulin loading control. b) Graphic representation of the quantitative analyses of the expression of myogenin relative to β-tubulin based on the densitometry values of the Western blot data, showing significantly greater myogenin expression in Glaxo dKO and Pfizer mdx/P2X7/− muscle compared to mdx (ANOVA, F=33.38, df=2, P<0.001, Tukey post-hoc). All western-blotting bands at expected weights (Table 2.3).
Figure 3.13. Western blotting, showing reduced embryonic myosin heavy chain (eMyHC) protein expression in Pfizer \textit{mdx}/P2X7\textsuperscript{−/−} muscle compared to \textit{mdx} at 4 weeks. \textbf{a}) Representative Western blot images of eMyHC and actin used as a protein loading control (Each lane contains protein from a different animal). \textbf{b}) The graphical representation of densitometric data derived from these immunoblots, showing significantly lower eMyHC expression in Pfizer \textit{mdx}/P2X7\textsuperscript{−/−} compared to \textit{mdx} (Mann Whitney, \(W=27.0\), \(\alpha=0.04\), \(P=0.0187\) adjusted for ties). eMyHC expression was not detectable in C57 samples. All western-blotting bands at expected weights (Table 2.3).
3.2.6 **Immunolocalisation and analysis of revertant fibre numbers in mdx and mdx/P2X7−/− muscle at 4 months**

A major feature of the dystrophic pathology in both *mdx* mice and DMD is the turnover of regenerating myofibres (Tanabe *et al.*, 1986; Wallace & McNally, 2009). One indication of an improved disease profile is the reduction in the number of degeneration-regeneration cycles. Analysis was undertaken to compare the number of these cycles in Pfizer and Glaxo *mdx/P2X7−/−* muscles to that of *mdx*.

Muscle fibres that gain dystrophin due to the restoration of the open reading frame of its transcript are known as revertant. The chances of somatic mutation or alternative splicing event responsible for such restoration increase with age and, particularly, the number of degeneration-regeneration events. Therefore, revertant fibre number have been used as an index for the numbers of such events that any skeletal muscle has suffered (Hoffman *et al.*, 1990; Yokota *et al.*, 2006; Young *et al.*, 2012). Analysis of anti-dystrophin labelled 4 month TA cryosections (**Figure 3.14**) showed that there was a decrease in the mean percentage of revertant fibres relative to total fibres, particularly in Pfizer *mdx/P2X7−/−* (0.997%) compared to *mdx* (1.306%). However this difference failed to reach statistical significance.
Figure 3.14. Immunofluorescence analysis, showing no difference in revertant muscle fibre numbers in Glaxo or Pfizer mdx/P2X7⁻/⁻ TA muscle compared to mdx at 4 months. a) Example images of 4 month TAs from all genotypes immunostained for dystrophin (green signal), highlighting revertant fibres (arrows) present in dystrophic genotypes. b) Graphical representation of revertant fibre counts, showing no significant difference in average values between genotypes at these N numbers (ANOVA, F=1.57, df=2, P=0.274).
3.2.7 Analysis of utrophin expression in *mdx* and mdx/P2X7<sup>−/−</sup> skeletal muscles at 4 weeks

We have previously identified an increase in centrally nucleated fibre size in mdx/P2X7<sup>−/−</sup> muscles. Gross morphological changes have been correlated with oxidative shift of fibre-types and utrophin overexpression (Ekmark *et al.*, 2003). Therefore, utrophin protein expression in GC muscles at 4 weeks was compared between Pfizer mdx/P2X7<sup>−/−</sup> and mdx mice (Figure 3.15). There was no significant difference in utrophin expression levels relative to β-tubulin between these genotypes at this age. C57 BL/10 samples were not included in the analysis because utrophin is known to be expressed at much lower levels in healthy muscles (Helliwell *et al.*, 1992; Pons *et al.*, 1994).

![Figure 3.15. Western blotting, showing no difference in Utrophin protein expression between mdx and Pfizer mdx/P2X7<sup>−/−</sup> GC muscle at 4 weeks of age.](image)

*Figure 3.15.* Western blotting, showing no difference in Utrophin protein expression between *mdx* and Pfizer *mdx/P2X7<sup>−/−</sup>* GC muscle at 4 weeks of age. **a)** Western blot image for utrophin (400kDa) and β-tubulin (43kDa) loading control. **b)** Graphical representation comparing average utrophin band intensity relative to β-tubulin, showing no significant difference between the genotypes (T-test, T= -0.87, df=3, P=0.446). All western-blotting bands at expected weights (Table 2.3).
3.2.8 Minimum Feret’s diameter and revertant fibre comparisons between aged, 20 month, mdx and mdx/P2X7−/− diaphragms

Mdx diaphragm muscle undergoes continuous degeneration. The immuno-histological comparisons of skeletal muscle regeneration indices were extended to see if improvements suggested at earlier time points translate into this more severely affected tissue. Using a semi-automated method of morphometry, it was confirmed that average minimum Feret’s diameter of diaphragm muscle fibres was increased in Pfizer-mdx/P2X7−/− compared to mdx at ~20 months of age (Figure 3.16a). The morphometric method could be adapted to work in even highly fibrotic old-dystrophic tissue (Figure 3.16b), and was employed to measure all muscle fibres in cross-section simultaneously (Figure 3.16c).

Moreover, through immuno-histological comparisons, significantly fewer revertant fibres in aged Pfizer-mdx/P2X7−/− compared to mdx were confirmed (Figure 3.16d).
Figure 3.16. Immunological and histological markers of regeneration are improved by P2X7 receptor ablation in aged dystrophic diaphragms. a) Graph showing increased average minimum Feret’s diameter of ~20 month diaphragm muscle fibres in WT (C57) and Pfizer-mdx/P2X7⁻/⁻ compared to mdx (ANOVA, F=40.72, P<0.001, df=3, n=3; Tukey post-hoc). b) An image depicting the method of semi-automated morphometry applied here, which could be used to measure all muscle fibres in an entire cross-section of a rolled diaphragm simultaneously (c, C57 shown in this example). d) There were also significantly fewer revertant fibres in Pfizer-mdx/P2X7⁻/⁻ compared to mdx ~20 month diaphragms (T-test, T=5.12, P=0.036, df=2, n=3).
3.3 Discussion

3.3.1 Generation and characterisation of mdx/P2X7−/− double mutant mice

The Glaxo P2X7 KO (Glaxo P2X7−/−) mouse was developed by using homologous recombination to disrupt the open reading frame by insertion of a promoterless lacZ construct into exon 1 of the mouse P2X7 gene and then backcrossed onto a C57 BL/6 wild-type background (Chessell et al., 2005). The Pfizer P2X7 KO (Pfizer P2X7−/−) mouse was made by insertion of a neomycin resistance cassette within exon 13 of the P2X7 gene, which caused an out-of-frame replacement within the C-terminus of the receptor sub-unit (Solle et al., 2001). Pfizer KO mice were also bred onto a C57/BL6 background. Both Glaxo P2X7−/− and Pfizer P2X7−/− mice were found viable but with slightly altered immune responses (see Chapter 4).

Using PCR and Western blotting the creation of two types of P2X7 mdx dKO animal strains was confirmed. The Glaxo dKO animal has a disruption in exon 1 of the P2X7 gene. The Pfizer dKO mouse was confirmed dystrophic, testing positive for the mdx mutation DNA by PCR and harbouring a neomycin resistance cassette displacing a region of exon 13 of the P2X7 receptor, resulting in the loss of P2X7 protein expression in Western blotting of muscle tissue extracts (Figures 3.2 and 3.3c). However, by immunolocalisation of cryosectioned TA tissue, a drastically diminished but still detectable expression of a P2X7 positive signal was evident in the double KO animal (Figure 3.4). The most likely cause for this signal is the previously identified splice variant of the P2X7 protein that can escape inactivation in the Pfizer KO strain (Taylor et al., 2009a). Thought only to be expressed in the T-cell subset of haematopoietic-derived immune cells, this variant was also found to be non-functional (Taylor et al., 2009a).

Perhaps more important as potential disease mediators are the other reported P2X7 splice variants, especially one describe in the Glaxo P2X7 KO mouse. First described by Nicke et al. (2009) with contribution from our laboratory, the P2X7k variant, possesses an altered N-terminal region that can actually enhance canonical large pore opening and subsequent cell death processes in response to ligand-receptor; ATP-P2X7 interactions. However, it has been found that the Glaxo mdx/P2X7−/− animal has no detectable P2X7 protein in muscle tissue extracts (Figure 3.3c).
There is also some controversy over the presence of functional splice variants preferentially expressed in regions of the central nervous system that may escape deletion in one or either of the two P2X7 KO mouse strains (Masin et al., 2012). The currently employed genetic PCR strategy for determining KO status does not discriminate between individuals likely to possess these alternative receptor transcripts. However, it may be possible that the P2X7 antibody used to confirm the absence of the full receptor from the dKO strains (Figure 3.3b) would recognise the K variant P2X7 receptor. In terms of suspected antibody-peptide interactions, it would be expected that the Synaptic Systems antibody (177 003), targeting a C-terminal region, may also recognise an N-terminally modified P2X7 peptide sequence. The antibody used therefore has the potential to recognise the two major functional P2X7 isoforms. The predominant ‘a’ and the gain of function ‘k’ splice variant differ by the substitution of the first 42 amino acids in the N-terminal domain of the P2X7 receptor (Nicke et al., 2009). The dominant negative-like 13b and 13c splice variants have also been shown to be expressed in Pfizer P2X7 KO tissues by us and others. The 13b and 13c isoforms are C-terminal truncated variants and were not expected to be recognised using the current antibody (Masin et al., 2012).

Another biological variable that could systematically affect disease phenotype and potentially bias further pre-clinical experimental testing is the differing background genetics of the experimental animals. For example, P2X7 genetic polymorphisms can influence key receptor functions. The common C57 BL/6 mouse strain has been shown to contain a proline to leucine amino acid substitution at position 451 of the P2X7 sequence that substantially reduces receptor activity (Adriouch et al., 2002). For this reason Adriouch et al. (2002) have warned against neglecting this variable when constructing pre-clinical experimental animal studies that involve the testing of the role of the P2X7 receptor. By being forced to use mdx and two P2X7 KO strains of mice, which are derived from C57 BL/10 and C57 BL/6 background strains respectively, we have introduced the potential for a systematic background mediated influence of P2X7 receptor polymorphisms contributing to an experimental bias. The proline 451 to leucine substitution is common to both strains (Adriouch et al., 2002). Therefore, by using these strains of mice, it is likely that we are underestimating the role of P2X7 in the pathogenic progression of muscular dystrophy. Interestingly in
humans, P2X7 polymorphisms that reduce receptor pore formation also shift immune cells to a lesser inflammatory state (Denlinger et al., 2005).

The C57 BL/10 strain of mouse is derived from the BL/6 type, minimising the chances for genetic background effects. Nevertheless, to address the issue of genetic heritage potentially influencing P2X7 function or mdx disease phenotypes, a control dystrophic P2X7+/− heterozygote (F1) heterozygote was used for comparison with the P2X7+/+ homozygous and mdx mouse strains. Since the F1 het mice were the resultant litter from an mdx female and a Pfizer P2X7 KO male, all males were expected to be dystrophic and to possess a fully functional P2X7 receptor on a mixed C57 BL/10 and BL/6 background. Subsequent differences between this control litter and the mdx mouse, according to key functional disease diagnostic tests including the level of serum CK (see Chapter 5), would therefore likely represent the effect of the mixed background influence. However, as shown in this section, the F1 het mouse possesses a significantly reduced expression of the P2X7 receptor compared to the mdx mouse (Figure 3.3c) that would ultimately alter the P2X7 influence on disease progression. Such an unexpectedly reduced expression of P2X7 on the heterozygous background has been previously reported for heterozygous immune cells (Chen et al., 2011). Clearly, the F1 heterozygotes could not be used as a positive control for the mdx/P2X7−/− KO mice but they serve as a good control for analyses of the effects of reduction of P2X7 levels in the dystrophic muscle. This could also explain the greater minimum Feret’s diameter of mdx/P2X7+/− muscle fibres compared to mdx (Appendix 9.4).

Moreover, the results from the Glaxo mdx/P2X7+/− mice could be used as a proof that the effects observed are due to P2X7 receptor knockout and not due to genetic background variation: both Glaxo and Pfizer are on the C57/Bl6 background but Pfizer mdx/P2X7−/− mice lacking all functional receptors showed significantly stronger effects than the Glaxo mdx/P2X7+/− mice, which retain one functional P2X7 isoform.

The present study shows that the P2X7 receptor localises to regions of monocellular infiltration, in the peripheral nervous system including nerve bundles, and at the sarcolemmal border of regenerating myotubes in mdx muscle (Figure 3.4). Sarcolemmal staining of P2X7 protein is consistent with previous lab findings for this receptor subunit (Yeung et al., 2006), for other P2X subunits (Jiang et al., 2005), and
the notion that P2X receptors may be necessary for, at least phases of, skeletal muscle myotube formation (Ryten et al., 2002; Araya et al., 2004). These latter groups have also shown P2X receptor localisation to developing and regenerating rat skeletal muscle in vivo (Ryten et al., 2001). However, our finding that myogenin expression may be upregulated upon the loss of P2X7 receptor activity in the mdx mouse model of DMD also partly contradicts the latter studies, in which P2X5 and P2X7 were shown to positively mediate skeletal muscle differentiation (Ryten et al., 2002; 2004). Myoblast to myotube terminal differentiation requires an increase in intracellular Ca\(^{2+}\). ATP is a stimulus that can induce this rise in intracellular Ca\(^{2+}\) in muscle cells (Collet et al., 2002; Cseri et al., 2002) but it must be possible for mouse skeletal muscle to achieve this Ca\(^{2+}\) threshold without the P2X7 receptor since no studies have reported a specific myogenesis defect in either Glaxo or Pfizer P2X7 KO animals.

### 3.3.2 Histology and regeneration marker comparisons between mdx and mdx/P2X7\(^{-/-}\) dKO muscles

Myogenesis is a recurring feature of mdx muscle, along with muscle fibre degeneration due to both apoptotic and necrotic processes, and the influx of inflammatory cell types. All these features are evident in histological preparations of dystrophic muscle. There was an obvious qualitative difference in representative H&E staining between mdx and C57 BL/10 TA muscle sections at both 4 weeks and 4 months (Figure 3.5). However, according to a qualitative assessment of these images, there was no clear difference between mdx and Pfizer mdx/P2X7\(^{-/-}\) dKO muscles at both ages. This early stage analysis also highlighted an important experimental concern, primarily related to two observed mdx disease factors. Firstly, there is consistent pathological heterogeneity within even a single muscle section in dystrophic mice, such that one area of muscle may appear healthy, whilst an adjacent area presents massive degeneration or immune cell infiltrations. Secondly, and consistent with the described differences between human and mouse pathology, there are often very few major areas of degeneration within a single muscle cross-section.

Exercise, or direct muscle intervention resulting in induced regeneration, is often used to exacerbate disease pathology in the mdx mouse model of DMD (Spurney et al., 2009; Kobayashi et al., 2012; Willmann et al., 2012). Since the mdx mouse does not
present disease symptoms as severe as DMD patients, mouse muscle challenge is an experimental attempt to evaluate therapies or probe pathways during extreme phases of the disease pathology, perhaps more similar to the general human condition. The substantial central nucleation and general lack of widespread degeneration in even 4 week TA muscles from the \textit{mdx} mouse (Figure 3.5) attests to a potential draw-back with using unchallenged mice in this study.

\textbf{Pax7, satellite cells, and muscle precursor cells}

Upon satellite cell self-renewal, Pax7 expression may increase coincident with a reduction in later stage myogenic progression. For instance, activation of the MRF, myostatin, may result in a reduced muscle fibre size via an ERK1/2 mediated enhancement of the Pax7 self-renewal pathway (McFarlane \textit{et al.}, 2008). Inflammatory second messengers might also increase the proliferation of muscle precursor cells at the expense of muscle fibre growth. IL-6 promotes satellite cell proliferation via a STAT3 signalling pathway (Toth \textit{et al.}, 2011). Satellite cells have traditionally been considered a quiescent muscle precursor cell type, with the transcription factor Pax7 becoming down-regulated inversely proportional to the differentiation status of the cell and the expression of a series of major MRFs, MyoD and Myf5 (Seale \textit{et al.}, 2000; Olguin & Olwin, 2004). It has been subsequently shown that Pax7 is expressed in satellite cells even into differentiation (Zammit \textit{et al.}, 2004; 2006; Collins \textit{et al.}, 2005), localising to centrally placed nuclei within muscle fibres and extracellular regions of muscle precursor cell fusion (Seale \textit{et al.}, 2000). This was confirmed here with the use of Pax7 immunofluorescence in 4 month \textit{mdx} muscles (Figure 3.6). Using a now validated antibody, it was thus decided to compare levels of the satellite cell marker, Pax7, between \textit{mdx} and both \textit{mdx}/P2X7 dKO TA and GC muscles at 4 weeks (Figure 3.7).

P2X7 has been implicated as a STAT3-related survival receptor in mouse embryonic stem cells (Thompson \textit{et al.}, 2012). Within undifferentiated E14tg2a cells, P2X7 receptor variants including the full-length ‘a’ and gain of function ‘k’, were found to mediate cell proliferation upon basal ATP stimulation but cell necrosis after chronic, or high-dose exogenous, ATP activation. In dystrophic muscle, local levels of ATP can reach very high levels and is persistently increased in extracellular spaces
consistent with chronic sarcolemmal instability. We have failed to identify a difference in the levels of Pax7 between mdx and mdx/P2X7−/− dKO mice at the acutely degenerative 4 week period (Figure 3.7). This may indicate that there is no phenotype associated with the loss of P2X7 on satellite cell numbers in the mdx mouse, may reflect the counterbalance between reduced proliferation/self-renewal and the cell death of muscle precursor cells, or might reflect localised changes in small muscle regions that are not translated to significant quantitative differences when tested at the level of whole muscle. To further probe for an effect of P2X7 ablation on muscle regeneration in the mdx mouse, more MRFs were considered along with a histological analysis of fibre size.

Enhancing muscle fibre size can increase resistance to contraction-induced damage and the deleterious effects of the dystrophic pathology (Gehrig et al., 2010). It is therefore interesting that we have seen an increase in the size of centrally nucleated muscle fibres in mdx/P2X7−/− dKO compared to mdx 4 week old animals (Figure 3.11). Since an increase in fibre size is generally considered beneficial in relation to the dystrophic pathology (Wagner et al., 2002; Gehrig et al., 2010; Vidal et al., 2012), a parallel qualitative increase in fibre size variation (CoV: Figure 3.10) should not necessarily be interpreted as negative, despite this also having been described as a marker correlated with disease severity (Briguet et al., 2004). Any derived marker of disease progression or amelioration should be evaluated within the context of the particular study. That our reported CoV values were similar to those reported by Briguet et al. (2004) for mdx muscles has reaffirmed the accuracy of the derived semi-automated morphometry method used here, something we have further confirmed visually, using an overlay of a analyse particles output mask upon the original image, as well as with an example manual versus automated analysis of the same tissue (Figure 3.9).

An increase in muscle fibre size can be mediated via multiple mechanisms. One of the best known muscle hypertrophy pathways involves the IGF-1/PI3K/Akt/mTOR cascade (Bodine et al., 2001b; Rommel et al., 2001). Indeed, experimental Akt overexpression was found to promote muscle myogenesis and improve disease outcome in the mdx model of DMD (Kim et al., 2011). The PI3/Akt/mTOR pathway is usually directly opposed by the atrophy related transcription factor FoxO3 and the genes atrogin-1, MuRF-1, and cathepsin-L (Bodine et al., 2001a; 2001b; Rommel et
al., 2001; Sandri et al., 2004; 2006). These atrophy factors negatively regulate the differentiation-inducing myogenic regulatory factors (Kitamura et al., 2007; Lokireddy et al., 2012). Without pinpointing the exact interaction, we can therefore conclude that loss of P2X7 promotes enhanced muscle fibre growth, possibly by enhancing the MRF myogenin (Figure 3.12), a marker for myoblast differentiation and fusion (Grounds et al., 1992; Dedieu et al., 2002; Vidal et al., 2012). A possible controversy relates to a recently published link between P2X7 receptor induction and PI3K/AKT in tumour cells (Bian et al., 2013), which, if translated into muscle, would be inconsistent with P2X7 loss and resultant muscle fibre growth.

Quiescent satellite cells do not express the MRFs MyoD or myogenin (Grounds et al., 1992). During differentiation of muscle precursor cells, Pax7 expression is inversely correlated with that of myogenin. Furthermore, an overexpression of Pax7 results in a downregulation of MyoD, and a subsequent reduction in myogenin (Olguin & Olwin, 2004). In a 9 time-point microarray and corroborative RT-PCR study focusing on regeneration-associated genes expressed in the mdx mouse from age 1-20 weeks, it was shown that the proliferation gene, Notch2, was persistently overexpressed in dystrophic hind-limb compared to wild-type control muscle (Turk et al., 2005). Myogenin was expressed at very similar levels between mdx and C57, until the 8 week period when dystrophic tissue showed an upregulation concomitant with a reduction in the wild-type animal. Altogether, these observations confirm the contingent nature of myogenic regeneration, with myoblast differentiation and fusion events following satellite cell activation, and myoblast proliferation. This result also indicates the enhanced therapeutic potential of modulating muscle myoblast differentiation, which is episodically regulated, over proliferation, which seems to be chronically activated in dystrophic muscle. Therefore, an increase in late-differentiation upon a reduction in P2X7 receptor activation, as indicted here by the increase myogenin expression in the mdx/P2X7−/− dKO compared to the mdx mouse, could be deemed an improvement in disease parameters.

Also important here is a consideration of the interaction of muscle precursor cells to their micro-environment and the interplay between one regenerative process and another. Using an in vitro model of myofibre myogenic regulatory factor expression, myogenin expression was shown to be inversely correlated with the level of pro-inflammatory cytokine TNFα (Szalay et al., 1997; Perdiguero et al., 2011).
Environmental stimuli that promote the proliferation of myogenic precursor cells, including myoblasts, seem to be generally antagonistic to signals that eventually promote the differentiation and fusion of these cells to (re)build muscle tissue. For instance, the pro-inflammatory cytokine TNF\(\alpha\) has been demonstrated to promote the proliferation of satellite cells \textit{in vitro} (Li, 2003; Tidball & Villalta, 2010), whereas it may inhibit later stage differentiation and fusion of these cells (Langen \textit{et al.}, 2001; 2002; 2004; Perdiguero \textit{et al.}, 2011). The cytokine IL-4 preferentially promotes the myoblast to myotube fusion (Horsley \textit{et al.}, 2003). The state of muscle precursor cells along a transition from proliferation, to differentiation, to fibre integration, is described via the expression of many other specific basic helix-loop-helix proteins and MRFs (see Boldrin \textit{et al.}, 2010; Dilworth & Blais, 2011).

Although it is not an MRF, the protein eMyHC is linked with skeletal muscle regeneration (Schiaffino \textit{et al.}, 1986), where it is transiently expressed in newly regenerated muscle fibres and has been associated with the maturation or fusion potential of muscle fibres (Martinello \textit{et al.}, 2011; Vidal \textit{et al.}, 2012). In this sense, the reduction of eMyHC in 4 week old \textit{mdx}/P2X7\(^{-/-}\) dKO muscle compared to \textit{mdx} (Figure 3.13) is contradictory to the increase in myogenin expression and their similar levels of central nucleation. However, in many published articles eMyHC expression has been correlated with disease severity at acute phases of muscle regeneration. For instance, eMyHC-positive small regenerating muscle fibres are more common in \textit{mdx}/utrophin double KO TA muscle than in \textit{mdx}, especially at the 4 week phase of disease progression (also used here), and concurrent with a more severe pathology in the \textit{mdx}/utrophin dKO mice (Grady \textit{et al.}, 1997). Additionally, despite the association of eMyHC with myotube maturation, other more directed studies have shown eMyHC expression to be limited to proliferating myoblasts (Rodgers, 2005) with a nuclear downregulation observed during myoblast differentiation (Silberstein \textit{et al.}, 1986). These latter studies comfortably marry the increase in myogenin expression in \textit{mdx}/P2X7\(^{-/-}\) dKO muscle to the decrease in eMyHC observed here.
Revertant fibres

A complementary analysis for monitoring muscle regeneration in relation to DMD disease severity is a histological measure of muscle dystrophin revertant fibre expression. Dystrophic muscle has the potential to re-express truncated dystrophin isoforms via random mRNA splicing events during regeneration. The numbers of these revertant fibres is therefore directly proportional to the amount of degeneration and regeneration events that has occurred in a single muscle tissue. Yokota et al. (2006) proposed the use of a histological measure of revertant fibre number, showing that reduced revertant fibre numbers was associated with improved disease features, including the stabilisation of a key DAPC binding partner, nNOS. Our current data failed to identify a significant decrease in total revertant fibre numbers between \textit{mdx} and either \textit{mdx/P2X7}\textsuperscript{-/-} dKO mouse in 4 months old TA muscle (\textbf{Figure 3.14}), although a qualitative decrease in mean revertant fibre percentage was evident for the Pfizer dKO mouse compared to \textit{mdx}. Base level revertant fibre comparisons could not be made between this study and the Yokota et al. (2006) paper on the basis that we studied 4 month old mice whilst Yokota et al. (2006) looked at a maximum age of 3 months. Additionally, we have presented revertant fibre numbers as a percentage of total fibres to maintain equivalency between individuals, whilst Yokota et al. (2006) instead presented their results as the mean of maximum revertant fibres. It is unclear how Yokota et al. (2006) normalised revertant fibre numbers between individuals, but a standard experimental approach would be to report mean number per a constant unit of area.

Our laboratory has previously used the measure of mean revertant fibre percentage to evaluate the effect of a P2X receptor antagonist, Coomassie-Brilliant Blue (CBB), on regeneration potential in the \textit{mdx} mouse at 4 months of age after a prolonged treatment schedule (125mg/kg every three days between 3 and 14 weeks of age) (Young et al., 2012). In that study, Young et al. (2012) successfully identified a reduction in \textit{mdx} mean revertant fibre percentage after CBB treatment (~2.4% in saline treated mice compared to ~1.5% in CBB treated mice). This result provided extra impetus to evaluate revertant fibre numbers in P2X7 dKO muscle compared to \textit{mdx}. However, despite recording revertant fibre numbers at the same 4 month period and adopting the same percentage method for reporting mean revertant fibre numbers between groups, our reported mean revertant fibre percentage for the \textit{mdx} animal is
~1.3% compared to ~2.4% for the saline treated group in the Young et al. (2012) paper. This difference could be for several reasons. Firstly, i.p. saline treatment could adversely affect DMD disease measures. This is unlikely, especially given a recent report that dietary sodium chloride supplementation may actually improve mdx disease outcome (Yoshida et al., 2006). Secondly, and more likely, Young et al. (2012) adopted a different sampling strategy, using a random microscope field-of-view approach to normalise between individuals, instead of a whole cross sectional analysis adopted here, likely to generate systematic differences in basal reported values.

Apart from experimental artefact leading to systemic differences in basal reported values, a more important observation is the reported significant difference in the Young et al. (2012) paper between saline and CBB treated mdx animals that we have failed to recapitulate at 4 months in a genetic model. In our study, the mdx/P2X7−/− dKO mouse only presented a qualitative decrease in revertant fibre number compared to mdx (Figure 3.14). The reason for this is unknown but is most likely related to not including enough individuals to reasonably achieve significance at the current N numbers of 3 and 4. The P2X7 inhibitor used in the Young et al. (2012) study, CBB, is selectively efficient at blocking rat P2X7, separate from other P2X receptors including P2X4 (Jiang et al., 2000), which may be co-assembled with P2X7 subunits (Yeung et al., 2004; Guo et al., 2007). Therefore, if a discrepancy does exist, it is unlikely to be related to a contribution of P2X4 to the dystrophic phenotype.

Utrophin expression

Utrophin is the structural analogue of dystrophin that is thought to provide partial functional replacement in the mouse model of DMD (Pons et al., 1994; Rafael et al., 1998; Baban & Davies, 2008). Utrophin is also thought to be differentially expressed across muscle fibre-types. Predominantly oxidative or glycolytic muscle fibres are also differentially distributed across muscle groups. Slow oxidative type muscle fibres express and retain more utrophin than fast type fibres (Gramolini et al., 2001; Chakkalakal et al., 2008). Notably, fast glycolytic type fibres have been shown more susceptible to dystrophic damage and death (Webster et al., 1988). Since utrophin expression is positively correlated with the expression of DAPC members including
β-dystroglycan, and endogenous utrophin downregulation at ~2 weeks of age coincides with the onset of necrosis in the \textit{mdx} mouse (Roma \textit{et al.}, 2004), it may be that utrophin has an ability to stabilise the sarcolemma via DAPC binding or, at least, is positively correlated with preferential sarcolemmal stabilisation in slow-type muscle fibres of the \textit{mdx} mouse. There is also some evidence for an association of slow-type muscle fibre development from myogenin mediated terminal differentiation, as opposed to the MRF myoD (Hughes \textit{et al.}, 1993; Talmadge, 2000). We have shown an increase in myogenin expression in P2X7 deficient muscle \textit{mdx} skeletal muscle (Figure 3.12). Partly for these reasons, we chose to compare utrophin levels between \textit{mdx} and \textit{mdx}/P2X7\textsuperscript{-/-} dKO hindlimb muscles at the 4 week acute degenerative period. From this analysis, we failed to identify a differing utrophin protein level between \textit{mdx} and Pfizer \textit{mdx}/P2X7\textsuperscript{-/-} dKO GC muscles at 4 weeks of age (Figure 3.15).

Utrophin has previously been defined as upregulated in \textit{mdx} mouse muscle compared to healthy controls, where an elevated expression is deemed compensatory and beneficial (Tinsley \textit{et al.}, 1998). From our data showing no difference between \textit{mdx} and Pfizer dKO utrophin expression levels, it could therefore be suggested that we have maintained this level of successful and endogenous compensation with the loss of the P2X7 receptor. Moreover, we have earlier shown an increase in centrally nucleated average muscle fibre diameter in \textit{mdx}/P2X7\textsuperscript{-/-} dKO muscle compared to \textit{mdx} perhaps indicating an enhanced muscle regenerative potential in the \textit{mdx} mouse upon the loss of P2X7 receptor activation. One important molecular pathway via which enhanced fibre growth could be effected is the Akt/mTOR cascade. In their study into the effects of valproic acid mediated α7 integrin upregulation on the \textit{mdx} pathology, Gurpur \textit{et al.} (2009) showed that Akt activation was linked to enhanced fibre diameter and a reduced susceptibility to apoptosis. Akt overexpression has also been shown to ameliorate disease features in the \textit{mdx} mouse coincident with an increase in the utrophin-glycoprotein complex (Kim \textit{et al.}, 2011). Similar to results presented here, Kim \textit{et al.} (2011) showed an increase in muscle fibre size without a statistically significant difference in numbers of centrally nucleated fibres. However, we have failed to find a related increase in utrophin expression between \textit{mdx} and \textit{mdx}/P2X7\textsuperscript{-/-} dKO muscle as their study found with improved pathology upon Akt overexpression. This may indicate that this is not the primary pathway effecting the
muscle fibre size increase in this study, with an increased utrophin-glycoprotein complex compensating by contributing an enhanced sarcolemmal stability to regenerating muscle.

It should also be noted that in several key measures of biological function, utrophin differs from dystrophin, with clinical implications regarding its compensatory effects. For instance, it has recently been shown that supra-physiological levels of forced utrophin expression did not increase the anchoring of otherwise delocalised nNOS to the sarcolemma in the mdx mouse (Li et al., 2010a).

Analysis of diaphragm from aged mice

In preceding analyses, two dystrophic age points were used in the mouse, 4 week and 4 month, to represent regenerative/degenerative and post-regenerative tissues respectively. These time points are relevant for pre-clinical and proof-of-principle studies into the effects of P2X7 ablation on the dystrophic background using recommended hindlimb muscles, in vivo (Table 3.1). However, it has been acknowledged that diaphragm represents a very suitable tissue for experimental pre-clinical testing, and perhaps more similar to the general human DMD pathology due to the enhanced severity of histologically-evident pathology in this dystrophic tissue compared to others (Table 3.1; Stedman et al., 1991). Furthermore, from a biological-mechanistic perspective, mdx diaphragm tissue undergoes continuous degeneration and tissue reorganisation relevant to observe longer-term effects of P2X7 ablation on muscle regenerative pathways and histological markers of disease progression, especially in aged mice. Therefore, comparing diaphragms from ~20 months old mice, at an age well-into the late-term whole body atrophy phase of mdx disease progression (Figure 1.5b), it was confirmed that the minimum Feret’s diameter of muscle fibres was significantly larger in Pfizer-mdx/P2X7−/− compared to mdx and intermediate in size between C57 and mdx (Figure 3.16). This result agrees with that obtained using 4 week TA muscles. With the inclusion of the Pfizer single-KO P2X7−/− diaphragm, we can now also state that removing P2X7 activity in vivo does not affect muscle fibre size under non-dystrophic conditions. Because the aged dystrophic diaphragm uniquely represents a severe model of perpetual degeneration and regeneration in the
mdx mouse, we can confidently apply this conclusion to less severely affected muscles and at younger ages.

Since revertant dystrophin-positive fibres arise through chance somatic reversions, their numbers in dystrophic tissue are positively correlated with time and, more specifically, the number of preceding cycles of degeneration and regeneration. In a severely disease-affected muscle like the diaphragm, and especially in aged animals, it may therefore be expected that inherent biological differences should be exaggerated with respect to underlying dystrophic severity. Significantly fewer revertant fibres were found in Pfizer-mdx/P2X7−/− compared to mdx ~20 month diaphragms (Figure 3.16). This result is consistent with previous reports published by our laboratory, using 4 month hindlimb muscle (Young et al., 2012), and lends further support to the notion that P2X7 receptor over-activity may be negatively influencing muscle regeneration in the mdx mouse. This is especially evident given the enhanced diaphragm-muscle fibre sizes from these ~20 months old Pfizer-mdx/P2X7−/− compared to mdx mice and the later mentioned other histological pathological markers measured in these same tissues and in heart (Chapter 4).

3.3.3 Conclusions

DNA and protein analysis techniques confirmed the generation of two mdx/P2X7−/− dKO mouse strains. The Pfizer mdx/P2X7−/− variant was preferred for most subsequent comparisons to mdx. This was primarily based upon a previously reported splice variant of P2X7, the K variant, which is known to escape inactivation in the Glaxo P2X7−/− KO mouse (Nicke et al., 2009). The background effect of differing wild-type mouse origins between both P2X7 KO strains and the mdx mouse was also deemed unlikely given the similarities between C57 BL/6 and BL/10, including the shared proline to leucine P2X7 amino acid substitution that renders the receptor substantially less active. While mdx/P2X7−/+ heterozygous mouse was found to be unsuitable as a true haplo-sufficient control based upon an intermediate expression of P2X7, in between mdx and C57, it was a good control for the impact of P2X7 expression levels on the mdx pathology.
P2X7 expression is greatly increased in dystrophic \textit{mdx} mice and localised to regions of mononuclear cell infiltrations and at the sarcolemma of regenerating muscle fibres. Without exacerbating disease features through exercise or exacerbation of muscle damage by other means, for instance, we have failed to identify obvious gross qualitative histological differences in pathology between \textit{mdx} and \textit{mdx}/P2X7\textsuperscript{-/-} dKO muscles at either 4 weeks of age, representing an acute phase of muscle degeneration in the \textit{mdx} mouse, or at 4 months, in post-degenerative skeletal muscle. However, a more detailed histological analysis using a semi-automated form of morphometry revealed that Pfizer \textit{mdx}/P2X7\textsuperscript{-/-} mice had a greater average minimum Feret’s diameter of centrally nucleated muscle fibres compared to \textit{mdx}, without significantly increased total central nucleation or a variation in fibre sizes across a whole cross-section. This was coupled to an increase in the levels of myogenin but not the satellite cell marker, Pax7. That the two could be normally negatively correlated relates to the observation that the cell cycle progression, necessary for myoblast proliferation, is usually directly opposed to the growth arrest, necessary for differentiation (Rodgers, 2005).

Irrespective of the mechanisms in play, it was clearly presented that these improved histological parameters also extended into the most severely disease-affected muscle in the \textit{mdx} mouse, the diaphragm, a tissue advised for use in pre-clinical and basic research studies because of its continuous degenerative profile. That it was also shown the increased minimum Feret’s diameter of \textit{mdx}/P2X7\textsuperscript{-/-} compared to \textit{mdx} was concurrent with fewer dystrophin-revertant fibres from this muscle, serves to highlight a potential link between the number of cycles of regeneration and muscle fibre size. Whilst larger muscle fibres are not necessarily beneficial in \textit{mdx} disease pathology, larger fibres have been shown to be more resistant to mechanical stresses in the \textit{mdx} mouse (Schertzer \textit{et al.}, 2007; Gehrig \textit{et al.}, 2010).
Effect of P2X7 receptor ablation on the immune cell phenotype in \textit{mdx} muscles

4.1 Introduction

4.1.1 Inflammation in DMD

Inflammation is an important aspect of DMD. For example, in a meta-analysis of microarray studies from a wide age range of DMD muscle biopsies, immune response genes were found consistently up-regulated (Kotelnikova \textit{et al}., 2012). Large immune cell infiltrations are found in DMD patients as well as in the \textit{mdx} mouse (Evans \textit{et al}., 2009), leading to the notion that the currently employed corticosteroid treatment in humans may be having at least some beneficial effect by reducing the cytotoxicity and inflammation in diseased muscles (Villalta \textit{et al}., 2009). Furthermore, treatments that limit immune cell activity have proven beneficial in ameliorating disease symptoms in \textit{mdx} mice (Spencer \textit{et al}., 2001). Mouse studies have shown steroids to be effective at reducing \textit{mdx} disease severity over short-term (Keeling \textit{et al}., 2007; Sali \textit{et al}., 2012) and longer term (Keeling \textit{et al}., 2007) treatments. In other longer term studies, however, it has also been observed that some features of \textit{mdx} pathology, like reduced muscle strength and heart fibrosis, may be worsened with regular steroid treatments (Sali \textit{et al}., 2012). It is currently difficult to dissociate these longer-term effects from the non-immune-modifying effects of steroid treatments, especially as these may differ in exercised and non-exercised muscles and with disease progression (Lim \textit{et al}., 2004). Moreover, it has been recently shown that nonsteroidal anti-inflammatory treatments in the \textit{mdx} mouse decreased levels of inflammatory cytokines concurrent with increases in muscle strength and reduced serum CK levels but, unlike steroids,
without negatively affecting key myogenic regulators (Huynh et al., 2013; Uaesoontrachoon et al., 2014). This suggests that anti-inflammatory treatments specifically targeting mechanisms involved in the pathological arm of the inflammatory response could modify the disease while avoiding the negative effects of long-term steroid treatment. However, some beneficial effect of steroids might be independent of immune response regulation. In the C. elegans model of DMD, prednisone reduced numbers of degenerating muscle cells (Gaud et al., 2004), while the worm possesses only a very primitive innate immune system that would not respond to prednisone in the same way as a mammalian does. Interestingly, when steroids were first used in DMD, the precise rationale for their supposed biological effect was not offered (Drachman et al., 1974).

As explained earlier, the form of inflammation in DMD is sterile, caused by danger signals and DAMPs, attracting and activating innate immune cell types, including macrophages and neutrophils. Due to the chronic nature of the degenerative process and the perpetual presentation of DAMPs human and mouse muscular dystrophy present features akin to autoimmune diseases (Tidball & Wehling-Henricks, 2005). In DMD carriers, this chronic inflammatory response can also occur and has led to misdiagnosis as polymyositis or viral hepatitis (Yoon et al., 2011).

A mix of innate and adaptive immune cells infiltrating dystrophic muscles contribute to cell debris clearance but also cell death and reparative processes. Macrophages make up the bulk of inflammatory cells in dystrophic muscle, with as many as 50,000 cells per mm³ (Wehling et al., 2001). Another major component of the inflammatory milieu are granulocytes and T-cells. CD8⁺ cytotoxic T-cells contribute to muscle degeneration via a perforin-mediated cytolysis. Increased T-cell numbers are also consistent with an autoimmune-like phenotype existing in DMD patients (Spencer et al., 1997).

The phenotype of infiltrating macrophages changes over the course of disease progression (Figure 4.1). As found in the mdx mouse, at an acute degenerative period around 4 weeks of age, M1 macrophages responsible for the cytolysis of muscle fibres and for T-cell activation predominate (Villalta et al., 2009). Later on, M2 (CD163 positive) macrophages responsible for reducing inflammation (Gordon, 2003; Martinez et al., 2008; Villalta et al., 2009) and inducing muscle regeneration but also
promoting scarring (Vidal et al., 2008) increase in numbers (Tidball & Villalta, 2010). In DMD, there is a continuum of M1 and M2 macrophages, with these phenotypes expressed at the same time in various regions of the continuously, albeit heterogeneously, degenerating muscle.

![Figure 4.1](Image)

**Figure 4.1.** Schematic representation of macrophage phenotypic transitions in relation to T-cell and MRF (myogenic regulatory factor) induction following muscle injury in mice. (Image adopted from Tidball & Villalta 2010). PMN (polymorphonuclear leukocytes: neutrophils); MEF2 (myocyte enhancer binding factor-2).

In summary, reducing total immune cell infiltrates may be problematic, since it also carries the potential to interfere with regenerative processes. However, interventions
that can change the ratio of competing immune cells towards pro-regenerative may benefit host health, ultimately reducing the severity of the disease pathology.

The P2X7 receptor presents an interesting target, potentially able to regulate the beneficial shifts in immune cell sub-types.

### 4.1.2 The P2X7 receptor and immune cell responses

ATP is now recognised as an important autocrine and paracrine signalling factor, influencing functions of inflammatory cells (Corriden & Insel, 2010). An example of this is ATPe enhancing IL-6 release by macrophages, via P2X- and P2Y-mediated Ca\(^{2+}\) currents (Hanley et al., 2004).

P2X7 is capable of inducing ATP-mediated cell death in macrophages via a Ca\(^{2+}\)-induced caspase/calpain activation (Hanley et al., 2012). In a flow cytometry study, P2X7 has been localised to most major immune cell populations. A predominant P2X7 protein surface expression was in monocytes, with weaker B-lymphocyte, T-lymphocyte, and natural-killer (NK) cell expressions (Gu et al., 2000). Neutrophil P2X7 surface expression was reported as negligible in this report (Gu et al., 2000) and deemed absent in another, more recent, study (Hanley et al., 2012) but identified by Christenson et al. (2008).

The induction of IL-1\(\beta\) release via P2X7 receptor activation is well established in a variety of infectious (Lee et al., 2012) and chronic inflammatory conditions (Arulkumaran et al., 2011). Although the canonical induction of IL-1\(\beta\) via P2X7 stimulation occurs in lipopolysaccharide (LPS)-primed macrophages, a model of pathogen-induced rather than sterile inflammation, it is known that P2X7 receptors can trigger IL-1\(\beta\) and TNF\(\alpha\) release via alternative activation routes such as by contact with microvesicles containing phospholipid secondary signalling mediators (Thomas & Salter, 2010), interaction with the ECM component biglycan (Babelova et al., 2009), or following phagocytosis of autophagic cells (Ayna et al., 2012). As a part of its general pro-inflammatory function, IL-1\(\beta\) has been shown to increase the expression of Th1-associated molecules including COX-2, IL-6, and osteopontin (OPN) (Uaesoontrachooon et al., 2008). IL-1\(\beta\) is also integral to the conversion of immunosuppressive T-reg into Th17 type cells participating in inflammation and
autoimmunity (Acosta-Rodriguez et al., 2007; Chung et al., 2009). TNFα, is an important inflammatory mediator in DMD (Lundberg et al., 1995; Grounds & Torrisi, 2004; Hodgetts et al., 2006; Pierno et al., 2007) and an elevated level of plasma TNFα has also been associated with cachexia (Nagaoka et al., 2006). TNFα was shown to inhibit the differentiation of muscle (Miller et al., 1988; Szalay et al., 1997; Layne & Farmer, 1999; Langen et al., 2001; 2002; 2004). TNFα and IL-1β significantly reduced the expression of myogenin in both murine and porcine myoblasts (Broussard et al., 2003; 2004).

With regard to the aforementioned macrophage sub-populations, P2X7 receptors may be differentially expressed across the M1 to M2 macrophage polarization gradient. Although protein levels and receptor membrane localisation appeared similar between M1 and M2 macrophages in vitro, P2X7 receptor gene expression was shown to be down regulated in M1 compared to M2 macrophages. However, in response to ATP, M1 macrophages preferentially release mature IL-1β (Pelegrin & Surprenant, 2009), which might be important in pathological progression of DMD.

4.1.3 Chapter summary

In summary, DMD muscle is chronically inflamed (Haslett et al., 2002; Porter et al., 2002), and treatments aimed at reducing this inflammation have ameliorated disease features in model organisms. The P2X7 receptor has been linked to the induction of inflammation pathways. It is known to be expressed on many immune as well as on dystrophic muscle cells (Ferrari et al., 1996; 1997; Gu et al., 2000; Di Virgilio et al., 2001; Gudipaty et al., 2001; Lenertz et al., 2009; Wewers & Sarkar, 2009). The P2X7 receptor can respond to DAMPs, including ATP, to shape the inflammatory response through release of pro-inflammatory cytokines such as IL-1β and TNFα or via influencing immune cell subtype selection. Therefore, we have used immunodetection methods to determine the expression of P2X7 protein in relation to major immune cell types in mdx and mdx/P2X7−/− skeletal muscle and to identify any potential shift in cell populations including between M1 and M2 macrophage subsets. qPCR analysis was undertaken to identify immunophenotype shifts resulting from P2X7 ablation, followed by immunodetection of key identified targets in mdx and mdx/P2X7−/− muscle samples. Finally, we extended our immunohistological analysis into aged dystrophic...
diaphragms and hearts, to see if positive modulation of inflammation by P2X7 ablation continued into later phases of disease progression, or whether disease features were in fact worsened.
Chapter 4: Effect of P2X7 ablation on *mdx* inflammatory phenotype

### 4.2 Results

#### 4.2.1 P2X7 co-immunolocalisation with immune cell subtypes in 4 week old *mdx* muscles

Specific antibodies have been used to detect CD11b, CD68, Ly6G, and OPN in C57, *mdx*, and Pfizer *mdx/P2X7<sup>−/−</sup>* muscle from 4 week old mice. These protein targets may be used to distinguish between major inflammatory cell types. CD11b is a protein subunit of α<sub>M</sub>β<sub>2</sub> integrin expressed on a variety of leukocytes including monocytes, granulocytes, macrophages, and NK cells (Ward *et al*., 2006). CD68, is a scavenger receptor type transmembrane glycoprotein expressed on monocytes and tissue macrophages (Ward *et al*., 2006). OPN, also known as secreted phosphoprotein 1 (SPP1), is a glycoprotein expressed by many cell types including macrophages, neutrophils, T-cells, dendritic cells, osteoblasts, and osteoclasts (Ashkar *et al*., 2000; O'Regan & Berman, 2000) (Suzuki *et al*., 2002). Lymphocyte antigen 6 complex [locus G] (Ly6G), also-known-as Gr-1, is expressed predominantly on bone marrow granulocytes and neutrophils. Ly6G has recently been linked with neutrophil ability to migrate to regions of tissue inflammation *in vivo* (Wang *et al*., 2012). The 4 week time point has been chosen as it is the period of active tissue degeneration/regeneration and major mononuclear cell infiltrations in the *mdx* mouse and, as such, closely resembles the human disease.

P2X7, in agreement with previous studies, localised definitively with two of these four immune markers, CD11b and CD68, in dystrophic *mdx* muscles *in vivo* (**Figure 4.2**: top two rows). A lesser degree of co-localisation was found between P2X7 and Ly6G (**Figure 4.2**: bottom row). The P2X7 receptor signal co-localised with OPN in a subset of cells but these two proteins appeared to have a mutually-exclusive pattern of expression in the cytoplasm and the nucleus (**Figure 4.2 and Figure 4.4**). Further higher magnification immunological analysis confirmed P2X7 co-localisation with CD68-positive monocytes (**Figure 4.3**).
Figure 4.2. Immunofluorescence, showing the P2X7 receptor (middle column) co-expressed with immune cell markers CD11b, CD68, and Ly6G in *mdx* TA muscle at 4 weeks. Note the inset image in the OPN panel showing strong P2X7 signal in the cytoplasm and OPN predominantly in cell nuclei.
Figure 4.3. P2X7 and CD68 co-localisation in 4 week *mdx* skeletal muscle. Z-stack slices highlight total co-localisation between P2X7 and macrophage marker, CD68.
Figure 4.4. P2X7 and OPN co-localisation in 4 week *mdx* GC muscle. Top image: OPN (red) heavily expressed in strongly P2X7-positive (green) areas of cellular infiltration. Bottom image: most interstitial P2X7-positive cells exhibit nuclear OPN expression (arrows). Orthogonal views provide Z-stack confirmation along the yellow intersect. The strongest OPN signal was associated with the myonuclei of small regenerating myofibres (yellow asterisks). Inset image showing co-localisation of the Hoechst (blue) signal with the OPN staining in some myonuclei (myofibres outlined in white) and interstitial immune cells.
4.2.2 *Comparisons of immune cell subtypes infiltrating mdx and mdx/P2X7<sup>-/-</sup> muscles*

Total GC cross-sections were immunohistochemically stained for a pan immune-cell marker CD11b. Generally, single areas of mononuclear CD11b<sup>+</sup> immune-cell aggregation were greater in 4 week *mdx* hind-limb tissue compared with Pfizer *mdx/P2X7<sup>-/-</sup>* (Figure 4.5). C57 control GC samples had only a slight CD11b<sup>+</sup> signal in locations corresponding with blood vessels at the perimysium borders.

To confirm the location and co-localisation prior to quantification, the pan macrophage markers CD68 and CD163, predominantly labelling all and M2 type macrophages respectively, were used for an immunofluorescence study. As observed by others (Deng *et al.*, 2012), both CD68 and CD163 antibodies labelled the cytoplasm of immune cells in the interstitial space of *mdx* muscles (Figure 4.6). CD163 immunofluorescence was present in a subset of CD68 positive cell bodies in 4 week GC (Figure 4.6).

In an attempt to characterise and compare the major immune cell infiltrate profiles in these two types of dystrophic muscles, *mdx* and *mdx/P2X7<sup>-/-</sup>* samples were stained using macrophage specific CD68 and F4/80 antibodies for immunofluorescence detection. Applying a colour pixel intensity analysis, no difference was found between *mdx* and Pfizer *mdx/P2X7<sup>-/-</sup>* for CD68 or F4/80 expression levels at 4 weeks (Figure 4.7). Individual cell bodies were inconsistently defined by either antibody, making the quantification of discrete cell numbers in cryosectioned tissue implausible. Additionally, the anti-F4/80 antibody from Abcam (ab74383) did not label interstitial cell bodies that define the traditional macrophage infiltrations in dystrophic mouse muscle tissue. F4/80 positive signal was evident in areas of obvious muscle degeneration, providing an indication that the anti-F4/80 antibody could be used as a macrophage associated marker for the semi-quantitative Western blotting and immunolocalisation pixel intensity distribution analysis.
Figure 4.5. Example immunohistochemical staining of CD11b-positive immune cells highlighting focal pathology in the GC mdx and Pfizer mdx/P2X7−/− at 4 weeks. Note, an absence of CD11b signal (red/brown) in C57 (WT: top) compared to both dystrophic samples (middle and bottom). Also note, the enhanced area of monocellular infiltration in mdx versus Pfizer mdx/P2X7−/− (dKO). Areas of disease-related inflammation were often heterogeneous across single muscle cross-sections and between individuals within the same genotype. Nuclear counterstaining was deliberately light to avoid masking primary signal in these images.
Figure 4.6. Immunofluorescence microphotographs, showing M2 macrophage sub-type marker CD163 expressed in a sub-set of CD68 positive cells in dystrophic skeletal muscle in vivo. The CD163 signal (green), predominantly found on the M2 subtype of macrophages, co-localises, with varying intensity, with a subset of CD68 expressing cells (yellow: bottom-right arrows and insert; orange: bottom-right arrowhead). Additional CD68 signal (red) that does not spatially coincide with CD163 agrees with the pan macrophage nature of this marker that may also be expressed on monocytes and fibroblasts. Cell nuclei are labelled with Hoechst (blue).
Figure 4.7. Immunofluorescence analysis, showing no difference in CD68 or F4/80 protein expression between \textit{mdx} and Pfizer \textit{mdx}/P2X7\textsuperscript{-/-} TA muscle at 4 weeks (also see Appendix 9.1; 9.2). Pixel intensity distribution of: \textbf{a)} CD68 and \textbf{b)} F4/80 showed no difference in average CD68 (T-test, $T=-0.16$, $P=0.884$, df=2, N=3) or F4/80 (T-test, $T=1.04$, $P=0.406$, df=2, N=3) fluorescence levels between 4 week \textit{mdx} and \textit{mdx}/P2X7\textsuperscript{-/-} TA muscles.
4.2.3 Western blotting analysis of immune cell markers between mdx and mdx/P2X7-/− muscles

Due to the complex staining pattern with the macrophage-specific antibodies and because of the inherent limitation of a method involving sampling at only a cross-sectional level, a Western blot assay was used to compare immune cell infiltrates in protein extracts from the whole 4 week TA or GC muscles of C57, mdx, and mdx/P2X7-/− mice.

The available antibodies recognizing CD4, CD8a, and Foxp3 antigens did not work under denaturing conditions used for Western blotting (data not shown). Using the F4/80 pan macrophage marker (~130 kDa), significantly lower intensity bands were found in GC from C57 and both mdx/P2X7-/− strains when compared to mdx, relative to the actin loading control (Figure 4.8). This indicates that total macrophage infiltrations may be reduced in mdx/P2X7-/− muscle compared to mdx, at 4 weeks.

The Western blotting analysis showed a significant reduction in the levels of F4/80, total macrophage marker, relative to CD163 in Pfizer mdx/P2X7-/− strains compared to mdx (Figure 4.8). There was no significant difference in this parameter between the hypomorph Glaxo mdx/P2X7-/− and mdx samples.
**Figure 4.8.** Western blotting, showing lower levels of F4/80 and F4/80 to CD163 protein expression in \(mdx/P2X7^{-/-}\) and C57 4 week GC muscle compared to \(mdx\). **a**) Representative immunoblots for F4/80, CD163, and actin (protein loading control). **b**) Graphical representation of the average F4/80 expression relative to actin (left) and F4/80 relative to CD163 (right), showing significantly less F4/80 protein in Pfizer, Glaxo, \(mdx/P2X7^{-/-}\) and C57 compared to \(mdx\) (ANOVA, \(F=9.93, P=0.001, \text{df}=3, N=4, \text{Tukey post-hoc}\)). F4/80 to CD163 protein ratio was significantly lower in C57 and also reduced in Pfizer but not Glaxo \(mdx/P2X7^{-/-}\) compared to \(mdx\) (ANOVA, \(F=4.05, P=0.033, \text{df}=3, N=4, \text{Tukey post-hoc}\), (Mann Whitney, \(W=26.0, P=0.0304\)). All western-blotting bands at expected weights (Table 2.3).
4.2.4 qPCR analysis of immune response gene expressions in skeletal muscles from mdx and mdx/P2X7−/− dKO mice

Numerous genes have been linked with the chronic inflammatory phenotype of the dystrophic mdx mouse. To address the question of the potential impact of P2X7 ablation on the key mediators of inflammatory responses in mdx skeletal muscles, a qPCR analysis was undertaken on C57, mdx, Glaxo mdx/P2X7−/− and Pfizer mdx/P2X7−/− muscle at the acute degenerative period of 4 weeks of age. mRNA samples were extracted, converted into cDNA and analysed by TaqMan qPCR method. This latter part of analysis was completed in collaboration with Prof. Pawel Kalinski and Dr. Ravikumar Muthuswamy, at the University of Pittsburgh, USA.

The quantitative PCR study included primer sets for the following transcripts: interferon-gamma (IFNγ), TNFα, CD4, CD8, perforin-1 (PRF1), granzyme-B (GZMB), Foxp3, retinoic acid related orphan receptor-C (RORC), granulocyte receptor-1 (GR-1; also known as subunit Ly-6G), COX2, indoleamine 2,3-dioxygenase 1 (IDO1), natural killer cell p46-related protein (NKP46), IL-12, IL-27, IL-17A, IL-10, chemokine [C-C] motif ligands: 5 (CCL5), CCL22, CXCL10, CXCL12, CX3CL1, and XCL1. The resulting qPCR data was analysed using ANOVA. The C57 values were not statistically analysed at ‘n=2’, and their mean is only reported for a general reference to the dystrophic groups. As expected, the levels of inflammatory marker mRNAs were lower in samples from C57 animals than in mdx animals.

Following statistical analysis of the qPCR data sets, Glaxo and Pfizer mdx/P2X7−/− mice showed a general trend towards a decreased inflammatory profile, compared to mdx samples (Figure 4.9). Four genes emerged as being of particular interest because of their statistically significant reduction in expression levels in mdx/P2X7−/− TA compared to mdx. Specifically, CX3CL1 and RORC mRNA levels were significantly reduced in both Glaxo and Pfizer mdx/P2X7−/− compared to mdx. TNFα mRNA was significantly reduced but only in Pfizer mdx/P2X7−/− 4 week TA compared with mdx. Finally, in Glaxo and Pfizer mdx/P2X7−/− 4 week TA tissue, Gr-1 mRNA expression was significantly reduced compared to mdx.

The general gene expression patterns for many of the other gene markers analysed correlated well with the potential shift away from a pro-inflammatory phenotype upon
P2X7 ablation (Figure 4.9). The pro-inflammatory prostaglandin-related COX-2 transcript had a lesser average expression value for Glaxo and Pfizer \( mdx / P2X7^{-/-} \) TA muscle compared to \( mdx \) samples. The same pattern was observed for PRF-1 gene expression. For FOXP3 and XCL1, there was a qualitative increase in gene product expression in Pfizer \( mdx / P2X7^{-/-} \) TA muscle compared to Glaxo \( mdx / P2X7^{-/-} \) and \( mdx \).
Chapter 4: Effect of P2X7 ablation on mdx inflammatory phenotype

Figure 4.9. Graphical representation of qPCR data comparing relative gene expression levels (2^{-ΔCt}) normalised to HPRT1, showing a reduced inflammatory mRNA profile in mdx/P2X7^{-/-} TA muscle compared to mdx. The results indicate a general trend towards a reduction of pro-inflammatory gene expression upon P2X7 receptor ablation with significantly lower expression levels of TNFα, CX3CL1, RORC, and GR-1 in P2X7 mdx/P2X7^{-/-} mice compared to mdx (ANOVA, F=8.19, 8.78, 6.39, 14.68, P=0.029, 0.010, 0.022, 0.002, df=2, respectively). Note: gene labels are above each graph.

Legend: Interferon-γ (IFNG), tumour necrosis factor-α (TNFA), perforin-1 (PRF1), scurfin/forkhead box P3 (FOXP3), retinoic acid related orphan receptor-C (RORC), granulocyte receptor-1 (GR1), cyclooxygenase-2 (COX2), indoleamine 2,3-dioxygenase 1 (IDO1), natural killer cell p46-related protein (NKP46).
4.2.5 **Comparison of Ly6G neutrophil marker and IL-1β expression in mdx and mdx/P2X7<sup>−/−</sup> muscles**

Neutrophils make up an important component of the innate immune response in dystrophic muscle (Hodgetts et al., 2006), and P2X7 receptor has been linked with pro-inflammatory cascades leading to local neutrophil accumulations (Christenson et al., 2008; Kawamura et al., 2012; da Silva et al., 2013). Since the Gr-1 (also known as Ly6G) gene was identified by the qPCR study as a factor reduced in both mdx/P2X7<sup>−/−</sup> mice compared to mdx, it was decided to compare levels of the encoded protein subunit, Ly6G, between genotypes using immunological methods. IL-1β is also closely linked to P2X7 activation and neutrophil migration and therefore IL-1β was included in this comparison.

Using immunofluorescence detection, it was found that an anti-Ly6G antibody labelled cells within regions of mononuclear infiltrations in mdx and Pfizer mdx/P2X7<sup>−/−</sup> 4 week TA muscles, consistent with it being expressed mainly on neutrophils but also on macrophages (Figure 4.10). The staining patterns indicated that Ly6G expression was more prevalent in mdx than in mdx/P2X7<sup>−/−</sup> muscle. To quantifiably confirm this, a Western blot comparison method was adopted. Ly6G protein expression was found to be significantly reduced in 4 week Pfizer mdx/P2X7<sup>−/−</sup> GC compared to mdx muscle (Figure 4.11).

The immunofluorescence experiments also showed increased IL-1β in mdx compared to Pfizer mdx/P2X7<sup>−/−</sup> muscle (Figure 4.10) but the experiment to confirm by Western blotting was not attempted. Finally, P2X7 co-localisation with Ly6G was undertaken and showed that this receptor is expressed in regions where neutrophils were present. (Figures 4.2 and 4.11b), which was contrary to one recent study (Hanley et al., 2012).
Figure 4.10. Example immunofluorescence images of Ly6G neutrophil marker (red) and IL-1β (green) in mdx and mdx/P2X7−/− TA muscles at 4 weeks. Within similarly located regions of mononuclear cell infiltrations, strongly Ly6G positive bodies appear more often in mdx (top) than in mdx/P2X7−/− (bottom). IL-1β cytoplasmic signal (green) was more evident in mdx than in Pfizer mdx/P2X7−/− muscle fibres. In the mdx sample, higher numbers of infiltrating cells were also less intensely Ly6G positive (e.g. yellow arrows), maybe presenting labelled myeloid cells. Also evident in the Pfizer mdx/P2X7−/− sample is a region of muscle fibre regeneration with less pronounced mononuclear cell infiltrations (line delineated area adjacent to asterisks). Cell nuclei (blue). See Appendix 9.6 for more examples.
Figure 4.11. Western blotting comparison and immunofluorescence staining, showing reduced neutrophil marker, Ly6G, expression in Pfizer \textit{mdx}/P2X7\textsuperscript{-/-} GC muscle compared to \textit{mdx} at 4 weeks. a) Western blot image for Ly6G and β-tubulin (loading control) in 4 week GC muscle. b) Graphical representation of average Ly6G band density relative to β-tubulin, showing significantly greater Ly6G protein levels in \textit{mdx} compared to Pf \textit{mdx}/P2X7\textsuperscript{-/-} tissue (T-test, T=4.14, P=0.006, df=6, n=4). c) Example Ly6G immunofluorescence comparison, illustrating P2X7 and Ly6G co-immunofluorescence in \textit{mdx}, lack of P2X7 signal and reduced total Ly6G staining in Pfizer \textit{mdx}/P2X7\textsuperscript{-/-} muscle compared to \textit{mdx}. Cell nuclei (blue). All western-blotting bands at expected weights (Table 2.3).
4.2.6 Evaluation of inflammation and fibrosis in aged dystrophic diaphragm and heart upon P2X7 ablation

By reducing inflammation at earlier stages of the disease, including potentially shifting macrophage sub-populations to the pro-regenerative M2 phenotype, we have created the potential to exaggerate fibrosis at later stages of mdx disease progression. To address this issue, we have monitored overall inflammation and measures of fibrosis, in aged dystrophic diaphragm and heart tissues. Both of these tissues, and especially diaphragm, are severely affected by the dystrophinopathy. They are thought to undergo continuous degeneration and enhanced fibrosis, more akin to the general human condition (Figure 1.5b).

Using collagen type-Ia immunofluorescence, trichrome staining, and CD11b immunolocalisation, we have confirmed the pathological increase in fibrosis in diaphragms and hearts as well as CD11b-positive immune-cell infiltrations in mdx tissues compared to WT (C57). Despite the above concerns, it was found that, with P2X7 ablation, inflammation was reduced even in aged dystrophic diaphragms and hearts (Figure 4.12a and Figure 4.13b, respectively). Moreover, collagen type-Ia deposition in diaphragms was not different from mdx (Figure 4.12b) and fibrosis in the heart was actually reduced (Figure 4.13a) in ~20 month Pfizer mdx/P2X7+/− mice compared to mdx. The diaphragm inflammation at ~20 months was also analysed in WT and Pfizer mdx/P2X7+/− using the anti-CD68 but at n=1 it could only be assessed qualitatively.

In aged diaphragms, similar collagen type-Ia depositions were also confirmed in Pfizer P2X7+/− compared to C57 (Figure 4.12b). Inflammation is qualitatively similar between Pfizer P2X7+/− and C57, using anti-CD68 immunology (Figure 4.12a, n=1).
Figure 4.12. An immuno-histological comparison of diaphragms from aged (~20 month) mice, showing continued reduced inflammation in Pfizer mdx/P2X7−/− versus mdx, without enhanced collagen type Ia deposition. a) Representative immunofluorescence of CD68 (right) with quantification and analysis in Pfizer mdx/P2X7−/− and mdx (left), showing reduced CD68 immune-cell infiltrations into diaphragms upon P2X7 ablation (T-test, $T=4.73$, $P=0.042$, df=2, n=3). A single WT (C57) and Pfizer-P2X7−/− tissue are presented for non-disease reference. b) Representative immunofluorescence of collagen type-Ia (right) with quantification and analysis of Pfizer mdx/P2X7−/−, mdx, WT, and Pfizer-P2X7−/− (left), showing reduced collagen deposition in WT and Pfizer-P2X7−/− compared to mdx, and no difference between mdx and Pfizer mdx/P2X7−/− (ANOVA, $F=19.39$, $P<0.001$, df=3, n=3; Tukey post-hoc).
Figure 4.13. An immuno-histological comparison of hearts from aged (~20 month) mice, showing reduced inflammation and fibrosis in Pfizer mdx/P2X7⁻/⁻ versus mdx. **a)** Representative Masson’s trichrome staining (left) with quantification and analysis of WT (C57), mdx, and Pfizer mdx/P2X7⁻/⁻ (right), showing reduced fibrosis in aged heart (blue staining in the heart tissue) from WT and Pfizer mdx/P2X7⁻/⁻ compared to mdx (ANOVA, F=166.29, P<0.001, df=2, n=3; Tukey post-hoc). **b)** Representative immunofluorescence of CD11b (left) with quantification and analysis of WT, mdx, and Pfizer mdx/P2X7⁻/⁻ (right), showing reduced CD11b immune-cell infiltrations into heart tissue upon P2X7 ablation, and in WT compared to mdx (ANOVA, F=19.65, P=0.002, df=2, n=3; Tukey post-hoc).
4.3 Discussion

4.3.1 P2X7 receptor expression in immune cells

In this study, P2X7 receptor expression has been analysed within the acute period of dystrophic muscle degeneration/regeneration and compared to expression of cell markers for specific immune cell subpopulations.

The general pattern of P2X7-receptor expression and the coincidence with immune cell marker expression observed in this study was consistent with published observations.

*CD11b*

P2X7 receptor expression was found to be coinciding with the CD11b subunit of the αMβ2 integrin receptor (Figure 4.2). CD11b, also known as integrin αM, is a protein subunit of the larger αMβ2 integrin complex expressed on a variety of leukocytes including monocytes, granulocytes, macrophages and NK cells and is a key receptor involved in phagocytosis, cell mediated cytotoxicity, chemotaxis, and cell activation leading to production of multiple pro-inflammatory cytokines, including TNFα. In mice with altered αMβ2 integrin-fibrinogen interactions in leukocytes, acute inflammatory responses were significantly diminished (Kitagawa et al., 2013). Another established function of the αMβ2 integrin is in promoting adhesion of neutrophils via its interaction with intercellular adhesion molecule-1 (ICAM-1) (McDonald et al., 2010). Interestingly, in clear relevance to this study, the sterile inflammation initiator used in this study was ATP and it was found acting through P2X7 receptor-mediated Nlrp3 inflammasome activation (McDonald et al., 2010).

*CD68*

P2X7 protein also localised to tissue regions expressing CD68 (Figure 4.2), a marker specific for the monocyte and macrophage subpopulations of leukocytes (Tidball & Villalta, 2010). True co-localisation was further confirmed by Z-stack analysis of immunolocalised CD68 and P2X7 by confocal microscope analysis (Figure 4.3). P2X7 receptor expression in macrophages and their CNS equivalent, microglia, has been previously described (Collo et al., 1997; North, 2002). Considering that ATPe acts as a danger signal helping to establish a state of chronic inflammation (Hanley et al., 2004), it is noteworthy that ATP can actively inhibit IL-1β release from
macrophages lacking the P2X7 receptor, even when these macrophages are presented with agents that normally elicit an IL-1β release-independent of P2X7 activation (Pelegrin & Surprenant, 2009). Similarly, ATP acting via the P2X7 receptor has been shown to reduce tolerogenic histocompatibility antigen class-1-G in human monocytes (Rizzo et al., 2009), reinforcing the notion of P2X7 as a predominantly pro-inflammatory receptor coupled to cells of the myeloid lineage.

**Osteopontin (OPN)**

OPN is a glycoprotein expressed by several activated immune cell types where it is an important mediator of chemotaxis, adhesion, and differentiation to pro-inflammatory phenotypes (Ashkar et al., 2000; Denhardt et al., 2001; Renkl et al., 2005). OPN expression is upregulated upon macrophage and T-cell activation but is not recognisably present in circulating monocytes (Krause et al., 1996). Furthermore, OPN expression has been shown to be upregulated in a wide range of dystrophic mouse muscles (Porter et al., 2002) and also in DMD patients (Vetrone et al., 2009), in agreement with its link to other chronic inflammatory conditions (O'Regan & Berman, 2000; Denhardt et al., 2001; Yumoto et al., 2002; Comabella et al., 2005; Agnholt et al., 2007; Kariuki et al., 2009). Perhaps most importantly, it has also been shown that P2X7 receptor inhibition can reduce the expression of OPN in LPS-stimulated macrophages in vitro (Hu et al., 1998). A potential decrease in OPN expression in mdx muscle as a function of the loss of P2X7 receptors may produce an anti-inflammatory immunophenotypic shift. Moreover, it may also affect muscle regenerative processes as OPN is expressed by myoblasts and can affect their proliferation (Uaesoontrachoon et al., 2008; Paliwal et al., 2012). The co-localisation study undertaken here showed P2X7 and OPN expression in close proximity but not clearly colocalising in many cells within regions of active mdx muscle pathology (Figure 4.2). This apparent lack of co-localisation in some cells was however restricted to, what appeared to be, myonuclei of small regenerating fibres (Figure 4.4). This is in agreement with aforementioned myonuclear OPN expression in regenerating muscle fibres (Uaesoontrachoon et al., 2008; Paliwal et al., 2012). Moreover, some of this apparent mis-localisation was due to a clearly different distribution of these two proteins in leukocytes as OPN signal was found in cell nuclei whereas P2X7 signal was present in the cytoplasm. This result therefore agrees with both OPN and the P2X7 receptor having a known expression in immune cells.
Nucleus-localised OPN has been reported previously in human embryonic kidney cells, where, interestingly it has been linked to cells in mitosis (Junaid et al., 2007).

**Ly6G**

The final marker that was monitored for its spatial expression relative to P2X7, was Ly6G, the protein product of the gene Gr-1. Ly6G is predominantly expressed on bone marrow granulocytes and neutrophils. Neutrophils represent an important component of the initial inflammatory infiltrate in acutely damaged or diseased muscle. In DMD, neutrophils contribute to the cytotoxic environment (Nguyen & Tidball, 2003b). This effect is, at least partly, mediated by myeloperoxidase released by neutrophils (Tidball & Villalta, 2010). The immunolocalisation study performed here showed that P2X7 may be expressed by at least some Ly6G-expressing cells (Figures 4.2 and 4.12c). Further work is needed to confirm to what extent Ly6G and P2X7 receptors are expressed in macrophages and neutrophils, respectively.

The overall level of P2X7 immunofluorescence shown here agrees with the existing literature describing the purinoceptor as predominantly expressed on cells linked with chronic states of immune responses. Having established the background expression profiles in the mdx samples and wishing to further explore the potential changes in these immune cells subpopulation in the absence of P2X7 receptor, comparisons of immune cell markers were made between the mdx and the mdx/P2X7<sup>-/-</sup> mouse.

### 4.3.2 Comparisons of macrophage subpopulations in mdx and mdx/P2X7<sup>-/-</sup> muscles

Used in other studies as a histological marker for disease severity (Spencer et al., 1997; Kim & Lawler, 2012), areas of CD11b aggregation, corresponding to immune cell infiltrates including monocytes, granulocytes, macrophages, and NK cells, were often smaller in area in P2X7 dKO muscle than in mdx (Figure 4.5). This qualitative observation was corroborated in the Western blotting using F4/80 macrophage marker, showing that these most numerous of immune cell infiltrating dystrophic tissue are reduced by P2X7 ablation (Figure 4.8). Since macrophages represent a major component of the CD11b-positive cellular infiltrate in dystrophic tissue, analysis was extended into immunolocalisation using the CD68 and F4/80 macrophage markers. While immunofluorescence intensity comparisons did not show...
significant differences (Figure 4.7), the more directly quantitative Western blotting confirmed that total macrophage loads were reduced in P2X7−/− double-knockout muscle compared to mdx (Figure 4.8).

A reduction in the total macrophage number has been shown to improve mdx disease manifestation independent of other factors. For instance, mdx soleus muscle depleted of macrophages using an i.p. injection of anti-F4/80 antibody administered from 1 to 4 weeks of age, reduced mdx muscle cell lysis by over 75% in vivo (Wehling et al., 2001). However, the possible impact of a long-term macrophage depletion is more complicated when one considers that macrophages are also important for reparative processes in injured muscle.

Depletion of macrophages at 4 days following muscle damage, decreased numbers of regenerating fibres, myoD expression, and caused a reduction in myofibre cross-sectional area in mice (Tidball & Wehling-Henricks, 2007). The population of macrophages present at later periods of muscle tissue regeneration may effect a shift towards myoblast proliferation over differentiation (Merly et al., 1999). This raises an interesting possibility that the increase in muscle fibre size in mdx/P2X7−/− muscle described earlier (Chapter 3: Figure 3.11) may be a result of increased myogenic differentiation or fusion events (Chapter 3: Figure 3.12) over proliferation and that this is, at least partly, mediated by a change in inflammatory phenotypes.

Macrophage population sub-types have been defined in terms of their activation, as either classical, M1, or alternative, M2. In the mdx mouse model of DMD, the cytolytic M1 type macrophage is known to be the major form expressed in acutely damaged tissue at the 4-6 week period, whilst the M2 type, although co-expressed at the 4-6 week period, is upregulated during the subsequent, predominantly regenerative, phases of disease progression (Villalta et al., 2009). By depleting macrophages at this later stage, Tidball & Wehling-Henricks (2007) may have preferentially depleted the M2 type macrophages, linked to repair. In another study by this group it has been shown that the increased activation of this subtype, via IFN-γ knock-out in vivo or by exogenous increase of IL-10 in vitro, also improved mdx disease outcome (Villalta et al., 2011a; 2011b). Because IL-10 production has been shown to be reduced by ATP acting on P2X7 (Rizzo et al., 2009) and because this receptor knockout has been linked to the reduction of active IL-1β (Mutini et al.,...
1999; Solle et al., 2001; Labasi et al., 2002) and TNFα (Chessell et al., 2005) this purinoceptor ablation could alter macrophage phenotypes. Within the M2 lineage in muscle there is also an established sub-division into M2a and M2c macrophages. Both of these M2 type cells produce the reparative cytokines IL-4 and IL-10 but the M2a type has been specifically linked to fibrosis in dystrophic mouse muscle (Vidal et al., 2008; Villalta et al., 2009) whilst the M2c type is especially linked with the regenerative phase (Tidball & Villalta, 2010).

Using markers for the M2c subtype of macrophage, CD163, and total macrophages, CD68, we have confirmed that CD163 labels a subset of CD68 positive cell bodies in 4 week mdx tissue (Figure 4.6). A Western blotting analysis of F4/80 to CD163 ratio was undertaken and a small shift towards the M2 phenotype was observed in mdx/P2X7−/− muscle compared to mdx, indicating a potential for a beneficial shift in macrophage composition in these muscles. However, such a shift could still lead to increased fibrosis (Zhang et al., 2012), a pathological implication we later address.

P2X7 receptor KO may influence the function of macrophages in addition to changing their phenotype. For instance, whereas M2 type macrophages tend not to activate caspase-1 in response to ATP stimulation, this activation pathway is otherwise important for the release of active IL-1β and the induction of macrophage pyroptosis, an apoptosis-independent form of cell death (Pelegrin & Surprenant, 2009). In sterile inflammation, IL-1β may be released upon caspase-1-induced Nlrp3 inflammasome activation, following tissue damage and the release of DAMPs like ATP (Iyer et al., 2009). In the mdx mouse, IL-1β is upregulated compared to control animals (Huang et al., 2009) while in another sterile inflammation study, monocyte infiltrations were reduced in IL-1β KO animals (Kono et al., 2012). In our study, P2X7 receptor ablation reducing the level of active IL-1β in the mdx mouse is consistent with the reductions in total macrophage numbers seen here.
4.3.3 Effect of P2X7 receptor ablation on inflammatory genes expression in mdx muscle

To measure key pro-inflammatory genes, which might be transcriptionally regulated upon P2X7 disruption, a qPCR study to compare relative mRNA levels in TA from 4 week old C57, mdx, Glaxo and Pfizer mdx/P2X7-/- animals was undertaken.

There was a general trend of decreased expression of pro-inflammatory genes noticeable even with a low sample number and several genes were differentially regulated across genotypes. It should be noted that the low number of biological replicates allows identification of major differences by qPCR analyses while small but still biologically significant differences may not be detected. Therefore, further qPCR analyses are being carried out in our laboratory to extend the preliminary findings of this study into both larger sample number and further inflammatory genes (see Future Studies). Of the differentially regulated genes TNFα, CX3CL1, RORC, and GR-1 (also known as Ly6G), were significantly lower in Pfizer mdx/P2X7-/- compared to mdx.

**TNFα**

Downregulation of this pro-inflammatory cytokine in the absence of P2X7 agrees with the known role of this receptor in TNF signalling.

TNFα has been strongly linked with the chronic inflammatory component of DMD pathology (Lundberg et al., 1995; Grounds & Torrisi, 2004; Hodgetts et al., 2006; Pierno et al., 2007). In an mRNA study of human muscle biopsies it was shown that levels of TNFα were higher in DMD patients than in another inflammatory myopathy, juvenile dermatomyositis (De Pasquale et al., 2012). Additionally, an anti-TNFα treatment in the mdx mouse resulted in a substantial reduction in muscle breakdown (Grounds & Torrisi, 2004). Furthermore, TNFα was inversely correlated with the antioxidant heme-oxygenase-1 expression in a myoblast cell culture model, showing the degenerative effects of oxidative stress in skeletal muscle (Vercherat et al., 2009).

**CX3CL1**

The chemokine CX3CL1 is expressed in a membrane bound form on a variety of tissue infiltrating monocytes where it has been linked to the trafficking of these cells and their interactions with cytotoxic lymphocytes in muscle-specific chronic
inflammatory disorders (Suzuki et al., 2005; 2012). Lymphocytes, including NK and CD8+ T-cells express CX3CR1, the native receptor for CX3CL1. In inflammatory conditions, it has been hypothesised that CX3CL1 could act as a major migration and recruitment factor (Nishimura et al., 2002). In patients with polymyositis, a muscle-specific chronic inflammatory disorder, levels of serum CX3CL1 were shown to positively correlate with disease activity and with reduced muscle strength (Suzuki et al., 2012). Similarly, CX3CL1 depletion in mice was shown to reduce several disease markers of an experimental autoimmune myositis, including TNFα, IFNγ, perforin levels, and the numbers of necrotic muscle fibres (Suzuki et al., 2005).

**RORC**

The orphan nuclear receptor RORγt, encoded by the gene RORC, is expressed in CD4+CD8+ cells where it is thought to induce their differentiation into the pro-inflammatory Th17 type, also downstream of IL-1β (Ivanov et al., 2006; Acosta-Rodriguez et al., 2007). RORC was upregulated along with numbers of IL-17 positive cells in a mouse model of experimental autoimmune encephalomyelitis, also coinciding with the induction by myeloid-derived suppressor cells and of Th17 from naïve CD4+ T-cells (Yi et al., 2012). This is another instance in which IL-1 may be inducing pro-inflammatory conditions, at least partly via the upregulation of a CD11b+/Gr-1+ myeloid cell population.

**Gr-1 (Ly6G)**

Granulocyte receptor-1 is expressed in bone marrow-derived granulocytes and tissue-infiltrating neutrophils. Neutrophils are an important cytotoxic cell type contributing to ROS generation and muscle degeneration in dystrophic tissue (Hodgetts et al., 2006). It was noted that in macrophages exposed to ATP released from dying autophagic cells there was activation of the NACHT, LRR, and PYD domains-containing protein 3 (NALP3) inflammasome, ultimately contributing to tissue recruitment of neutrophils (Ayna et al., 2012).

Many of the other gene expression levels reflected the shift to a lesser inflammatory state upon P2X7 receptor ablation. These included IFNG, encoding IFNγ, involved in a Th1 T-cell induction profile and the activation of macrophages (Villalta et al., 2011a).
PRF-1 is the gene encoding the enzyme, perforin-1 in cytotoxic T-cells (Trapani & Smyth, 2002). Perforin and granzyme-B are major enzymes effecting the apoptotic function of cytotoxic lymphocytes. COX-2 is responsible for the eicosanoid inflammatory signalling. Knock-out of this gene reduced macrophage invasion into injured muscle and also lowered myoblast proliferation (Bondesen et al., 2004) and recent evidence has localised COX-2 expression to necrotic and fibrotic lesions in mdx muscle (de Oliveira et al., 2013a). The chemokine XCL1 has been shown to enhance the regulatory function of CD4^+CD25^High T-reg cells in allergic asthma (Nguyen et al., 2008). The increase in the mRNA expression profile for the XCL1 gene in Pfizer mdx/P2X7−/− TA compared to mdx is therefore consistent with the similarly patterned expression of Foxp3. Foxp3 is a transcription factor involved in the specification of immunosuppressive T-cells (Hori et al., 2003). IL-6 was shown to potentiate the Th17 pro-inflammatory cell population through the negative regulation of T-reg Foxp3 via P2X7 receptor (Schenk et al., 2011). Th17 cells are a common feature of the autoimmune inflammatory milieu and are also found upregulated in DMD (De Pasquale et al., 2012; Marwaha et al., 2012). As mentioned previously, RORγt (encoded by RORC), promotes the induction of the Th17 T-cell population while Foxp3 can directly inhibit RORγt target transcription and subsequent Th17 induction (Zhou et al., 2008). CXCL12 is a small protein involved in chemotaxis and a pro-inflammatory cytokine cascade. From even a pre-symptomatic phase of DMD, several CC- and CXC-class chemokines, including CXCL12, have been found to be upregulated in this disease (Pescatori et al., 2007). During sterile inflammation CXCL12 is thought to complex with high mobility group box 1 (HMGB1), released from damaged and necrotic cells, to interact with its native receptor, CXCR4, expressed on a variety of leukocytes (Venereau et al., 2012). In injured muscle, this CXCL12-HMGB1 heterocomplex was found to promote inflammatory cell recruitment (Schiraldi et al., 2012).

P2X7 activation has also been linked to pro-inflammatory T-cell activation (Tsukimoto et al., 2009) and the negative selection of tolerogenic CD25^+ T-regulatory cells in vivo (Daniel et al., 2010). Importantly, there is evidence of autoimmune disease features in DMD and mdx muscles (Karpati et al., 1988b; Tidball & Wehling-Henricks, 2005). Pharmacological inhibition of P2X7 has limited autoimmune T-cell activation and proliferation (Lang et al., 2010) and P2X7-mediated T-regulatory cell
selection in a heart muscle rejection model has recently been shown (Vergani et al., 2013). It is, therefore, interesting to consider whether P2X7 blockade could have similar beneficial effect in mdx muscle.

4.3.4 Neutrophils and the P2X7 receptor

Altered GR-1-gene expression indicated reduced neutrophil infiltrations, which are known to contribute to the dystrophic muscle pathology, into mdx/P2X7−/− muscle compared to mdx muscle. These cells induce muscle cell lysis but, unlike macrophages, are not thought to aid in regenerative processes (Pizza et al., 2005). It is, therefore, unsurprising that muscle necrosis was reduced upon depletion of neutrophils in the mdx mouse (Hodgetts et al., 2006). Decreased GR-1/Ly6G expression in mdx/P2X7−/− mice could contribute to the alleviation of disease features described here. Consistent with these data, neutrophil numbers were also reduced in bronchoalveolar lavage samples from smoke-challenged P2X7−/− KO mice compared to wild-type controls (Eltom et al., 2011).

Mechanisms connecting P2X7 receptor ablation to reduced neutrophil numbers may include reduced macrophage to neutrophil co-operation, lower levels of active IL-1β and TNFα, and reduced neutrophil lifespan due to a lower resistance to apoptosis. Furthermore, ATP-induced macrophage ROS production can trigger macrophage inflammatory protein-2 synthesis, leading to neutrophil migration (Kawamura et al., 2012). IL-1β is also important for recruitment of neutrophils into inflamed tissues, including in sterile inflammation (Netea et al., 2010; Kono et al., 2012), and IL-1β is a known downstream product of P2X7 receptor activation. Therefore, P2X7 ablation would have a potential to modify neutrophil as well as macrophage functions. Macrophages and neutrophils together are more efficient at cell lysis than either cell population alone by using a NO-dependent, superoxide-independent, mechanisms (Nguyen & Tidball, 2003a). Therefore, due to this interdependency, treatment strategies that can reduce either neutrophil or cytotoxic macrophage numbers may be beneficial for the amelioration of DMD progression.

Immunolocalisation analysis presented here suggests that P2X7 expression could co-localise with neutrophils, which agrees with some previous data (Christenson et al.,
2008). Furthermore, in rheumatoid arthritis, P2X7 stimulation has been shown to impart an anti-apoptotic property to neutrophils (Christenson et al., 2008). In another study, receptor expression could not be confirmed in human neutrophils using a variety of experimental approaches, including RT-PCR, Western blotting, immunostaining, and patch-clamp electrophysiology (Martel-Gallegos et al., 2010). Therefore, it is unclear whether P2X7 receptor ablation could impact neutrophil functions directly.

Because the presence of one immune cell type can influence the proliferation and activation of another, it is difficult to predict, but also important to know, the precise effect of P2X7 ablation on the mdx inflammatory cascade.

The dual effects of low (tonic) and high (toxic) P2X7 receptor activation may also be important for inflammatory cells. Amoroso et al. (2012) showed a link between P2X7 function and the induction of aerobic glycolysis in fast-growing cells, also linked to hypoxia inducible factor 1α. Immune cells would directly benefit from the alternative energy source provided under conditions of acute hypoxia in areas of tissue damage.

### 4.3.5 Effect of P2X7 receptor ablation on inflammation and fibrosis in aged mdx diaphragm and heart

Further studies were undertaken to confirm the positive effects of P2X7 ablation on dystrophic muscle and to exclude the possibility that a skewed macrophage population might have a negative impact on DMD pathology through enhanced fibrosis. The data presented earlier as well as results showing reduced myofibroblast number and collagen deposition in the absence of P2X7 in a mouse model for tubulointerstitial disease (Goncalves et al., 2006) were encouraging. This effect was coincident with a reduction in the pro-fibrotic cytokine TGFβ. TGFβ and collagen type-I deposition were significantly attenuated in mdx mice diaphragms in which TNFα was blocked (Gosselin & Martinez, 2004). Since we have evidence of a reduction in TNFα gene expression in the mdx/P2X7−/− mouse at 4 weeks, it seems plausible that TGFβ and collagen expression may be limited via this same pathway.

While TGFβ analysis was not undertaken as part of this study, we could otherwise confirm that reducing P2X7 activation did not exacerbate fibrosis in either aged ~20
month \textit{mdx} diaphragms or hearts (\textbf{Figures 4.12b, 4.13a}). In fact it reduced fibrosis in \textit{mdx}/P2X7\textsuperscript{-/-} compared to \textit{mdx} aged heart tissue (\textbf{Figure 4.13a}). Furthermore, inflammatory cell infiltrations were reduced in both diaphragms (\textbf{Figure 4.12a}) and hearts (\textbf{Figure 4.13b}) from \textit{mdx}/P2X7\textsuperscript{-/-} mice, consistent with results for the 4 week hindlimb muscles presented earlier. These data are also consistent with published findings indicating the positive correlation between general immune-cell infiltrations and cardio-fibrosis in the adult \textit{mdx} mouse. Fibrosis was significantly reduced in hearts from 10-12 month \textit{mdx} mice in which the adapter molecule myeloid differentiation primary response gene 88 (myd88) was knocked-out (Henriques-Pons et al., 2013). Myd88 expression is positively correlated with macrophages and is involved in their full-scale activation in response to cytokines like IFN-\textgamma (Shi et al., 2003). In another study with a direct relevance for P2X receptor mediation of this response, the pan P2 receptor antagonist, suramin, was used in 11 month old \textit{mdx} mice to reduce cardiac fibrosis concomitant with reduced inflammatory cell infiltrations (de Oliveira Moreira et al., 2013b). In another similar study, but in 6 month \textit{mdx} mice, suramin reduced serum CK and fibrosis in TA, biceps brachii, and diaphragm muscles but not heart, following a daily 1 hour down-hill exercise regime, for a period of 7 weeks (Taniguti et al., 2011).

\subsection{Conclusions and further studies}

Corroborating published observations, we have shown that P2X7 expression is co-localised with a variety of immune cell sub-types in dystrophic mouse muscle \textit{in vivo}. Furthermore, the loss of P2X7 receptors resulted in the reduction of pro-inflammatory macrophages and neutrophils, coincident with a reduction of expression of pro-inflammatory genes.

This study therefore represents the first known analysis of an effect of P2X7 ablation on chronic inflammation in skeletal muscle. Outside of skeletal muscle, this association has already been established for brain (Sharp et al., 2008; Chu et al., 2012; Kimbler et al., 2012), alimentary tract (Schenk et al., 2011), kidney (Goncalves et al., 2006), salivary gland (Woods et al., 2012), and heart (Vergani et al., 2013).

Macrophages make up the majority of infiltrating immune cells in dystrophic muscle such that their modulation has great potential to influence disease parameters. Another
myeloid cell type recruited to muscle during early stages of inflammation and collaborating with macrophages to effect tissue damage are neutrophils. Neutrophil numbers were also significantly decreased in mdx/P2X7−/− muscles and this effect can be a direct as well as indirect one. However, the P2X7 receptor has been linked with many more aspects of innate and adaptive immune response than we have tried to monitor. For instance, based on the known function of the P2X7 receptor in T-cells, a proper analysis of lymphocyte populations is required.

Also interesting in this regard, is work on P2X7 in dendritic cells, where receptor activation resulted in dendritic cells with lower potential to activate T-helper cells through antigen presentation (Mutini et al., 1999). Therefore, quantification of T-cell subtypes is lacking. Attempts were made to determine CD4 and CD8 numbers by immunohistochemistry and flow-cytometry but were unsuccessful due to low antibody specificity.
5

Functional analysis of \textit{mdx} and \textit{mdx/P2X7\textsuperscript{-/-}} dKO mice

5.1 \textit{Introduction}

DMD is characterised by a loss of muscle integrity, leading to reduced muscle force generation. Other major symptoms include a mild to severe cognitive impairment in many patients and an osteopenic, bone resorption, phenotype. The primary aetiology that leads to impaired learning capacity (Hinton \textit{et al.}, 2004) may be contributing to cognitive impairment but it is now known that the loss of dystrophin may lead to neuronal and blood brain barrier dysfunction directly, due to the specific roles for dystrophin and the associated DAPC in the CNS (Man \textit{et al.}, 1991; Gorecki \textit{et al.}, 1992; Austin \textit{et al.}, 2000; Aleman \textit{et al.}, 2001; Lien \textit{et al.}, 2012). Osteopenia is a far less studied feature of DMD despite a high penetrance and the severe clinical implications for patients.

Although the loss of P2X7 receptor activation in the \textit{mdx} mouse has so far seemed to ameliorate disease pathology through anti-inflammatory and pro-myogenic effects, functional pre-clinical measures are required to assess the effective outcomes of molecular pathway changes on some of the major symptoms of DMD.

Sarcolemmal breakdown leading to muscle fibre degeneration and the enhanced release of immune-stimulating cytoplasmic components is a primary dysfunction downstream of dystrophin absence. The DAPC effectively mechanically stabilises the sarcolemma by coupling the intracellular cytoskeleton to extracellular matrix components, such that the loss of dystrophin function will directly compromise myofibre viability (Petrof \textit{et al.}, 1993; Dudley \textit{et al.}, 2006). Second messenger led
pathways, including proteolytic and apoptotic pathways downstream of elevated intracellular Ca^{2+}, are now also considered integral to membrane destabilisation.

Weakening of muscle force is a primary symptom of DMD. It is a factor representing the convergence of multiple cellular and molecular dysfunctions inherent to the disease, including the exaggerated degeneration and leakiness of muscle fibres.

5.1.1 Muscle force in DMD and mdx

Muscle force production is a single functional output measure of organism health, directly relevant as a clinical therapeutic measure of treatment efficacy in muscular dystrophy studies. The reduction of muscle force in DMD patients is mainly related to the loss of muscle fibres over time. Muscle fibres reduce in number and are replaced by fatty-fibrous deposits, resulting in few capable force-producing elements and a tissue ultrastructure less optimal at translating or sustaining that force. In the \textit{mdx} mouse, total muscle force is remarkably well maintained across the life span of the animal due to very successful compensatory regeneration. When muscle force is adjusted relative to muscle size, it has been established that there is a ‘specific’ muscle-force deficit in different muscle groups (Lynch \textit{et al.}, 2001; Goldstein & McNally, 2010). For example, WT extensor digitorum longus muscle showed a recorded maximum specific isometric tetanic force of \approx 250 \text{kN/m}^2 compared to \approx 220 \text{kN/m}^2 for \textit{mdx} at 6 months, with the soleus muscle also presenting similar values (Lynch \textit{et al.}, 2001). In other studies, the C57 diaphragm presented a maximum specific isometric tetanic force of \approx 195 \text{kN/m}^2 compared to \approx 150 \text{kN/m}^2 for \textit{mdx} at 6 months (Schertzer \textit{et al.}, 2007; Gehrig \textit{et al.}, 2012). Typical maximum specific force values for isolated diaphragm have not been published.

Another feature of dystrophic muscle is a susceptibility to stretch-induced damage. In \textit{mdx} mouse studies, this feature of the disease can be tested and compared using an eccentric contraction assay. Following the stretching of muscle past a maximum tetanic length, subsequent excitation-force traces can be mapped, usually resulting in more pronounced healthy versus diseased tetanic force differences, \textit{in vivo} or \textit{in vitro}, as the dystrophic muscle characteristically worsens over repeated eccentric stretches (e.g. Blaauw \textit{et al.}, 2008). Eccentric contraction assays are particularly useful when
testing therapies hypothesised to improve the mechanical stability of the sarcolemma, for instance following pharmacological or genetic approaches to reinstate functional dystrophin (Gilbert et al., 2003). Their sensitivity to intermediate differences in muscle function along a continuum of disease severity means that eccentric contraction assays are useful to map functional improvements following most treatment strategies.

In addition to structural protein factors, the specific force deficit of the \textit{mdx} mouse, derived through either tetanic or eccentric force experimentation, also attests to a fundamental metabolic aspect of disease biology, which is also likely shared by human DMD patients. In addition to the loss of muscle fibres, several cellular-biochemical mechanisms can contribute to reduced muscle force production in DMD. These include an altered energy handling capacity, a shift in muscle oxidative phenotype, an increase in ROS/RNS generation, and a systematic change in neuromuscular innervation. All of these phenomena could involve purinoceptor signal transmission via P2X7, as shall be discussed below.

\textit{Energy handling and fibre-type shifting in muscular dystrophy}

Skeletal muscle is an important site of glucose metabolism and glycogen storage. In DMD, skeletal muscle has been shown to contain significantly less glucose than in non-dystrophic subjects (Sharma et al., 2003). This seems to indicate a fundamental energy handling deficit in DMD that could affect muscle function. Muscle metabolic potential is also reflected in oxidative capacity or muscle fibre-type, a parameter also known to be compromised in DMD.

Using an exercise mimetic compound, AICAR, to exogenously activate the ATP:AMP cellular sensor, AMPK, in the \textit{mdx} mouse has been shown to shift myofibres to the type I oxidative phenotype, more resilient to dystrophic challenge (Jahnke et al., 2012). Part of this effect was mediated by a reduction in Foxo1 transcription. Inversely, it has been shown that Foxo1 over-expression resulted in fewer type I fibres and smaller type I and II fibres (Kamei et al., 2004). If P2X7 is contributing to autophagic induction in DMD muscle, this could result in reduced muscle force potential and may also help to explain the earlier defined increased
average minimum Feret’s diameter values for the mdx/P2X7−/− dKO hindlimb muscle compared to mdx.

Larger muscle fibres tend to produce greater force, but this effect is also normally correlated with muscle fibre-type. Fast-glycolytic muscle fibres tend to be larger and to produce greater maximum force. For instance, mdx mice treated long-term with an aptamer inhibitor for all IGF-binding proteins, NBI-31772, resulted in larger muscle fibres capable of producing more specific force, less susceptible to contraction induced sarcolemmal breakdown, and shifted to a fast-glycolytic, type-IIb, fibre-type (Schertzer et al., 2007).

**Increased ROS/RNS production**

Persistently increased intracellular Ca\(^{2+}\) causes muscle damage and muscle force reduction via activation of Ca\(^{2+}\)-dependent calpain proteases. Recent work suggests that this may be heavily influenced by an accompanying increase in ryanodine receptor S-nitrosylation affecting sarcoplasmic reticulum Ca\(^{2+}\) handling (Bellinger et al., 2008a; 2008b; 2009). Others have also reported a decrease in specific muscle force production upon increased ROS/RNS generation (Li et al., 2011b).

The origin of these enhanced levels of detrimental ROS/RNS is multi-focal. Recent evidence suggests that above normal iNOS expression, associated, for instance, with increased immune cell infiltration, may not represent a major cause for reduced muscle force in DMD, or even the majority of nitrotyrosination in dystrophic muscle (Li et al., 2011a). Instead, the intracellular location of native nitrogen and oxygen donors may be more important than their total absence or presence. At the cell membrane, NO donated by nNOS is thought essential for promoting satellite cell activation and fusion but when delocalised it may contribute to overall nitrosative stress leading to nitrosylative protein modifications and the loss of muscle force (Eu et al., 2003; Bellinger et al., 2008a; 2008b; 2009).

A link to enhanced cellular ROS/RNS with P2X7 over-activation in muscle is not immediately obvious but has been described for at least two forms of P2X7-mediated neuronal cell death (Nishida et al., 2012; Apolloni et al., 2013). In both cases, P2X7 activation was described as mediating increased ROS generation via activation of
NADPH oxidase. Moreover, recent work has also identified the P2X7 receptor as responsible for ROS generation in an erythroid cell line (Wang & Sluyter, 2013). That P2X7-dependent ROS generation in these cell types is shared with macrophages (Martel-Gallegos et al., 2013) perhaps indicates the ubiquity of this cellular cascade.

Neuromuscular synaptic transmission

P2X receptors, including P2X7, have been shown to be linked to ACh co-release at synapses (Sun & Stanley, 1996; Hong & Chang, 1998; Salgado et al., 2000) and this is a means by which the receptor may affect muscle force generation. It has also been shown that this co-release may be a mechanism by which inactive heterosynaptic inputs may be protected from an imposed loss of synaptic efficacy (Jia et al., 2007). In this latter study, P2 receptors were considered the likely ATP targets effecting this protection since the P2 blocker, suramin, promoted substantial activity-dependent neuromuscular synaptic losses. Of particular note, 100 μM ATP, instead of protecting synapses, reduced synapse strength (Jia et al., 2007). This activation level threshold behaviour is characteristic of the P2X7 receptor and may be related to the multiple open states this receptor is known to possess, including the large pore formation (Rassendren et al., 1997; Browne et al., 2013).

5.1.2 Dystrophic bone phenotype

Given the clear musculoskeletal interactions, bone structure abnormalities found in DMD patients and mdx mice were believed to have muscle origins. However, rather than being an effect of asymmetric loss of muscle force alone, these abnormalities have recently been found to be independent disease manifestations.

Bone is a plastic tissue, able to adapt its structure in reaction to age-related biological changes (Hamrick et al., 2006), metabolic disorders, and animal immobility (Kaneps et al., 1997). In DMD patients, long-term steroid therapy may contribute to the loss of bone mineral density (Bianchi et al., 2003; Mayo et al., 2012).

Muscle weakness can influence bone mineralisation by reducing shear stress forces necessary to activate osteoblasts (Hillam & Skerry, 1995; Yan et al., 2012), although
it is likely that other intrinsic factors, different in dystrophic compared to healthy organisms, also greatly contribute to osteopenia in the disease state. For instance, Nakagaki et al. (2011) found a characteristic bone phenotype in the femur of 3 week old mdx mice without evidence of accompanying quadriceps pathology. Similarly, osteoblasts from healthy humans did not mineralise bone after contact with serum from DMD patients, indicating the presence of inhibitory paracrine factors in DMD serum (Rufo et al., 2011).

Hormonal imbalance linked to chronically lower testosterone levels and hypogonadism are thought to heavily propagate lower bone mineral density in DMD patients (Cruz Guzman Odel et al., 2012). Based on available literature evidence, it is also likely that inflammatory cytokines significantly influence the osteopenic phenotype of DMD patients. For instance, IL-6 was found to enhance osteoclastogenesis and to be increased in DMD and mdx serum. In the Pfizer P2X7\(^{-/-}\) KO mouse, IL-6 was one cytokine initially found to have a more limited expression compared to wild-type controls (Solle et al., 2001).

However, perhaps the best reason for studying the effects of P2X7 ablation on the osteopenic phenotype of the mdx mouse is to determine if there is a compound exacerbation in bone resorption. P2X7 function has been previously positively linked to activity-dependent bone formation, whilst loss-of-function polymorphisms have been linked to increased fracture risk in humans and bones of lower density in mice (Gartland et al., 2003; Ke et al., 2003; Orriss et al., 2010; Syberg et al., 2012; Wesselius et al., 2013). Therefore, P2X7 gene disruption could affect mdx bone positively by changing the dystrophic phenotype but also negatively by impacting on bone resorption.

5.1.3 Chapter summary

The DMD pathology is multi-facetted. Humans and mice share many disease symptoms including a compromised sarcolemmal stability, reduced muscle force and endurance potential, as well as osteopenia.

To test the therapeutic potential of targeting P2X7 receptors to treat DMD, we have sought to test several basic parameters of muscular dystrophy pathology in the mdx
compared to the *mdx/P2X7^-/-* dKO mouse. To do so, we have followed established methods of pre-clinical study, including an analysis of the levels of serum CK across dystrophic genotypes at 4 weeks, a complimentary histological IgG study in GC muscle at 4 weeks, an *in vitro* diaphragm tissue-bath study measuring maximum isometric tetanic force at 4 weeks, and a μCT analysis of trabecular bone morphometry across 6 month *mdx, mdx/P2X7^-/-* dKO, and Pfizer KO mice. μCT samples have been prepared by the candidate and analysis was performed by collaborators, Prof. Anna Teti and Dr. Andrea Del Fattore, University of L’Aquila, Italy.

Serum CK level is an indication of muscle protein extravasation following sarcolemmal compromisation. Serum CK is a standard clinical diagnostic marker for DMD, also used in mouse model studies (Hess *et al.*, 1964; Coulton *et al.*, 1988). Mouse IgG immunohistochemistry was used to determine sarcolemmal instability by measuring the levels of blood borne immunoglobulins that are normally excluded from the muscle cytosol. In this sense, IgG staining could be correlated with serum CK data, although the latter is a more global measure of membrane compromisation.

For a preliminary analysis, it was also decided to test the potential of using a drug-based P2X7 antagonist for therapeutic intervention. *Mdx* mice were injected i.p. with the P2X7 antagonist CBB (125mg/kg) or saline negative control every day from postnatal day 2 (P2) until collection at 4 weeks. Serum CK analysis was then performed.
5.2 Results

5.2.1 Serum CK comparisons between mdx and mdx/P2X7\textsuperscript{+/−} dKO mice

Mouse serum was collected and using a commercial enzymatic CK detection kit, it was confirmed that the mdx mouse had a much higher level of serum CK compared to the non-dystrophic C57 at 4 weeks of age. The C57 and Pfizer mdx/P2X7\textsuperscript{+/−} dKO mouse had a significantly lower average serum CK level than age-matched mdx (Figure 5.1). There was a large spread in the data about the mean for each dystrophic group, necessitating large ‘n’ numbers before statistical significance could be attained. The Pfizer mdx/P2X7\textsuperscript{+/−} dKO mouse presented an average serum CK, 796.9 IU/L, almost half that of mdx, 1461.6 IU/L. The spread of individual scores about the mean was also greater for mdx (StDev=848) than for Glaxo dKO (StDev=591) or Pfizer dKO (StDev=561) mice. Glaxo mdx/P2X7\textsuperscript{+/−} dKO mouse serum CK was qualitatively intermediate but not significantly different from either mdx or Pfizer mdx/P2X7\textsuperscript{+/−} dKO serum CK levels.

![Figure 5.1](image)

**Figure 5.1.** Serum CK levels are significantly lower in both C57 and Pfizer mdx/P2X7\textsuperscript{+/−} dKO serum relative to mdx at 4 weeks. Mouse serum CK levels showed significantly lower average values for C57 and Pfizer P2X7\textsuperscript{+/−} dKO compared to mdx at 4 weeks (ANOVA, \( F=6.94 \), \( P<0.001 \), \( df=3 \), Tukey post-hoc).
5.2.2 Muscle membrane permeability in mdx and mdx/P2X7\textsuperscript{-/-} dKO muscle

Serum CK analysis gives an indication of total muscle fibre damage. To assess individual fibre membrane damage within specific muscles a histological measure of fibre permeability was used to quantify and compare mdx and mdx/P2X7\textsuperscript{-/-} dKO GC muscles. As a corollary for serum CK a tracer was employed that can only enter muscle fibres when sarcolemma integrity is compromised.

An anti-mouse IgG antibody conjugated to a fluorophore was used to label blood-derived immunoglobulins in GC cryosections at 4 weeks. Based on a pixel density distribution of the immunolocalisation signal as a percentage of total cross-sectional area, significantly lower values were found in Pfizer mdx/P2X7\textsuperscript{-/-} dKO GC (1.47\% ±0.9) compared to mdx (6.34\% ±2.5) (Figure 5.2). Whilst not statistically significantly different from either genotype, Glaxo mdx/P2X7\textsuperscript{-/-} dKO GC muscle presented an intermediate average IgG percentage value (3.1\% ±2.08). Non-dystrophic controls did not present IgG infiltration (Figure 5.2a), so were not measured.

Using the IgG antibody, muscle fibres present one of two main forms of infiltration, differentiated by the levels of tracer intensity, either low or high.
Figure 5.2. Immunofluorescence analysis, showing absent infiltrating mouse IgG in C57 and a significantly reduced IgG in Pfizer mdx/P2X7−/− dKO GC muscle fibres compared to mdx at 4 weeks. a) Example whole 4 week GC muscle cryosections presenting mouse IgG Immunofluorescence (red: indicative of individual muscle fibre membrane being compromised) in C57, mdx, and Pfizer dKO mice. Note, insert showing higher and lower tracer intensity within a single cluster of permeable membranes. b) Graphical representation of average mouse IgG signal as a percentage of total muscle area for mdx, Glaxo dKO, and Pfizer dKO, showing a significant difference in mouse IgG positive signal between mdx and Pfizer mdx/P2X7−/− tissues (ANOVA, F=5.52, P=0.031, df=2).
5.2.3 **Analysis of isometric tetanic force in mdx and mdx/P2X7−/− dKO diaphragms in vitro**

Significantly reduced absolute muscle force is a hallmark of DMD; a symptom also shared with the *mdx* mouse (Lynch *et al.*, 2001; Goldstein & McNally, 2010). Using an *in vitro* diaphragm organ bath setup to measure isometric tetanic force evoked by electrical field stimulation, muscle force was compared between C57, *mdx*, Glaxo dKO, and Pfizer dKO mice at 4 weeks and 4 months.

A triangular section of excised muscle was taken from the mid-section of the whole diaphragm (Figure 5.3a). A voltage stimulus required to elicit a maximal isometric twitch (P$_t$) response (Figure 5.3b) was determined during diagnostic testing (data not shown), with the value of 140V being chosen as being well within the plateau range. A stimulus frequency of 100Hz was used to achieve a maximal isometric tetanic force (P$_o$) (Figure 5.3b). This value was based on published data and also confirmed during testing (data not shown). All tissue bath experiments were performed with the tissue in a Krebs buffer maintained at a physiological temp. of 37°C. After the stimulus-response recording the tested muscle was wet-weighed for normalisation per milligram of tissue and force in Newtons derived by multiplication by 0.0098.

At 4 weeks of age, average maximal isometric tetanic force per gram of tissue was significantly increased in the Pfizer *mdx/P2X7−/−* dKO diaphragm compared to *mdx* (Figure 5.3c). The mean value of 12.544 N/g force production for Pfizer dKO was intermediate between *mdx*, 5.1 N/g, and C57, at 32.34 N/g, and similar to that of the Glaxo dKO diaphragm.

At 4 months of age, average maximal isometric tetanic force per gram of tissue was significantly increased in both Glaxo and Pfizer P2X7−/− dKO diaphragms compared to *mdx* (Figure 5.3d). The mean values of 17.5 N/g and 23.4 N/g force production for Glaxo dKO and Pfizer dKO, respectively, were again intermediate between *mdx*, at 12.6 N/g, and C57, at 32.34 N/g.
Figure 5.3. Diaphragm tetanic muscle force is increased in *mdx*/*P2X7−/−* dKO muscle compared to *mdx* at 4 weeks and 4 months. Example experimental setup, stimulus-force trace, and graphical representation of *in vitro* diaphragm tissue bath data comparing average isometric tetanic force (N/g) between C57, *mdx*, Glaxo dKO, and Pfizer dKO at 4 weeks and 4 months. a) Image depicting a setup used for measuring diaphragm muscle force *in vitro*. The triangular muscle segment has been secured to the plastic clamp via a rib attachment maintained *in situ*. b) An example force trace indicating maximal isometric twitch tension (P<sub>t</sub>) and tetanic tension (P<sub>o</sub>), elicited by 140V stimuli applied singularly or in 100Hz trains, respectively. c) At 4 weeks, C57 presents a significantly greater average isometric tetanic force than *mdx* (ANOVA, F=7.79, P=0.002, df=3, Tukey post-hoc), with Pfizer dKO also presenting increased force compared to *mdx* via T-test (T=-2.4, df=8, P=0.043). d) At 4 months, C57 and both Glaxo dKO and Pfizer dKO diaphragms present greater average isometric tetanic force upon electrical field stimulation at 100Hz (ANOVA, F=102.55, P<0.001, df=3, Tukey post-hoc).
5.2.4 Bone histomorphometry comparisons in mdx and mdx/P2X7<sup>−/−</sup> dKO mice at 6 months

6 month old C57 BL/10, mdx, Pfizer mdx/P2X7<sup>−/−</sup> dKO, and Pfizer P2X7 KO mice had their femurs removed after death and were prepared for μCT analysis. Four parameters, described as some of the basic minimum recommendations for quantitatively assessing trabecular bone morphology (Bouxsein et al., 2010), were then derived from micro-CT data (Figure 5.4). The ratio of bone volume to trabecular volume (BV/TV, %) is a measure of bone density. Interestingly, BV/TV was significantly higher in C57, Pfizer mdx/P2X7<sup>−/−</sup> dKO, and Pfizer P2X7 KO mice compared to mdx. Furthermore, the Pfizer mdx/P2X7 dKO mouse had a BV/TV % similar to C57 wild-type and Pfizer P2X7 KO animals. Trabecular thickness (Tb.Th) was again lower in mdx, although only Pfizer P2X7 KO presented a significantly higher average. Trabecular spacing/separation (Tb.Sp) is a measure of the distance between trabeculae. C57, Pfizer mdx/P2X7<sup>−/−</sup> dKO, and Pfizer P2X7 KO mice all presented lower average Tb.Sp compared to mdx. Trabecular number (Tb.N) is a measure of the number of trabeculae per unit of area or length. Mdx mice presented a lower average Tb.N than other genotypes, although only C57 and Pfizer P2X7 KO animals were significantly higher. Altogether, these measures indicate a rescued osteopenic phenotype in mdx/P2X7<sup>−/−</sup> dKO mice compared to mdx at 6 months.
Figure 5.4. Micro-CT bone histomorphometry comparisons, showing lower bone resorption in wild-type, P2X7−/− KO, and mdx/P2X7−/− dKO femurs compared to mdx at 6 months. Four parameters were compared between genotypes, including bone volume/total volume percentage (BV/TV %) (ANOVA, F=8.07, df=3, P=0.003, Tukey post-hoc); trabecular thickness (Tb.Th μm) (ANOVA, F=4.013, df=3, P=0.029, Tukey post-hoc); trabecular space (Tb.Sp μm) (ANOVA, F=49.59, df=3, P<0.001, Tukey post-hoc); and trabecular number (Tb.N/mm²) (ANOVA, F=6.7, df=3, P=0.006, Tukey post-hoc). All statistical comparisons are shown relative to mdx, with significantly different averages denoted by an asterisk.
5.2.5  **P2X7 receptor pharmacological antagonism study in mdx mice**

Serum CK levels did not significantly differ between CBB injected, saline injected, or non-injected *mdx* mice following a 4 week treatment, at the current N numbers (Figure 5.5). The average serum CK value for CBB injected *mdx*, 883 ±481.6 IU/L, was qualitatively lower than that presented by *mdx*, 1462 ±847.9 IU/L, and similar to Pfizer *mdx/P2X7-/-* dKO, 797 ±560.8 IU/L. Similarly, saline injected *mdx* mice presented an increased average serum CK, 1506 ±1014.2 IU/L, which was not significantly different from CBB injected *mdx* (Figure 5.5b). There was a large spread in the injected *mdx* individual data points that was qualitatively similar to non-injected mice (Figure 5.5a). Data from this CBB and saline treatment CK study was added to previous CK data for comparative purposes.
Figure 5.5. Preliminary P2X7 receptor antagonist study, showing qualitatively, but not significantly, lower serum CK levels in CBB injected compared to mdx and mdx saline injected mice at 4 weeks of age. a) Analysis of mouse serum CK, comparing 4 week mdx with C57 BL/10, both mdx/P2X7<sup>−/−</sup> dKOs, and Coomassie Brilliant Blue (CBB) injected mdx. Only C57 BL/10 and Pfizer dKO mice presented serum CK that was significantly different from mdx (ANOVA, F=5.99, P<0.001, df=4, Tukey post-hoc). Also note, saline treated mdx were added to the mdx group for this analysis. Individual value points for serum CK (red spots). b) Serum CK from 4 week CBB and saline injected mdx did not significantly differ (T-test, T=-1.41, P=0.208, df=6).
5.3 Discussion

5.3.1 Sarcolemmal permeability differences between mdx and mdx/P2X7^/- dKO mice

Lower average serum CK in Pfizer mdx/P2X7^/- dKO compared to mdx mice indicates reduced total muscle pathology related to P2X7 ablation (Figure 5.1). The fact that the Glaxo mdx/P2X7^/- dKO mouse did not present a significantly reduced serum CK at 4 weeks, perhaps reflects the hypomorph (incomplete P2X7 KO) status of this mouse (Nicke et al., 2009). This is also interesting as an explanation for the repeated qualitatively intermediate outcome for this genotype in multiple measures of disease pathology. As was the case for multiple inflammatory transcripts (see Chapter 4), the Glaxo mdx/P2X7^/- dKO mouse present an average serum CK intermediate between that for the Pfizer mdx/P2X7^/- dKO and the mdx mouse.

Average serum CK levels were similar between mice at 4 weeks and 4 months (not shown). This observation is inconsistent with there being less muscle inflammation and degeneration at 4 months than at 4 weeks. However, CK is a metabolic enzyme and it should be expected that overall levels would vary as a function of age and size of muscle. Therefore, the similar levels of serum CK from 4 month old animals compared to 4 weeks, perhaps reflects the larger sizes of animals at 4 months compared to 4 weeks. In one recent example of an analysis of mdx serum CK, levels were actually higher in 6 month animals compared to 4 week (Baltgalvis et al., 2011).

Also, the dystrophic animals in this study were not exercised; a method often used to exacerbate disease pathology for pre-clinical therapeutic testing (Spurney et al., 2009; Kobayashi et al., 2012; Willmann et al., 2012). Without exercising or challenging the muscle, natural conservative behaviours may be adjusting to limit disease expressivity, or metabolic and molecular disease pathways may be otherwise limited by natural controls. These compensatory factors may be beneficial for the well-being of affected animals but may also mask the sometimes subtle effects of altering an experimental variable, such as the removal of P2X7 receptor function, especially in vivo. The average CK values reported here for 4 week old mdx mice, 1461.6 ±848IU/L, is much less than published values for exercised mdx mice (Kobayashi et al., 2012) but similar to that shown in un-challenged animals, for example 1148±477 IU/L in 8 week old mice (Sacco et al., 2010).
Mouse-IgG

Levels of GC fibre permeability using the anti-mouse IgG antibody, complemented the serum CK data by showing significantly less IgG signal in Pfizer mdx/P2X7<sup>−/−</sup> dKO myotubes compared to mdx (Figure 5.2). Also paralleling the findings of the serum CK assay, Glaxo mdx/P2X7<sup>−/−</sup> dKO mice again presented a disease marker severity qualitatively greater than that for the Pfizer mdx/P2X7<sup>−/−</sup> dKO mouse and less than mdx. In the IgG histological assay of fibre permeablisation it should also be noted that muscle fibres positive for tracer infiltration presented either a strong or a weak cytoplasmic staining. This experimental effect has been noted elsewhere (Straub et al., 1997; Wooddell et al., 2010), although the biological cause is as yet not fully understood.

A total understanding of the biological consequences of sarcolemmal IgG permeability is difficult to ascertain from current literature but the association with muscle damage and a subsequent positive correlation with dystrophinopathy disease severity seems obvious from seminal papers (Straub et al., 1997; Wehling et al., 2001; Sacco et al., 2010; Wooddell et al., 2010). However, it should also be mentioned that a compromised or damaged sarcolemma does not always result in the loss of that cell. Muscle from healthy or dystrophic subjects can become damaged before a process of repair (for a recent discussions see Han and Campbell, 2007; Swaggart et al., 2014). Furthermore, in one correlative study in mdx mice, the authors could not establish a clear histological correlation between the dye, Evans blue, and H&E evident features, like necrosis (Straub et al., 1997). Whilst necrotic fibres always stained positively for Evans blue, dye positive skeletal muscle fibres sometimes co-localised with morphologically normal fibres in the corresponding H&E stain.

5.3.2 Specific diaphragm force differences between mdx and mdx/P2X7<sup>−/−</sup> dKO mice

Muscle force generation is mediated by multiple cellular and biochemical factors that are differentially regulated in the DMD disease state. Upon P2X7 ablation, we have shown an increase in diaphragm muscle specific force at 4 weeks, using a non-multivariate analysis, and at 4 months, by standard ANOVA (Figure 5.3). Although the role of iNOS in reducing muscle force generation in the mdx mouse has been
contested (Li et al., 2011a), inflammatory infiltrate probably still influences force production. For instance, neutrophils are uniquely capable of rapidly releasing superoxide, which is cytotoxic (Toumi et al., 2006) and able to reduce muscle force production (Li et al., 2011b). In addition to this, we have previously also shown that \textit{mdx/P2X7\textsuperscript{-/-} dKO} mice have an increased centrally nucleated fibre size (Figure 3.11) and reduced sarcolemmal fragility (Figure 5.1; 5.2), which are obvious other factors that could contribute to an increased tetanic force production in \textit{mdx/P2X7\textsuperscript{-/-} dKO} mice compared to \textit{mdx}. In the diaphragm an attempt was made to normalise muscle force to muscle mass between genotypes but muscle fibre size was not quantitatively appraised for comparison. A standard approach for inferring diaphragm cross-sectional area requires applying the calculation \(\text{CSA}(\text{cm}^2) = \frac{\text{diaphragm wet mass (g)} \times \text{fibre length (cm)} \times 1.056 (\text{g/cm}^3)}{\text{1.056 g.cm}^{-3}}\), where \(1.056 \text{ g.cm}^{-3}\) represents the constant for a pre-determined average muscle density (see Staib et al., 2002). However, muscle fibre density may not be uniform along the length of a single diaphragm, especially in a DMD disease state. Another important consideration is that maximum specific force varies as a function of myosin heavy chain content in even the diaphragm, with slow-type muscle fibres producing less force than the fast-type (Geiger et al., 2000). Considering that fibre-type can change as a function of disease severity, it may be possible that normalising muscle force to diaphragm cross-sectional area or weight would not adjust relative to this important factor. Despite this, it would still be expected that greater force producing muscle should be deemed healthier, even as a function of preserved fast-type fibre integrity. Finally, the normal approach for inferring diaphragm cross-sectional area uses a constant density-value \((1.056 \text{ g/cm}^3)\) that has been shown to change as a function of aging processes, contributing to sarcopenia (Greising et al., 2013). P2X7 receptors may be involved in aging related cellular and molecular events and DMD otherwise involves similar forms of muscle atrophy, usually defined in terms of cachexia.

In other studies, it has been shown that genetic ablation of TNF\(\alpha\) production significantly improved maximal isometric diaphragm force in the \textit{mdx} mouse (Gosselin et al., 2003) and TNF\(\alpha\) application reduced diaphragm force due to the direct blunting of myofilament Ca\(^{2+}\) activation (Reid et al., 2002). These results are consistent with the increase in isometric tetanic force presented for the \textit{mdx/P2X7\textsuperscript{-/-} dKO} diaphragm compared to \textit{mdx} (Figure 5.3), since we have also confirmed a
reduction in TNFα mRNA expression in 4 week TA muscle (Figure 4.9). It should be noted, however, that this comparison has been made between diaphragm force and hindlimb cytokine expression and thus is indirect. Nevertheless, all other parameters were constant in leg and diaphragm muscles and therefore it is justified on the basis of shared purinergic utilisation and basic musculoskeletal biology.

It was mentioned in the general introduction (1.6) that the Glaxo and Pfizer P2X7−/− KO mice are similar, for instance related to absent mature IL-1β induction in macrophages following LPS-priming (Solle et al., 2001; Chessell et al., 2005) but they also differ, for instance related to the differing bone phenotypes observed in either strain (Ke et al., 2003; Gartland et al., 2003b). It is known that the Glaxo P2X7−/− KO mouse possesses a gain-of-function splice variant, the P2X7 ‘K’ variant, that may be contributing to identifiable functional differences. However, it is prematurely speculative to expect an exaggerated disease severity in dystrophic mice carrying the gain-of-function ‘K’ variant of P2X7, even if the normal full-length purinoceptor can be linked to disease pathogenesis or progression. In this study, the Glaxo mdx/P2X7−/− dKO mouse also presents a qualitative increase in diaphragm tetanic force at 4 weeks (Figure 5.3a) and a significantly greater tetanic force at 4 month (Figure 5.3b) compared to mdx, and this may be consistent with other improved disease features, such as elevated myogenin protein levels (Figure 3.12) or reduced total macrophage infiltration (Figure 4.8) and immune marker mRNA (Figure 4.9) in 4 week hindlimb muscle. We have not otherwise attempted a systematic analysis of P2X7 K-variant influence. The wider significance of the qualitative pattern of intermediate Glaxo to Pfizer mdx/P2X7−/− disease marker presentation often repeated in this work shall be further mentioned in the general discussion section.

5.3.3 Bone remodelling differences between mdx and mdx/P2X7−/− dKO mice

Previous positive data, indicating a reduction in skeletal muscle inflammatory phenotype, a potential improvement in muscle regenerative potential, and the improvement in maximum isometric diaphragm muscle force generation coupled to a decreased muscle membrane permeability, may translate to an improved bone phenotype in the mdx/P2X7−/− dKO mouse compared to mdx. However, this must also be counter-balanced by a well-defined osteopenia phenotype associated with the
P2X7 KO mouse (Gartland et al., 2003; Ke et al., 2003; Orriss et al., 2010; Syberg et al., 2012; Wesseliuss et al., 2013). The P2X7 receptor is thought to be expressed on both osteoblasts and osteoclasts (for review see Orriss et al., 2010) but it has been described as having alternate functions in either cell population. In osteoblasts, the P2X7 receptor has been mainly described as promoting bone formation (Panupinthu et al., 2007; 2008), including a fluid-flow mediated ERK1/2 phosphorylation with subsequent osteogenic gene activation (Okumura et al., 2008). Additionally, one of these osteogenic genes was osteopontin (OPN), also a pro-inflammatory factor found to be chronically upregulated in DMD and linked with enhanced counter-productive fibrosis (Vetrone et al., 2009; Zanotti et al., 2011). In osteoclasts, the role of P2X7 is varied with a preference towards promoting the apoptosis of this cell type, effectively limiting bone resorption (Ohlendorff et al., 2007; Wesseliuss et al., 2013).

Neither of the predominant effects on osteoblasts or osteoclasts fit our data (Figure 5.4), except when we consider information, perhaps more relevant to complicated chronic diseases. In one study, it was found that P2X7 expressed by osteoclasts may also function to promote the formation of multinucleated osteoclasts, and that this may be inhibited by P2X7 receptor blockade (Gartland et al., 2003). Importantly, Gartland et al. (2003) showed that P2X7 activation had the potential to promote osteoclastogenesis in addition to the apoptosis of this cell type, as if helping to balance skeletal homeostasis. In healthy adult bone, osteoclasts are normally low in number. In the DMD disease state, in which skeletal homeostasis is already chronically perturbed by a host of factors, it is difficult to automatically assume that an over-expressed P2X7 receptor may still be predominately effecting bone formation over resorption, or that the removal of this receptor would not benefit bone turnover.

In DMD, many factors will disturb bone homeostasis, including long-term glucocorticoid use (Bianchi et al., 2003), reduced bone stress contributed by more limited musculoskeletal movements (Hillam & Skerry, 1995; Yan et al., 2012), and chronic skeletal muscle inflammation. Focusing on this last factor, it was recently shown that IL-6 was one of several candidate proteins that adversely affected bone parameters in the mdx mouse (Rufo et al., 2011). That we have earlier indicated a reduction in the inflammatory phenotype of mdx/P2X7−/− dKO muscle compared to mdx, including a potential reduction in type-M1 macrophages known to preferentially
release IL-6, creates the further possibility that bone osteopenia may be reduced in the dKO mouse by this mechanism.

Additionally important in terms of this study, is evidence that higher levels of free fatty acids have been found to promote P2X7 receptor expression via MAPK induction in PC12 neuroblastoma cells, leading to enhanced IL-6 release (Xu et al., 2013). Perhaps more relevant in bone formation, P2X7 receptor activation was also found to promote IL-6 release in bone-joint located synoviocytes in a model of rheumatoid arthritis (Caporali et al., 2008). Finally, in an original analysis of the Pfizer P2X7 KO mouse, receptor ablation was found to reduce total levels of circulating IL-6 (Solle et al., 2001).

Even if P2X7 ablation did rescue the mdx bone resorption phenotype, it is still difficult to explain our P2X7 KO data from animals without muscular dystrophy. Our data showed P2X7 KO mice to have BV/TV and Tb.N values very similar to C57 mice, indicating no osteopenic phenotype in P2X7 KO mice according to these parameters (Figure 5.5).

None-the-less, the current finding that P2X7 receptor ablation may also rescue the muscular dystrophy bone phenotype in addition to the aforementioned benefits to sarcolemmal stability, diaphragm muscle strength, reduced muscle inflammation, and an improved muscle regeneration potential, warrants further study because of the potential for P2X7 inhibitors to effect an holistic therapeutic intervention in DMD, acting on multiple aspects of disease pathology simultaneously.

5.3.4 The effects of pharmacological antagonist CBB on serum CK levels (preliminary study)

Administration of broad purinoceptor antagonists have shown some impact in the mdx model of DMD (Taniguti et al., 2011; de Oliveira Moreira et al., 2013b). A proof of principal study was carried out to look into the effects of the specific P2X7 antagonist CBB on the level of a standard pre-clinical diagnostic marker of DMD, serum CK.

CBB is a non-competitive inhibitor of the P2X7 receptor, with around 1000-fold more potency at the rat-receptor than the most closely related subunit, P2X4 (Jiang et al.,
On this basis, CBB is a highly specific antagonist and information gleamed from its use may be used to support the involvement of the P2X7 receptor in the pathogenesis of DMD, even over the closely related and sometimes co-expressed P2X4 receptor. Furthermore, CBB has already been used in mouse studies to challenge the P2X7 receptor and to improve animal health (Gandelman et al., 2010).

Using CBB to antagonise P2X7 receptor function in vivo, qualitatively lower serum CK levels were attained compared to mdx, and similar to the Pfizer mdx/P2X7−/− dKO mouse, that did not reach statistical significance. If more than the current number of 10 animals were tested, it may have been possible to attain a significant difference between saline-injected or non-injected mdx and CBB-injected mdx mice. This claim is corroborated by a power analysis study, indicating a lower than required number of CBB injected animals to be confident in the outcome of determined difference between this and other groups (Appendix 9.10: with an assumed group standard deviation of 550 there is a ~56% chance that we can correctly identify a difference at N=10, if that difference exists). The pattern is none-the-less promising because of similar results obtained from the KO study. Moreover, CBB has a slow association rate and does not competitively block agonist activation (Michel et al., 2008). We are currently testing new generation, increased affinity, competitive antagonists that block all P2X7-mediated functions, including Ca2+ influx, pore formation, and IL-1β release [36], which therefore are significantly more effective.

If the P2X7 receptor is eventually recognised as a useful target in the treatment of DMD, it is likely that other drug-based compounds will be necessary to safely challenge receptor function in vivo. One of the current advantages of challenging the P2X7 receptor is that several highly-specific antagonists have already been developed that, in several cases, have already passed human clinical-trial safety testing for the treatment of other human disorders (Arulkumaran et al., 2011), creating a potential fast-track avenue to explore secondary medicinal applications.
The current study has sought to investigate the role of the P2X7 purinoceptor in the pathogenesis of DMD using the \textit{mdx} mouse model of this disease. Molecular and functional parameters were compared in \textit{mdx} to two specific strains of \textit{mdx/P2X7}\textsuperscript{-/-} double-mutant mouse and it was found that P2X7 ablation in this most widely used animal model of DMD produced significant improvements observed both in leg muscles at 4 weeks as well as in diaphragms and hearts at 20 months. At these stages and in these muscles, \textit{mdx} disease features mimic DMD pathology. In addition to amelioration of key molecular and functional parameters of muscle damage, decreased inflammation and a reduction of non-muscle symptoms were also evident. This wide impact reflects convergence of P2X7 ablation on multiple pathological mechanisms, thus revealing a potential new strategy for treating this debilitating disease (see below).

The main comparison was performed between the \textit{mdx} animal and the Pfizer \textit{mdx/P2X7}\textsuperscript{-/-} mouse strain. The Pfizer P2X7 KO mouse varies from the Glaxo strain through differing targeted modifications of the P2X7 gene such that in the Glaxo P2X7 KO mouse a low expression but gain of function splice variant, the P2X7k, escapes inactivation (Nicke \textit{et al.}, 2009). The P2X7k variant can contribute to enhanced receptor mediated pore formation and enhanced context specific cell-death compared to the predominant P2X7a variant (Nicke \textit{et al.}, 2009). Interestingly, the differential expression of the gain-of-function P2X7k variant in the Glaxo P2X7 KO mouse has been suggested as a potential reason behind differing bone resorption across the two knockout strains (Syberg \textit{et al.}, 2012), even though its overall expression is much lower than of the normal P2X7a isoform (Masin \textit{et al.}, 2012).
Throughout this study, a pattern has emerged in which the Glaxo \textit{mdx/P2X7$^+/-$} dKO mouse has presented qualitatively intermediate phenotype between \textit{mdx} and the Pfizer \textit{mdx/P2X7$^+/-$} mouse (Table 6.1). For example, in a measure of total body muscle membrane permeability, Glaxo \textit{mdx/P2X7$^+/-$} serum CK was qualitatively intermediate between \textit{mdx} and Pfizer \textit{mdx/P2X7$^+/-$} levels. This was corroborated by a non-significant but intermediate muscle cytoplasmic IgG staining levels found in the Glaxo \textit{mdx/P2X7$^+/-$} mouse compared to \textit{mdx} and the Pfizer variant. Also compelling are the many intermediate values in the panel of inflammatory genes expression, including IFN$\gamma$, TNF$\alpha$, and Ly6G, also coupled to the muscle protein analysis of F4/80 and CD163 by western blotting. Finally, intermediate skeletal-muscle protein expressions were found for the myogenic factors eMyHC and myogenin.

These partial effects of P2X7 ablation observed in Glaxo \textit{mdx/P2X7$^+/-$} and also observed in heterozygous \textit{mdx/P2X7$^{+/+}$}, which were found to have intermediate P2X7 levels, suggest that even incomplete inhibition of this receptor may have some therapeutic effects. Indeed, broad purinoceptor antagonists showed some impact in the \textit{mdx} model of DMD (Taniguti et al., 2011; de Oliveira Moreira et al., 2013b) and the short-term administration of CBB antagonists in this study also gave promising results. As mentioned earlier, CBB does not competitively block agonist activation (Jiang et al., 2000) and suramin used in other studies is non-specific not only with respect to its receptor antagonism but also regarding the off-target effects. Therefore, future studies should concentrate on high affinity, competitive antagonists, which are significantly more effective.

Moreover, as the Pfizer \textit{mdx/P2X7$^+/-$} dKO mouse consistently presented values further from \textit{mdx} and more indicative of amelioration of disease parameters, it provided justification for preferential use of this strain in most of the subsequent comparisons.

Regarding mechanisms by which P2X7 ablation produced muscle recovery, these could involve both direct effects in muscle cells (Yeung et al., 2006; Young et al., 2012; 2013; 2015; Valladares et al., 2013) as well as the reduced inflammation (Porter et al., 2002; Vidal et al., 2012; Idzko et al., 2014).
### 4 week C57 BL/10 Pfizer dKO Glaxo dKO mdx

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>C57 BL/10</th>
<th>Pfizer dKO</th>
<th>Glaxo dKO</th>
<th>mdx</th>
</tr>
</thead>
<tbody>
<tr>
<td>General Histology</td>
<td>Qualitative</td>
<td>Qualitative</td>
<td>Qualitative</td>
<td>Qualitative</td>
</tr>
<tr>
<td>Pax7 expression</td>
<td>n.s.</td>
<td>n.s.</td>
<td>Dystrophic baseline</td>
<td>Dystrophic baseline</td>
</tr>
<tr>
<td>Central nucleation</td>
<td>Qualitative</td>
<td>n.s.</td>
<td>Dystrophic baseline</td>
<td>Dystrophic baseline</td>
</tr>
<tr>
<td>Myofibre Feret’s CoV</td>
<td>n.s.</td>
<td>Dystrophic baseline</td>
<td>Dystrophic baseline</td>
<td>Dystrophic baseline</td>
</tr>
<tr>
<td>Muscle min. Feret’s diameter</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>Dystrophic baseline</td>
<td>Dystrophic baseline</td>
</tr>
<tr>
<td>Myogenin expression</td>
<td>P&lt;0.005</td>
<td>P&lt;0.005</td>
<td>Dystrophic baseline</td>
<td>Dystrophic baseline</td>
</tr>
<tr>
<td>eMyHC expression</td>
<td>Qualitative</td>
<td>P&lt;0.05</td>
<td>n.s.</td>
<td>Dystrophic baseline</td>
</tr>
<tr>
<td>Revertant fibres</td>
<td>None</td>
<td>Qualitative but n.s.</td>
<td>n.s.</td>
<td>Dystrophic baseline</td>
</tr>
<tr>
<td>Utophin expression</td>
<td>n.s.</td>
<td>Dystrophic baseline</td>
<td>Dystrophic baseline</td>
<td>Dystrophic baseline</td>
</tr>
<tr>
<td>Macrophage infiltration</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>Dystrophic baseline</td>
</tr>
<tr>
<td>M1/M2 MØ ratio</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>Qualitative but n.s.</td>
<td>Dystrophic baseline</td>
</tr>
<tr>
<td>Key inflammatory mRNAs</td>
<td>4 genes P&lt;0.05</td>
<td>3 genes P&lt;0.05</td>
<td>Dystrophic baseline</td>
<td>Dystrophic baseline</td>
</tr>
<tr>
<td>Neutrophil infiltration</td>
<td>P&lt;0.05</td>
<td>Dystrophic baseline</td>
<td>Dystrophic baseline</td>
<td>Dystrophic baseline</td>
</tr>
<tr>
<td>Serum CK</td>
<td>P&lt;0.005</td>
<td>P&lt;0.05</td>
<td>Qualitative but n.s.</td>
<td>Dystrophic baseline</td>
</tr>
<tr>
<td>Mouse IgG infiltration</td>
<td>P&lt;0.05</td>
<td>Qualitative but n.s.</td>
<td>Dystrophic baseline</td>
<td>Dystrophic baseline</td>
</tr>
<tr>
<td>Diaphragm tetanic force</td>
<td>P&lt;0.005</td>
<td>P&lt;0.05</td>
<td>Qualitative but n.s.</td>
<td>Dystrophic baseline</td>
</tr>
</tbody>
</table>

Table 6.1. Summary table showing a relative distribution of phenotypes across genotype at 4 weeks. In each case a colour coding system is employed to indicate positive difference (green: along with indicated P value) from the dystrophic baseline (peach) presented by the mdx mouse. Positive difference represents statistical and qualitatively determined differences between individual means that have been considered healthier. n.s. = non-significant. Where a qualitative difference in continuous variable was observed that was not statistically significant, that box was coloured yellow-green (chartreuse). Boxes are shaded-out where variables were not recorded for a particular genotype. Note, the general pattern in which the Pfizer mdx/P2X7−/− dKO mouse presents more variables that are different from mdx than the Glaxo mdx/P2X7−/− animal. See Appendix 9.10 for a more detailed version of this summary table.
Dystrophin-deficient muscles chronically release ATP together with other DAMPs and resulting immune cell infiltrations contribute to disease progression (Porter et al., 2002; Villalta et al., 2009). Deletion of P2X7, with its unique capability to respond to high ATPe levels and to shape the inflammatory pathway through release of pro-inflammatory cytokines would be expected to have an impact. Indeed, in \textit{mdx/P2X7\textsuperscript{\textminus\textminus}}, there was a clear overall decrease in the muscle inflammatory signature. In particular, there were lower total macrophage specific F4/80 marker expressions and an indication of a shift in macrophage subtype from an M1 to an M2 (pro-regenerative) phenotype. Importantly, this shift did not result in exacerbated fibrosis in either aged mdx hearts or diaphragms. Moreover, 4 week \textit{mdx/P2X7\textsuperscript{\textminus\textminus}} dKO mouse muscle consistently presented lower expressions values of pro-inflammatory transcripts compared to mdx. These included markers such as TNF\textalpha and RORC, both of which are positively associated with Th1 and Th17 T-cell induction. Consistent with the reduced level of the mRNA and protein levels of the granulocyte marker Ly6G in \textit{mdx/P2X7\textsuperscript{\textminus\textminus}} dKO muscle compared to \textit{mdx}, there also were reduced numbers of infiltrating leukocytes.

It is therefore interesting to explore two aspects of P2X7-evoked modulation of DMD biology. These include an effect on DMD inflammation and a potentially fundamental interaction with cellular pathways.

A typical feature of DMD is the sterile inflammatory response that is also autoreactive and, at least partly, T-cell mediated. CD4 and CD8 positive cells were amongst the first immune cells to be recognised as contributing to myotube cytolysis in DMD (Spencer et al., 1997; 2002). The autoimmune features of DMD, such as abnormal major histocompatibility complex I and II expression and chronic inflammation, are gaining attention (Tidball & Wehling-Henricks, 2005). P2X7 is a likely candidate to effect a T-cell mediated autoimmunity in DMD. For example, in a sterile form of inflammation following cardiac muscle transplantation, P2X7 expression was elevated and found to promote Th1 and Th17 cell induction in a STAT3-dependent manner (Vergani et al., 2013). Moreover, certain populations of T-cell are known to regulate macrophage cell sub-types. For instance, CD4+CD25+ T-cells (including T-regs) promote M2 macrophage induction \textit{in vivo} (Liu et al., 2011). In one recent study, this time directly applicable to the mdx mouse, the P2 receptor antagonist suramin, applied for 8 months, reduced inflammation, fibrosis, and necrosis in the heart (de
Oliveira Moreira et al., 2013b). This result directly parallels the reduced heart fibrosis and inflammation observed in 20 month Pfizer mdx/P2X7/−/− compared to mdx mice presented earlier, adding much greater support to the hypothesis of a leading purinergic mechanism involved in mdx pathogenesis. TGFβ levels did not change in the heart after the 3 month suramin treatment of the de Oliveira Moreira (2013b) study but did increase in the diaphragm. TGFβ in particular is associated with fibrosis pathways, so it was important to explore the possibility of increased diaphragm fibrosis upon P2X7 ablation. Fibrosis has been linked to the activity of regenerative M2-type macrophages, which were also increased relative to M1-type macrophages, at least in earlier stages of the dystrophinopathy in mdx/P2X7/−/− compared to mdx mice. However, despite concerns raised by both of these previous observations, fibrosis was not increased in aged 20 month Pfizer mdx/P2X7/−/− compared to mdx diaphragms, whilst reduced inflammation was maintained.

Another important finding in this thesis has been the reduction in TNFα levels in mdx/P2X7/−/− muscle compared to mdx at 4 weeks, since TNFα has been previously linked to the key P2X7 function as well as to DMD disease progression. The inflammatory cytokine TNFα is known to promote satellite cell activation and proliferation (Li, 2003; Tidball & Villalta, 2010) whilst also inhibiting the differentiation and fusion of myotubes (Miller et al., 1988; Szalay et al., 1997; Layne & Farmer, 1999; Langen et al., 2001; 2002; 2004), providing some mechanistic support for the enhanced muscle fibre diameters observed in 4 week hindlimb and 20 month diaphragms in Pfizer mdx/P2X7/−/− compared to mdx mice. Enhanced proliferation, as is found in DMD, may contribute to satellite cell replicative senescence (Decary et al., 2000) and fibrosis (Alexakis et al., 2007) whilst enhanced differentiation, without a large decline in satellite cell or myoblast proliferation, may result in the larger myotubes and reduced fibrosis as seen in the mdx/P2X7/−/− mouse compared to mdx. Reduced TNFα expression due to reduction of macrophage numbers also correlated with improved insulin sensitivity in a human study (Di Gregorio et al., 2005). A similar effect on muscle metabolic potential has been found for another pro-inflammatory mediator down-stream of P2X7 receptor activation, namely IL-1β. In a human study of type-2 diabetes, it has been shown that IL-1β blockade could improve outcome by reducing inflammation and increasing cellular glucose levels (Larsen et al., 2007; 2009). Finally, TNFα and IL-1β are both elevated
from early stages of DMD (Kumar & Boriek, 2003), and both have been found to interfere with IGF1 signalling in dystrophic muscles (Broussard et al., 2003; 2004; Strle et al., 2004). This factor also impinges on myogenic potential and muscle fibre size. Of interest in the debate regarding macrophage polarisation, the generally anti-inflammatory and M2 macrophage-associated cytokine IL-10 limits IGF1 interference by IL-1β (Strle et al., 2008).

It has been suggested that the degenerative nature of the dystrophic muscle pathology may also be associated with the deterioration of the function of myogenic precursor cells over repeated cycles of proliferation and self-renewal (Bigot et al., 2008). As has been mentioned previously, this could also be linked to the increased systemic and tissue level inflammation present in dystrophic muscle. In the mdx mouse model, disease phenotype is less severe and does not result in the marked chronic degeneration and drastically premature mortality seen in human patients, with the possible exception of diaphragm muscle. When telomere length is artificially shortened by experimental intervention to remove the RNA component of telomerase, the mdx pathology is significantly more severe and also presents an obvious degenerative profile (Sacco et al., 2010), hinting at a fundamental compensatory advantage in the mouse compared to human and also evidence for a muscle-cell-specific challenge in DMD.

That targeting of P2X7 could be used to challenge even the directly muscle specific origins of the DMD pathology has been hinted at by our laboratory and others. Previous work by our laboratory has discovered ERK1/2 phosphorylation to be downstream of P2X7 receptor activation in dystrophic muscle (Young et al., 2012). ERK1/2 is a member of the mitogen activated protein kinases (MAPKs), whose activation is also linked to myoblast proliferation. Upon MAPK inhibition, it has also been shown that there was a corollary increase in the differentiation of skeletal muscle cells in vitro (Coolican et al., 1997), indicating the potential reciprocal relationship between the ERK1/2 – proliferation pathway with, in that case, a PI3K/p70S6K – differentiation pathway. By here showing an increased muscle fibre size and myogenin expression upon P2X7 receptor loss in the mdx mouse, it is possible that regenerative emphasis was shifted towards differentiation by limiting ERK1/2 activation in vivo. It should also be noted that in the Coolican et al. (1997) study, it was shown that myogenin, whilst important, was not sufficient to induce
differentiation in the absence of factors such as MEF-2. Without having monitored for many of the other MRFs, we cannot therefore exclude the possible involvement of these proteins in the increase of myofibre size upon reduced P2X7 activation. In terms of a potential benefit upon dystrophic disease pathology, there is evidence to suggest that mdx skeletal muscle is pathologically weighted towards hyper-proliferation of muscle precursor cells over their eventual differentiation; an observation with obvious impact on a supposed satellite cell replicative senescence. For instance, at an epigenetic level it was shown that mdx skeletal muscle expresses higher levels of a class I histone deacetylase 2 (HDAC2) than non-dystrophic controls (Colussi et al., 2008). This enzyme was shown to limit the formation of multi-nucleated myotubes via the regulation of the follistatin-myostatin pathway. Part of the cause for enhanced HDAC2 activity in dystrophic muscle is the loss of key DAPC members, resulting in reduced NO mediated S-nitrosylation of HDAC2 (Colussi et al., 2008). In more global measures of protein mediated genetic regulation, it was further shown that wider ranging histone modifications were potentiating proliferative and inflammatory cell pathways in humans and mice, also partly via reduced NO production in DMD muscle (Colussi et al., 2009).

The P2X7 receptor is a particularly interesting disease target in this regard since it too has been linked with proliferation, including of muscle precursor cells. In one study, purinergic receptors including P2X7 were shown to promote ERK1/2 mediated, intracellular Ca^{2+} dependent, satellite cell proliferation in vitro (Banachewicz et al., 2005). In another more recent study, it was shown that ATPe promoted C2C12 muscle myoblast proliferation via purinergic receptor activation, except at excessive levels when it became cytotoxic (Martinello et al., 2011). Also important for the current hypothesis, was their claim that myoblasts had an inherent reduced resistance to ATP due to a lower ATP hydrolysing capability compared to C2C12 myotubes. If true of regenerating mouse muscle fibres in vivo, this reduced NTPDase activity in myoblasts may partly explain the presence of increased centrally nucleated muscle fibre size in the mdx/P2X7\textsuperscript{-/-} dKO mouse compared to mdx. Regenerating muscle fibres mostly recapitulate developing muscle cells. Therefore, P2X7-deficient and regenerating fibres may be less susceptible to the higher levels of ATPe that Martinello et al. (2011) defined as cytotoxic. This raises the alluring possibility that targeting of the P2X7 receptor in DMD may increase muscle fibre re-growth potential and
sarcolemmal stability, perhaps concomitant with a decreased susceptibility to apoptotic and necrotic cell death via Ca\(^{2+}\) dependent and independent mechanisms.

Without eliminating satellite cell proliferation and self-renewal, a shift towards enhanced differentiation seems likely to alleviate DMD disease severity, concomitant with a reduction in the hyper-proliferation of myogenic precursor cells normally observed in dystrophic muscle. Another interesting reason for this, may be related to the exaggerated fibrosis also seen in human, and to a lesser extent mouse, dystrophinopathy. Specifically, it has been shown that dystrophic myogenic cells themselves express the extracellular matrix component collagen type-I inversely proportional to their expression of the differentiation MRF myogenin (Alexakis et al., 2007). This early collagen expression was also correlated with the later expression of non-myogenic cells in aged muscle, indicating the hyper-proliferation of satellite cells as a self-perpetuating mechanism of collagen overproduction linked to an ultimate myogenic defect (Alexakis et al., 2007). The effect of P2X7 ablation on dystrophic fibrosis, including collagen-Ia deposition, is an aspect of pathological mediation that has been addressed here.

Specifically, cardiac fibrosis was reduced in the \(mdx/P2X7^{-/-}\) dKO mouse compared to \(mdx\) at ~20 months. In relation to the above discussion, it was interesting that levels of myogenin were higher in younger \(mdx/P2X7^{-/-}\) compared to \(mdx\) hindlimb muscle, perhaps indicating a shift away from hyper-proliferative myogenesis upon P2X7 receptor ablation, subsequently coinciding with reduced long-term collagen production and fibrosis. The generally activatory and proliferative effect of immune cells on MPCs has also been described, providing an alternative or adjacent mechanism by which P2X7 could affect myogenesis, and finally mediating fibrosis. That aged hearts and diaphragms from Pfizer \(mdx/P2X7^{-/-}\) compared to \(mdx\) mice also showed reduced immune cell infiltrations, strongly supports the correlation even if it does not prove causality. A likely explanation for the improvement in these histological markers of dystrophic pathology, probably involves the interruption of purinergic mediation of the communication between the complex milieu of cells present in dystrophic muscle, even if single disease pathways may be more important than others. Exaggerated skeletal and cardiac muscle fibrosis is a defining histological feature of dystrophinopathy; present in the most common mammalian models, the \(mdx\) mouse and the Golden Retriever muscular dystrophy dog, and DMD (Willmann
et al., 2009; Nakamura & Takeda, 2011). Therefore, irrespective of the precise mechanisms leading to the reduced fibrosis and inflammation in aged hearts and diaphragms from mdx/P2X7−/− compared to mdx mice, these results should be seen as highly significant. The improvements found in 20 month old hearts and diaphragms of mdx/P2X7−/− demonstrated that similar mechanisms operate in both skeletal and cardiac muscles and that these mechanisms remain active throughout the mdx pathology at least in muscles undergoing continuous, severe degeneration/ regeneration akin to human disease. The cardiac improvement is also of clinical importance because with prolonged survival (due to advances in general care) heart failure becomes a more common cause of death in DMD.

At the cellular level, and without relying on its modulation of immune cells only, the P2X7 receptor may be modifying mdx and DMD disease outcome in the following additional ways. As already mentioned, the P2X7 receptor has been linked to ERK1/2 activation in dystrophic muscle cells (Young et al., 2012). ERK1/2 has recently also been shown to reciprocally-negatively interact with AMPK to induce a form of muscle specific cellular insulin resistance (Hwang et al., 2013). It is therefore plausible that P2X7 over-activation may also result in skeletal muscle metabolic instability, which is linked to a general state of cell stress and chronic intracellular Ca2+ elevation (Park et al., 2011; 2013) as seen in DMD and the mdx mouse (Figure 6.1). More directly, high ATPe acting on P2X7, which is over-expressed on dystrophic muscle cells (Young et al., 2012; 2013; Valladares et al., 2013), could activate both ion channel and large pore opening, producing an efflux of the intracellular content and activation of signalling cascades involving both ERK phosphorylation and increases in intracellular Ca2+ levels, which contribute to deregulation of Ca2+ homeostasis in mdx fibres. Therefore, P2X7 ablation or inhibition would eliminate Ca2+ influx occurring via this receptor and that can also trigger secondary modulation of other Ca2+ channels; effects that have been described in mdx myofibres (Friedrich et al., 2004; Vandebroutck et al., 2006). Thirdly, given that P2X7-activation in dystrophic muscle cells has recently been linked to autophagy induction, then this may be a primary mechanism through which enhanced purinergic signalling may be effecting DMD atrophy, metabolic instability, and muscle fibre susceptibility to cell death processes (Jahnke et al., 2012). Indeed, our laboratory has recently shown P2X7 large pore formation to induce a novel form of autophagic cell...
death in \textit{mdx} myoblasts and myotubes, which involves heat-shock proteins (Young \textit{et al.}, 2015). This mechanism may contribute both to fibre loss and exhaustion of the pool of muscle-resident stem cells required for regeneration (Sacco \textit{et al.}, 2010). A seeming contradiction is that has been suggested that autophagy is a generally beneficial process in muscular dystrophy via elimination of defective mitochondria (De Palma \textit{et al.}, 2012; Pauly \textit{et al.}, 2012). However, while P2X7 ablation reduced autophagy in 4 week old \textit{mdx} TA, it is not clear whether, or to what extent, it affected muscle or immune cells. Future research is needed to clarify the cell-type dependency as well as the reported shift between positive and negative roles for autophagy in young and old dystrophic muscles, respectively (Hindi \textit{et al.}, 2014).

Another interesting observation is that glucocorticoid therapies for DMD, including the widely used prednisone, may actually activate autophagy by interfering with glucose metabolism, thus potentially decreasing muscle oxidative potential in the chronic disease state (Louard \textit{et al.}, 1994; Short \textit{et al.}, 2009). Specifically addressing this issue, Huynh \textit{et al.} (2013) have compared prednisolone treatment, with a non-steroidal glucocorticoid receptor modulator, in \textit{mdx}. Both treatments were successful at reducing key inflammatory markers associated with disease severity but the non-steroid compound resulted in significantly less osteopontin, Foxo1, and Foxo3 induction compared to prednisolone. Yet more evidence indicating that autophagy is generally detrimental for skeletal muscle in DMD come from a study in which sarcolemmal-delocalised nNOS was shown to enhance muscle atrophy by upregulating Foxo3-mediated atrogin-1 and MuRF1 (Suzuki \textit{et al.}, 2007). Delocalised nNOS is recognised as a fundamental feature of DMD, contributing to disease pathogenesis and a reduced muscle force potential (Li \textit{et al.}, 2010a; Li \textit{et al.}, 2011b, Figure 6.1).

Finally, targeting the P2X7 receptor is, to our knowledge, the first clinically-relevant treatment for the non-muscle symptoms of DMD. The P2X7 receptor plays significantly different roles in bone physiology and in disease states (Kvist \textit{et al.}, 2014). Importantly, ablation of P2X7 in \textit{mdx} model did not exacerbate but improved the dystrophic bone phenotype. As bone abnormalities in \textit{mdx} mice have been linked to chronic inflammation (Abou-Khalil \textit{et al.}, 2014), the reduced inflammatory signature in \textit{mdx/P2X7} muscles may also translate into reduced bone loss in these mice.
Figure 6.1. A Schematic representation of potential roles for the P2X7 receptor in the pathological progression of DMD. Absence of dystrophin and resulting loss of the DAPC leads to reduced cell membrane stability with a chronic increase in Ca\textsuperscript{2+}, ROS, and RNS causing protein damage, apoptosis, necrosis and metabolic instability. Damaged muscle releases large quantities of ATP and DAMPs, which trigger a chronic inflammatory response. P2X7 purinoceptor up-regulation on dystrophic myofibres could be involved in several pathogenic pathways: 1) intracellular Ca\textsuperscript{2+} build-up; 2) PKC mediated p47phox translocation and subsequent ROS generation; 3) sterile activation of immune cells leading to sarcolemmal breakdown, cell death, and reduced myocyte differentiation. High extra-cellular ATP levels may skew responses of muscle macrophages by promoting the M1 over the pro-regenerative M2 population while P2X7 purinergic over-activation in dystrophic myoblasts (not shown) may contribute to their death and thus reduce muscle regeneration further still.

NADPH oxidase may be expressed at the skeletal muscle membrane where it can contribute significantly to ROS generation (Whitehead et al., 2006). ATP is now a recognised extracellular signal promoting the translocation of NADPH oxidase subunits via secondary signalling molecules such as PKC (Cheng et al., 2013). In terms of cellular ROS generation, the NADPH oxidase complex may be a greater contributing factor than even acute inflammatory cells. Although the NADPH oxidase organizer subunit p47phox is shown in the figure based on recent evidence of P2X receptor involvement in A549 epithelial cells, gp91phox and p67phox subunits have been highlighted as increased in even pre-symptomatic mdx TA muscle compared to wild-type controls (Whitehead et al., 2010).

Chronically elevated cytosolic Ca\textsuperscript{2+} is a classical feature of DMD leading to calpain activation and increased Ca\textsuperscript{2+} uptake into mitochondria. The P2X7 receptor is capable of passing high Ca\textsuperscript{2+} levels into cells but has also been linked to the release of Ca\textsuperscript{2+} from intracellular stores via inositol-1,4,5-triphosphate (IP3) receptor (IP3R) activation, at least in neuronal cells (Chao et al., 2012).

Recent evidence has shown that long-term increased levels of intracellular Ca\textsuperscript{2+} has an inhibitory effect on the metabolic energy sensor AMPK in muscle via CaMKII and protein phosphatase 2A (PP2A) induction (Park et al., 2011; 2013). Reduced AMPK activation will lower available intracellular ATP.

Linking endoplasmic reticulum stress to the pathogenesis of insulin resistance and type2 diabetes, recent work has highlighted an inhibitory cross-talk between AMPK and ERK signalling pathways in skeletal muscle (Hwang et al., 2013). Since we have previously established ERK1/2 phosphorylation as a downstream effect of P2X7 over-activation in mdx muscle (Young et al., 2012), it may be that this is also contributing to tissue specific insulin de-sensitisation, resulting in the reduced cytosolic glucose levels found in DMD muscle.
Many aspects of the current model for the potential involvement of the P2X7 receptor in the pathology of DMD (Figure 6.1) are currently speculative, based partly on findings presented here and partly on literature-based evidence. As well as important clinical therapeutic concerns associated with targeting of the P2X7 receptor, possible future studies therefore represent experimental testing of model claims, as suggested below:

- **Extend the analysis of inflammatory mediators in mdx/P2X7<sup>−/−</sup> dKO mice compared to mdx, to include IL-1β, TGFβ, and T-cell marker expression.** Including the previously mentioned TGFβ, all of the above factors have been shown to be modulated by P2X7 receptor activation. IL-1β is a pro-inflammatory cytokine whose maturation from zymogen and secretion is effected by a secondary stimulation of human macrophages by ATP, or other agonists, acting on the P2X7 receptor (Ferrari et al., 1997; Elssner et al., 2004). Despite the consistent observation that this P2X7-dependent IL-1β release requires priming of macrophages with LPS, IL-1β levels are still elevated in DMD without pathogenic cause (Lundberg et al., 1995; Kumar & Boriek, 2003). With recent data indicating that P2X7 receptor activation has a fundamental impact on T-cell subset selection (Hubert et al., 2010; Schenk et al., 2011), it would be especially interesting to see if reducing P2X7 receptor activation has any impact on T-regulatory cells within mdx skeletal muscle. qPCR transcript comparisons in the current study already qualitatively indicated that Foxp3 may be elevated in 4 week old mdx/P2X7<sup>−/−</sup> dKO muscle compared to mdx but this study needs extending to statistically support or to deny this claim.
• **Immunophenotype primary-cell extracts by flow cytometry.** A standard approach to quantify and to compare immunophenotype between experimental groups is via flow cytometry. A focus would be made on macrophage subtype and total neutrophil infiltrations. Due to T-cell sub-type also emerging as a promising P2X7-related immune regulatory factor, in addition to the conventional association of CD4 and CD8 positive lymphocytes with pathological progression in DMD (Spencer *et al.*, 1997; 2001), this cell type would also be a focus for flow cytometry.

Preliminary studies have already been carried out, evaluating a primary cell extraction method for the isolation of immune cells from homogenised skeletal muscles (data not shown).

• **Perform pathway analysis on P2X7 inflammatory response.** Whilst above normal Ca\(^{2+}\) influx may be important in the complicated disease pathology of dystrophic muscle, downstream of P2X7 over-activation (*Fig 6.1*), it is also worth considering adjuvant or alternative cellular processes such as the reduction of intracellular K\(^+\) also associated with inflammatory response and proteolysis. In this pathway it has been shown that the inflammatory effect of an efflux of intracellular K\(^+\) operates *via* the NALP3 inflammasome (that protein complex controlled by the NLRP3 gene) rather than *via* the IPAF (ICE [interleukin-1β converting enzyme]-protease activating factor), ASC (apoptosis-associated speck-like protein containing caspase activation and recruitment domain) independent, inflammasome in both mouse and human monocyte cultures (Petrilli *et al.*, 2007). Interestingly, the K\(^+\) efflux activated NALP3 inflammasome may be, at least partly, triggered by P2X7 pore-forming activation in macrophages (Perregaux & Gabel, 1994) or elsewhere.

• **Extend the pharmacological study with the use of modern P2X7 antagonists, including A740003, A438079, and A804598 from Tocris bioscience.** The current study has administered only CBB. It is an effective P2X7 receptor antagonist which has been used to aid recovery by reducing inflammation in an animal model of acute spinal injury (Peng *et al.*, 2009). However, it does not have pharmacological properties of a clinical drug. Alternative specific P2X7 antagonists include GSK314181A, AZ10606120, AZ11645373, AZD-9056, CE-224535, and EVT-401
One should also consider the use of sub-cutaneous drug-delivery implantation devices to administer P2X antagonists, reducing stress-inducing injections. To additionally evaluate the clinical therapeutic potential of these P2X7 antagonists for the treatment of DMD, it may be necessary to administer P2X7 antagonists for the \textit{mdx} mouse simultaneous with steroid treatments. Without a significant compound benefit over steroid-only treatment, the use of P2X7 antagonists may not be justifiable considering that most DMD patients already use glucocorticoids to manage their condition.

- **Challenge other P2X receptor subtypes.** The pan-P2X receptor blocker suramin has been recently used to reduce \textit{mdx} cardiomyopathy (de Oliveira Moreira \textit{et al.}, 2013b). Although the improvement using suramin could be significantly mediated by a reduction in P2X7 receptor activation, it is also worth considering the involvement of other P2X receptors, many of which are more potently antagonised by suramin than is the case for P2X7 (North & Surprenant, 2000; Gever \textit{et al.}, 2006). It should also be remembered that P2X7 is not the only P2X subunit to be expressed in muscle tissue. During various stages of development skeletal muscle fibres have been shown to express P2X1, 2, 4, 5, 6, and 7 receptors (Ryten \textit{et al.}, 2001; 2004; Jiang \textit{et al.}, 2005; Yeung \textit{et al.}, 2006), indicating their importance during proliferation and differentiation phases of muscle (re)generation. This is additional to the expression of P2X receptors on numerous other infiltrating cell types, including immune cells, smooth muscle, fibroblasts, and nervous tissue.

In relation to other P2X receptors, P2X4 has been shown to have a similar expression profile to P2X7, even heterotrimerising with it to form a functional purinoceptor (Guo \textit{et al.}, 2007). Interestingly, analyses of publically available microarray datasets have failed to confirm P2X7 as being significantly upregulated in dystrophic mouse compared to healthy samples (one representative study shown in Figure 7.1). This result is difficult to explain other than as an experimental inadequacy given that P2X7 is well-established to be expressed on macrophages and its presence on these cells infiltrating \textit{mdx} muscles has been shown in this study. It is likely that the microarray results reflect low sensitivity of this assay. Nevertheless, P2X4, which is known to co-localise with P2X7 in macrophages has been found upregulated in \textit{mdx} skeletal hindlimb muscle (GC/soleus) compared to wild-type samples at multiple ages and
correlated with the macrophage specific marker CD68 (Figure 7.1). Moreover, P2X4 expression correlated with macrophage infiltration, a finding previously published by our laboratory (Yeung et al., 2004). Considering these findings, it might be worth investigating the effects of P2X4 inhibition in mdx mice or even in mdx/P2X7−/− double mutants. In a limited adjacent western blotting study, P2X4 expression was qualitatively lower in Pfizer mdx/P2X7−/− compared to mdx GC muscle at 4 week, without reaching statistical significance (Appendix 9.7). Regarding other P2X receptors, it has been recently proposed that P2X2 and P2X5 can also heterotrimerise to form a P2X7-like receptor (Compan et al., 2012). The significance of this interaction has not been explored in the current study but might also be tested in future studies.

- **Further bone remodelling experiments.** It is very interesting that this study has shown reduced bone mineral resorption in mdx/P2X7−/− mice compared to mdx at 6 months of age, since osteopenia is a ubiquitous disease feature in DMD that may be exacerbated with conventional therapies, such as glucocorticoids (Mayo et al., 2012). As well as extending the mdx/P2X7−/− to mdx comparison to include a greater N number, it may also be beneficial to test the possible pathways involved in bone resorption and rescue. For instance, controlling levels of IL-6 may elucidate the endogenous role of this cytokine in inflammation mediated bone mineral loss in DMD and the induction by P2X7 activation.
Consider some of the off-target effects of reducing the activation of P2X7 in vivo. There are recorded mood-stabilising properties of P2X7 antagonists and genetic ablation in mice (Basso et al., 2009; Csolle et al., 2013a; 2013b). This off-target effect, if proven reliable in humans, may have obvious therapeutic and psychological
benefits for human DMD patients. However, as a more immediate consideration, mood improvement in mice is often defined by reduced immobility, a behaviour that could influence DMD disease features. P2X7 receptor pore formation is already impaired in mice on certain genetic backgrounds, including the BL/6 and BL/10 strains, due to a nucleotide polymorphism that results in a proline to leucine amino acid substitution at position 451 (Adriouch et al., 2002). This was a factor that was earlier considered as potentially influencing our study of P2X7 receptor function in DMD. When considering that P2X7 receptor antagonists are also the focus of clinical trials for chronic pain therapy, we must consider the possibility that \( mdx/P2X^{-/-} \) mice used in the current study may sympathetically and parasympathetically experience fewer dystrophic sensations than \( mdx \) mice, in parallel with a modified biology that may or may not be physically attenuating cellular and molecular disease pathology. Whilst ostensibly good for the well-being of the animal, this may also mask improvements as measured by standard operating procedures. For instance, if an \( mdx/P2X7^{-/-} \) mouse is moving about more in their cage and entertaining other normal physical pursuits, like fighting siblings, with a greater tendency than the \( mdx \) mouse, then they may be artificially elevating disease markers, like sarcolemmal tear and elevated serum CK. Therefore, further studies should also make some effort to record and to control for this potential disease model masking.
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ATP. Alteration of this signal in Mdx mice is a likely cause of dystrophy". *PLoS One*, 8(11), e75340.


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Appendix 9.1. CD68 and F4/80 immunofluorescence in 4 week *mdx* TA. Three microscope fields of view are shown (rows) for each of three individual animals (columns). Note, CD68 labels all macrophage subsets.
Appendix 9.2. CD68 and F4/80 immunofluorescence in 4 week mdx/P2X7\(^{-/-}\) dKO TA. Three microscope fields of view are shown (rows) for each of three individual animals (columns). Note, CD68 labels all macrophage subsets.
Appendix 9.3. Macro used for Fiji semi-automatic morphometry.
Appendix 9.4. Average minimum Feret’s diameter comparison between mdx, mdx/P2X7+/- and mdx/P2X7+/− F1 heterozygous mice. Centrally nucleated muscle fibres from mdx/P2X7+/- F1 heterozygous mice showed a similar size distribution to samples from mdx/P2X7+/−.
Appendix 9.5. RNA quality control by agarose gel electrophoresis and spectrophotometry. a) Extracted total RNA was electrophoretically run on a non-denaturing agarose gel to infer the quality of RNA from rRNA 28S (top band) and 18S (bottom band) subunits. Representative example images shown. b) Summary table, including spectrophotometry data used to determine RNA concentration and non-nucleic acid contaminant content, measured by comparison of OD values recorded at 260/280 and 260/230 wavelength.
Appendix 9.6. Ly6G (red) and IL-1β (green) co-localisation in 4 week GC muscles of *mdx* (left) and *mdx/P2X7<sup>-/-</sup>* (right) mice. *Mdx* muscle seemed to present a greater concentration of Ly6G positive signal in muscle than presented in *mdx/P2X7<sup>-/-</sup>* samples. IL-1β expression appeared increased in *mdx* compared to *mdx/P2X7<sup>-/-</sup>* muscles but comparative quantitative studies are required for confirmation.
Appendix 9.7. Comparison of P2X4 receptor expression in *mdx, mdx/P2X7<sup>-/-</sup>* and C57 (wild type) 4 week GC muscles. The difference between P2X4 expression in *mdx* and Pfizer *mdx/P2X7<sup>-/-</sup>* did not reach statistically significant levels (T-test, T=1.17, P=0.307, df=4).
Appendix 9.8. Eosin and Alcian Blue staining of mast cells in *mdx* tibialis anterior at 8 weeks. Note, infrequent mast cell signals (blue) in endomysial regions at the end of an acute degenerative period in the *mdx* mouse.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>General histology</th>
<th>Pax7 expression</th>
<th>Central nucleation</th>
<th>Myofibre Feret’s CoV</th>
<th>Muscle fibre size (min Feret’s diameter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57 BL/10</td>
<td>4W,+; 4M,+</td>
<td></td>
<td>Negligible</td>
<td>4W, 15.4%</td>
<td>4M, 535AU</td>
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<td></td>
<td>18M, dia, 20.37μm, P&lt;0.001</td>
</tr>
<tr>
<td>mdx</td>
<td>4W, x 4M, x</td>
<td>4W, TA, 0.57AU</td>
<td>4M, 63.7%</td>
<td>4W, 369AU</td>
<td>4W, TA, 22.89μm, 4M, TA, 32.43μm, 18M, dia, 12.72μm</td>
</tr>
<tr>
<td>Pfizer dKO</td>
<td>4W, x 4M, x</td>
<td>4W, TA, 0.98AU, P=0.139</td>
<td>4W, 24.5%, P=0.309, 4M, 63.1%, P=0.825</td>
<td>4W, 400AU, P=0.162, 4M, 548AU, P=0.475</td>
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<td></td>
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<td>4W, GC, 0.71AU</td>
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<td>4W, GC, 0.85AU, P=0.406</td>
<td>21M, dia, 15.83μm, P=0.011</td>
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<tr>
<td>Glaxo dKO</td>
<td>4W, x 4M, x</td>
<td>4W, TA, 0.54AU, P=0.925</td>
<td>21M, dia, 15.83μm, P=0.011</td>
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<td>4W, GC, 0.87AU, P=0.366</td>
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<tr>
<th>Genotype</th>
<th>Myogenin expression</th>
<th>eMyHC expression</th>
<th>Revertant fibres</th>
<th>Utrophin expression</th>
<th>Macrophage infiltration</th>
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</thead>
<tbody>
<tr>
<td>C57 BL/10</td>
<td>Not expected</td>
<td>None (n=2)</td>
<td>None</td>
<td>4W, 0.003AU, P=0.0304</td>
<td></td>
</tr>
<tr>
<td>mdx</td>
<td>4W, 0.221AU</td>
<td>4W, 0.876AU</td>
<td>4M, TA, 1.31%, 18M, 22.61 (μm⁻²)</td>
<td>4W, 1.33AU</td>
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<tr>
<td>Pfizer dKO</td>
<td>4W, 1.17AU, P=0.001</td>
<td>4W, 0.05AU, P=0.0187</td>
<td>4M, TA, 1.0%, P=0.197</td>
<td>4W, 1.62AU, P=0.415</td>
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<td></td>
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<td>21M, 11.29 (μm⁻²), P=0.036</td>
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<tr>
<td>Glaxo dKO</td>
<td>4W, higher, 1.04AU, P=0.001</td>
<td>4W, 0.336AU, P=0.0933</td>
<td>4M, TA, 1.26%, P=0.772</td>
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<tr>
<td>Genotype</td>
<td>M1/M2 MØ ratio</td>
<td>Key inflammatory mRNAs</td>
<td>Neutrophil infiltration</td>
<td>Serum CK</td>
<td>Mouse IgG infiltration</td>
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<tr>
<td>C57 BL/10</td>
<td>4W, 0.04AU, P=0.0304</td>
<td>4W (n=2)</td>
<td>Not expected</td>
<td>4W, 150.83 IU/L, P&lt;0.001</td>
<td>Negligible</td>
</tr>
<tr>
<td>mdx</td>
<td>4W, 2.96AU</td>
<td>4W</td>
<td>4W, 0.97AU</td>
<td>4W, 1461.6 IU/L</td>
<td>4W, 6.34%</td>
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<td>Pfizer dKO</td>
<td>4W, 0.109AU, P=0.0304</td>
<td>4W, <strong>+</strong></td>
<td>4W, 0.29AU, P=0.006</td>
<td>4W, 796.85 IU/L, P=0.012</td>
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<tr>
<td>Glaxo dKO</td>
<td>4W</td>
<td>4W</td>
<td>4W, 0.002-0.029</td>
<td>4W, 1.47%, P=0.02</td>
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</table>

P<0.001
### Appendix 9.9

Summary of the comparisons of disease phenotypes between C57, *mdx*, and both *mdx*/P2X7<sup>−/−</sup> double mutants, including statistical details. For each disease feature and genotype, a colour is given to denote the direction of the statistically significant change from the dystrophic (*mdx*) baseline (also denoted by the P value given): blue = increase; pink = decrease. For continuous variables, specific values and units of measurements are given, sometimes indicating qualitative differences, where significant difference cannot be determined.

The following is a summary of the individual statistical tests used for each disease feature: Pax7 – equal variance T-test; Central nucleation – T-test; Myofibre Feret’s CoV – equal variance T-test; Muscle fibre size – equal variance T-test; Myogenin – equal variance T-test; eMyHC Mann Whitney non-parametric; Revertant Fibres – equal variance T-test; Urophin expression – equal variance T-test; Macrophage infiltration – Mann Whitney non-parametric; M1/M2 macrophage ratio – Mann Whitney non-parametric; Neutrophil infiltration – equal variance T-test; Serum CK – equal variance T-test; Mouse IgG – equal variance T-test; Diaphragm tetanic force – equal variance T-test; Aged inflammation – equal variance T-test; Aged fibrosis – equal variance T-test.

Note, for the qPCR array, only the genes that gave significantly different expression levels are presented: ++++ = 4 significantly reduced genes (TNFA, RORC, GR1, CXC3L1), +++ =

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Table 9.9</th>
<th>Bone histomorphometry</th>
<th>Aged inflammation</th>
<th>Aged fibrosis</th>
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<tr>
<td>dKO 0.557AU, P=0.1124</td>
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<tr>
<td>Genotype</td>
<td>Diaphragm tetanic force</td>
<td>BV/TV, Tb.N., Tb.Sp</td>
<td>6M</td>
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<td>C57 BL/10</td>
<td>4W, 32.38N/g, P&lt;0.001</td>
<td>18M, dia (CD68), 1157.5 (.mm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>261.72 (.mm&lt;sup&gt;2&lt;/sup&gt;)</td>
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<td>4M, 32.36N/g, P&lt;0.001</td>
<td>18M, heart (CD68), 24.4 (.mm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>1.424 (%)</td>
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<td>[N=1]</td>
<td>P=0.004</td>
<td>18M, heart (CD11b)</td>
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<td>18M, heart (trichrome)</td>
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<td>38.1 (%)</td>
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<td>18M, heart (trichrome)</td>
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<td>6.957 (%)</td>
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<td>18M, heart (trichrome)</td>
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<td>3.091 (%)</td>
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<td>18M, heart (trichrome)</td>
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<td>3.091 (%)</td>
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<tr>
<td>Pfizer dKO</td>
<td>4W, 12.57N/g, P=0.049</td>
<td>21M, dia (CD68), 810 (.mm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>101 (.mm&lt;sup&gt;2&lt;/sup&gt;)</td>
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<td>4M, 23.43N/g, P&lt;0.001</td>
<td>21M, heart (CD68), 33 (.mm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>3.091 (%)</td>
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<td>18M, heart (trichrome)</td>
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<td>39.16 (%)</td>
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<td>P=0.32</td>
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<td></td>
<td></td>
<td>21M, heart (trichrome)</td>
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<td>3.091 (%)</td>
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<td>P=0.001</td>
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<td>18M, heart (trichrome)</td>
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<tr>
<td>Glaxo dKO</td>
<td>4W, 13.16N/g, P=0.135</td>
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<td></td>
<td>4M, 17.57N/g, P=0.011</td>
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3 reduced genes (RORC, GR1, CXCL1). Ages of samples represented by W=weeks; M=months.
Appendix 9.10. Power analysis of serum CK analysis, including CBB injected mice, showing higher and lower estimates for numbers of animals required to achieve set powers. 

a) An analysis required for higher (left) and lower (right) N-number estimates using two observed group standard-deviations of 800 and 550, respectively. The expected maximal difference between groups was set based on the average serum CK difference observed between mdx and Pf-mdx/P2X7−/− samples. 

b) A re-representation of data in (a) with power plotted against N number. Assuming there is a difference, for the CBB injected mice, we have between ~25-55% chance to identify a 665 unit difference at the current N=10.

This analysis was undertaken with the hope of achieving an a posteriori power analysis intended to refine future N number use, as well as to assess confidence in current estimates and the potential for type-ii error.

# FORM UPR16
Research Ethics Review Checklist

Please complete and return the form to Research Section, Quality Management Division, Academic Registry, University House, with your thesis, prior to examination.

<table>
<thead>
<tr>
<th>Postgraduate Research Student (PGRS) Information</th>
<th>Student ID: 4716 55</th>
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<tbody>
<tr>
<td>Candidate Name: ANTHONY SINADINOS</td>
<td></td>
</tr>
<tr>
<td>Department: PHARMACY AND BIOMEDICAL SCIENCES</td>
<td>First Supervisor: PROF. DAREK GÖRECKI</td>
</tr>
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<td>Start Date: OCTOBER 2009</td>
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<th>Study Mode and Route:</th>
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<th>Integrated Doctorate (NewRoute)</th>
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<td>Roy Doc (PD)</td>
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<td>PhD</td>
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</table>

| Title of Thesis: | PLX7 RECEPTOR KNOCKOUT ALLEVIATES THE PATHOLOGY IN THE MDX MOUSE MODEL OF DUCHENNE MUSCULAR DYSTROPHY |

| Thesis Word Count: (excluding ancillary data) | 44,713 |

If you are unsure about any of the following, please contact the local representative on your Faculty Ethics Committee for advice. Please note that it is your responsibility to follow the University’s Ethics Policy and any relevant University, academic or professional guidelines in the conduct of your study.

Although the Ethics Committee may have given your study a favourable opinion, the final responsibility for the ethical conduct of this work lies with the researcher(s).

# UKRIO Finished Research Checklist:
(If you would like to know more about the checklist, please see your Faculty or Departmental Ethics Committee rep or see the online version of the full checklist at: [http://www.ukrio.org/what-we-do/code-of-practice-for-research/](http://www.ukrio.org/what-we-do/code-of-practice-for-research/))

| a) Have all of your research and findings been reported accurately, honestly and within a reasonable time frame? | YES/NO* |
| b) Have all contributions to knowledge been acknowledged? | YES/NO* |
| c) Have you complied with all agreements relating to intellectual property, publication and authorship? | YES/NO* |
| d) Has your research data been retained in a secure and accessible form and will it remain so for the required duration? | YES/NO* |
| e) Does your research comply with all legal, ethical, and contractual requirements? | YES/NO* |

*Delete as appropriate

UPR 16 (2013) – November 2013
Candidate Statement:

I have considered the ethical dimensions of the above named research project, and have successfully obtained the necessary ethical approval(s)

<table>
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<tr>
<th>Ethical review number(s) from Faculty Ethics Committee (or from NRES/SCREC):</th>
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<th>Date:</th>
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</table>

If you have *not* submitted your work for ethical review, and/or you have answered 'No' to one or more of questions a) to e), please explain why this is so:

The main ethical consideration associated with this project relates to the use of transgenic animal models for pre-clinical life-science research. An independent ethical review has been conducted to allow for this research, in compliance with UK Home Office standards governing the use and treatment of experimental animals.

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