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The P2X7 purinoceptor in pathogenesis and treatment of dystrophino- and sarcoglycanopathies

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Abstract

Dystrophinopathy and sarcoglycanopathies are incurable diseases caused by mutations in the genes encoding dystrophin or members of the dystrophin associated protein complex (DAPC). Restoration of the missing dystrophin or sarcoglycans *via* genetic approaches is complicated by the downsides of personalised medicines and immune responses against re-expressed proteins. Thus, the targeting of disease mechanisms downstream from the mutant protein has a strong translational potential. Acute muscle damage causes release of large quantities of ATP, which activates P2X7 purinoceptors, resulting in inflammation that clears dead tissues and triggers regeneration. However, in dystrophic muscles, loss of α-sarcoglycan ecto-ATPase activity further elevates extracellular ATP (eATP) levels, exacerbating the pathology. Moreover, seemingly compensatory P2X7 upregulation in dystrophic muscle cells, combined with high eATP leads to further damage. Accordingly, P2X7 blockade alleviated dystrophic damage in mouse models of both dystrophinopathy and sarcoglycanopathy. Existing P2X7 blockers could be re-purposed for the treatment of these highly debilitating diseases.

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Muscular dystrophies are a group of inherited neuromuscular diseases. The most common of these is Duchenne muscular dystrophy (DMD) in which loss of full-length dystrophin (dystrophinopathy) is associated with disruption of the membrane dystrophin-associated protein complex (DAPC), comprising of dystroglycans and sarcoglycans. DMD shares molecular abnormalities with Limb Girdle Muscular Dystrophies (LGMD). LGMD type 2C to LGMD type 2F are sarcoglycanopathies caused by mutations in the γ, α, β and δ sarcoglycan genes respectively, which cause the loss of the tetrameric sarcoglycan sub-complex of DAPC [1•]. The absence of dystrophin/DAPC affects muscle development [2••] and these abnormalities are later recapitulated in a pathological vicious cycle in adult dystrophic muscle, with altered asymmetric division of dystrophic satellite cells [3••], affecting myoblast functions indispensable for muscle regeneration [4••], and where the lack of dystrophin in differentiating myotubes results in dysfunctional myofibers unable to resist physiological contraction-induced stress [5•]. These defects lead to progressive muscle wasting, disability and death of young men [6]. Sarcoglycanopathies also lead to muscle damage, albeit heterogeneous in onset and severity [1•]. In both DMD and LGMDs, the cycles of muscle degeneration and regeneration are aggravated by sterile inflammation. There is no disease-modifying treatment for any of these debilitating diseases.

Aberrant purinergic signalling in dystrophic muscle

Both DMD and LGMD are associated with abnormalities in purinergic signalling. Skeletal muscle (SM) requires ATP to function, indeed there is a particularly high intracellular concentration of ATP within muscle [7]. In physiological conditions, only small amounts of ATP are released [8] and such extracellular ATP (eATP) becomes a signalling molecule acting *via* a family of purinergic receptors. Activation of either the ionotropic (P2X) or metabotropic (P2Y) purinoceptors results in increased intracellular Ca^{2+} and a range of downstream effects (reviewed in Burnstock 2020 [9•]).

Muscle damage results in millimolar concentrations of eATP being released, whereupon its function changes. High eATP becomes a key danger/damage associated molecular pattern (DAMP) molecule. It activates a specific receptor, P2X7, that only fully responds to high eATP concentrations, suggesting that it works primarily in pathological conditions. Hence, P2X7 is dubbed the danger/damage receptor and expressed by all immune cell types, responsible for triggering the inflammatory

response [10]. Upon prolonged activation by high eATP, P2X7 can form a large pore and plasma membrane permeabilization [11].

In acute muscle damage, inflammation contributes to the removal of dead cells and prepares tissue for repair. The excessive accumulation of eATP is prevented by extracellular hydrolysing enzymes (nucleotidases). In healthy muscle tissue, the key membrane-bound ATP hydrolase responsible for 25% of eATP degradation is α-sarcoglycan [12••,13•]. Being a DAPC member, α-sarcoglycan is lost from the sarcolemma in DMD and LGMDs. Therefore, in these dystrophies, the imbalance between ATP release and degradation worsens dramatically. The resulting high eATP over-activates purinoceptors on muscle cells and can contribute to abnormal intracellular Ca^{2+} homeostasis found in dystrophic muscle [14] and also triggers chronic inflammatory responses, which exacerbate disease symptoms.

Inflammation and immune responses in dystrophino- and sarcoglycanopathies

Chronic muscle inflammation is an important factor in the pathogenesis of both DMD and LGMD. In DMD muscle, inflammatory changes precede the dystrophic muscle damage [15,16••] and muscles of the *Dmd* ^{mdx} mouse model of DMD contain 20 times more macrophages and 7 times more dendritic cells than are found in healthy individuals [17]. In some sarcoglycanopathies, inflammation can exceed that in dystrophin-deficient muscles [18]. Concurrently, treatments that reduced dystrophic muscle inflammation, significantly improved the dystrophic phenotype in patients and mouse models of dystrophino- and sarcoglycanopathies [19–25]. Yet, while targeting infiltrating immune cells alleviated symptoms, total ablation exacerbated the phenotype [20], in agreement with the role these cells play in muscle repair, where specific inflammatory cytokines activate muscle stem cells and ultimately regeneration [26,27]. Therefore, there is a need for more targeted approaches to quell these inflammatory responses. Given that the mechanism underlying dystrophic inflammation involves P2X7 expression by all muscle-infiltrating immune cells, this purinoceptor is an attractive therapeutic target to diminish damaging inflammation and promote muscle repair.

Therapeutic effects of P2X7 blockade in dystrophino- and sarcoglycanopathies

Indeed, the therapeutic impact of genetic ablation and pharmacological blockade of P2X7 was clearly demonstrated in mouse models of dystrophino- and sarcoglycanopathy. Ablation of P2x7 in *Dmd* ^{mdx} mice (*Dmd* ^{mdx}/*P2rx7* $^{-/-}$) resulted in a significant attenuation of dystrophic symptoms including reduced inflammatory and pro-fibrotic molecular signatures. There was a shift in immune cell populations, with an overall decrease in macrophage infiltration and a significantly lower ratio of pro-inflammatory to pro-regenerative macrophages in muscle from *Dmd* ^{mdx}/*P2rx7* $^{-/-}$ mice to *Dmd* ^{mdx} controls. Moreover, there was a shift towards T regulatory (T_{reg}) cells denoted by significantly increased Foxp3 and IL-12α expression in *Dmd* ^{mdx}/*P2rx7* $^{-/-}$ muscles [28••]. Thus, *P2x7* ablation not only ameliorated tissue inflammation but also promoted T_{reg} cell expansion, known to suppress dystrophic muscle damage [29]. These improvements were evident at the peak of disease severity and also at 20 months in leg, diaphragm and cardiac muscles, the latter effect having particular importance as patients who survive longer eventually die of cardiac failure. Notably, reduced inflammatory parameters corresponded with improved muscle structure and increased muscle strength *in vivo* [28••].

Moreover, pharmacological blockade with P2X antagonists such as Coomassie Brilliant Blue G (CBB) and oxidised ATP (oATP) [28••] [30], produced improvements, even following a short-term treatment of *Dmd* ^{mdx} mice. This therapeutic effect also included reduced tissue inflammation and increased number of T_{reg} cells [30]. Also, in α-sarcoglycan null mice, *in vivo* treatment with a broad-spectrum P2X receptor antagonist reduced inflammation and promoted T_{Reg} expansion in muscles. Again, this dampening of the inflammatory response, and alteration of the adaptive immune component of muscle infiltrating cells, was associated with reduced necrosis and fibrosis and increased muscle strength [31•]. While in this study using a broad spectrum P2X blocker oATP, these effects could also be attributed to the P2X4 purinoceptor blockade, highly significant improvements were subsequently reported with the selective P2X7 purinoreceptor antagonist A438079. After a long-term treatment, muscle strength recovered to almost wild type levels [32•].

These data identify P2X7 purinoceptor blockade as an attractive target for translational approaches in dystrophino- and sarcoglycanopathies. Importantly, selective P2X7 inhibitors have been developed (e.g. GSK1482160; CE-224,535 and AZD9056) and proven safe in clinical trials in inflammatory pain, rheumatoid arthritis and Crohn's disease [33–35]. Such drugs would offer the most specific treatment effect, but none have been approved as medicines yet and none tested in children. Therefore, identification and repurposing of existing drugs blocking P2X7 activity may be closer to the clinics. Indeed, Zidovudine (azidothymidine, AZT), the well-known nucleoside reverse transcriptase inhibitor (NRTI), was found to bind to the same allosteric site [36] as the "big pharma" P2X7 compounds [37••] and to be a potent P2X7 blocker [38]. Even a short-term treatment of *Dmd* ^{mdx} mice with AZT attenuated key disease abnormalities, including decreased inflammation in leg and heart muscle and reduced sarcolemma permeability. It also increased muscle strength and the treatments did not cause any detectable side effects

[37••]. Given these results in dystrophy combined with findings that this drug can reduce age-related macular degeneration *via* a P2X7-dependent mechanism [39], AZT with its established pharmacological profile, particularly in the paediatric population, appears to be a prime candidate for rapid re-purposing for the treatment of these debilitating and still incurable diseases. It is also a very low-cost treatment, which is not insignificant given that the cost effectiveness was a reason behind the recent NICE recommendation to no longer provide the Translarna (Ataluren) treatment.

P2X7 blockade might have an additional, potentially important application. Dystrophin replacement using genetic approaches is the main, currently pursued treatment. However, dystrophin re-expressed in genetically deficient skeletal muscle, an immunogenic location *per se*, further enhanced by dystrophic inflammation, triggers immune responses. These negatively affect treatment efficacy [40••], therefore prevention or suppression of immune responses should be considered. Induction of tolerance to dystrophin would maximize the treatment impact whilst minimizing the risks associated with immunosuppression. Interestingly, a few cases of spontaneous longer-term re-expression of dystrophin were associated with increased numbers of Treg cells in treated muscles [41–43]. This agrees with Treg upregulation being an established immune tolerance mechanism. As mentioned, P2X7 blockade induced Treg cells in dystrophic muscles [28••, 30] and it was found to prolong transplant survival [44–48], also by expanding TRegs [44,49]. Therefore, P2X7 blockade during dystrophin re-expression could combine direct beneficial effects against dystrophic pathology with improved transgene expression *via* Treg expansion. This could be a very significant advantage.

Altered P2X7 expression and function in dystrophic cells

Importantly, the role of P2X7 in dystrophic muscles extends beyond the activation of inflammation due to high eATP levels. Studies have revealed a purinergic phenotype in a range of dystrophic cells with upregulated expression of this receptor in muscle biopsy samples from DMD patients [30,50] and in muscle from mouse models of dystrophinopathy [50,51] and α-sarcoglycanopathy [31•]. In addition to muscle cells, significant functional abnormalities of this receptor have also been described in DMD lymphoblasts [52]. This might indicate a DMD-evoked purinergic abnormality being common across species and also cell types.

Dystrophic *Dmd* ^{mdx} myoblasts exposed to high levels of eATP *in vitro*, responded with increased cytosolic Ca^{2+} influx [35], whereas treatment with apyrase, an eATP degrading enzyme, reduced intracellular Ca^{2+} levels in *mdx* cells [53]. The P2X7 dependency of this effect was confirmed by the response being evoked by BzATP, an agonist preferentially activating P2X7 and conversely, lost in the presence of its blockers. ERK phosphorylation and the opening of the large pore, further confirmed P2X7 activation [50]. Moreover, under some conditions, P2X7 activation in cultured dystrophic muscle cells led to cell death *via* necrosis or even a unique mechanism of autophagic cell death [54]. Interestingly, exposure of *Dmd* ^{mdx} myoblasts to high eATP concentrations dramatically increased cell migration [55•], even though dystrophic myoblast chemotaxis is generally reduced compared to unaffected cells [4••]. The impact of eATP on myoblast migration differs depending on their muscle of origin and is further confounded by the co-expression of other purinoceptors (submitted).

Thus, large amounts of eATP released from damaged dystrophic muscle cells, combined with reduced degradation of eATP due to loss of ecto-ATPase activity of α-sarcoglycan and the increased expression of P2X7 in dystrophic cells result in an environment consistent with over-activation of ATP receptors. Such a strong and prolonged activation of P2X7, with its large pore opening, can further increase permeability of the dystrophic sarcolemma, contributing to the death of dystrophic myofibers, while death and abnormal migration of dystrophic myoblasts can further reduce the repair of dystrophic muscles and therefore contribute to inefficient regeneration [4••].

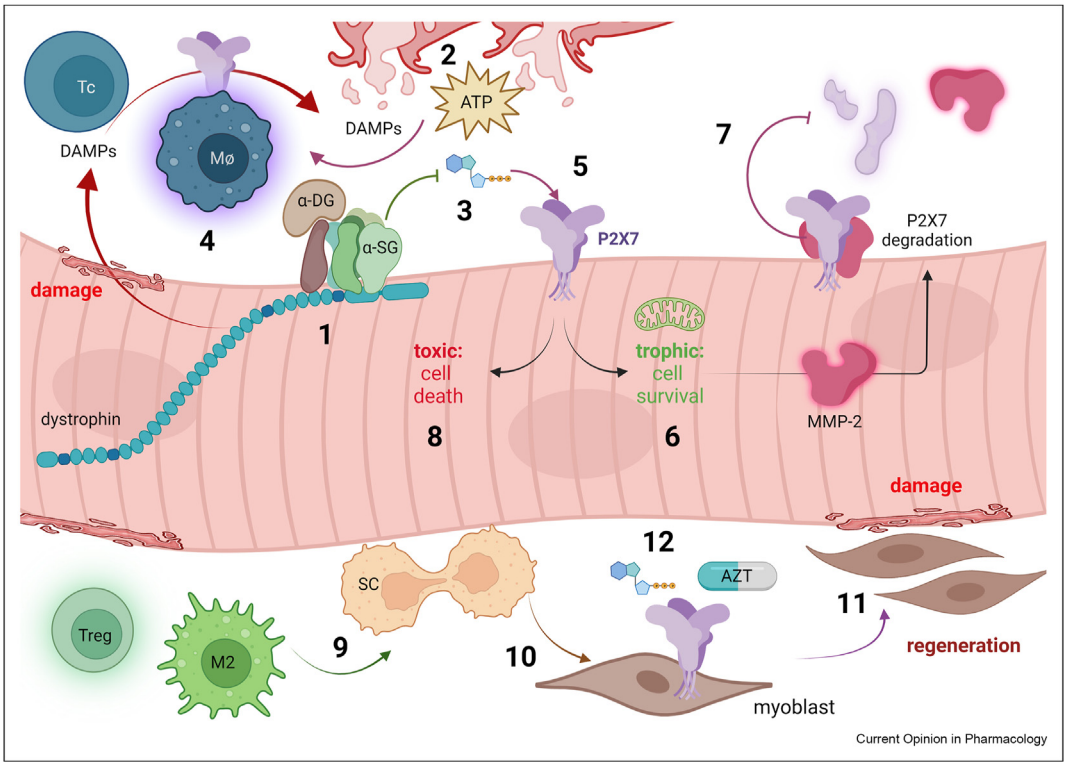
There is some evidence that ATP-mediated Ca^{2+} entry through the P2X4 receptor may modulate skeletal muscle contractility [56]. Given the common co-expression and cooperation of P2X7 with P2X4 in different cells [57–59], the question of their functional interactions and even heteromerization leading to a specific P2X receptor phenotype in the dystrophic muscles could arise. However, complex molecular, biochemical, pharmacological and electrophysiological analyses revealed that P2X4 and P2X7 subunits can only form functional homomers. Although P2X4 and P2X7 may form heterotrimeric P2X4/P2X7 receptors, these do not possess specific, discernible properties [60,61]. Furthermore, analysis revealed that while there is increased P2X4 expression within Duchenne muscle, this is a result of P2X4 presence on infiltrating macrophages [62] rather than an upregulation in dystrophic muscle cells, as is the case for P2X7 [51]. Thus, P2X4 in dystrophic muscle appears to be just another feature of the dystrophic inflammatory response.

P2X7 upregulation: dysfunction or compensation?

An interesting but unanswered question relates to the mechanism of P2X7 upregulation in dystrophic cells. The obvious one, based on the well-established role of dystrophin, is that the loss of dystrophin and/or DAPC scaffolding affects P2X7 localization and this triggers its overexpression and affects function. Yet, myoblasts (and also lymphoblasts [52]) that show P2X7 upregulation do

not express significant levels of dystrophin and DAPC, and abnormalities found in myoblasts are likely to be epigenetic [4••]. Furthermore, there is no evidence of an interaction between P2X7 and dystrophin and DAPC members. So, the alternative may be that this purinoceptor is upregulated not as a result of the dystrophic pathology but as a compensatory mechanism in adaptation to unfavorable conditions: reactive oxygen species, inflammation and metabolic abnormalities, which are present in dystrophic muscles. It may resemble the seemingly paradoxical overexpression of P2X7 on cancer cells, where its high levels also coincide with the elevated levels of eATP, with the combined effect that should lead to death of cancer cells. Instead, different tumours seem able to take advantage of the low-level tonic stimulation that provides significant benefits such as increases in growth, the Warburg effect, migration and invasion (reviewed in [63]), without triggering the P2X7 cell death cascade. Also, stimulation of P2X7 enhances proliferation/differentiation of satellite cells and improves myofiber metabolism and the metabolic mechanism in DMD may be analogous to that found in SOD1 mice, whereby P2X7 signaling was found to inhibit glycogen synthesis in favor of glucose consumption and also improve mitochondrial respiration in muscle fibers [64]. Indeed, the impacts of DMD on muscle cell energetics have been described [4••,65,66] and given that there are questions about whether mitochondrial alterations in DMD are a causative metabolic defect or adaptive reprogramming (Reviewed in [67]), P2X7 modulation might be one of the mechanisms compensating for dystrophic abnormalities. Another indication that the P2X7 purinoceptor may have a protective role, is that it is required to prevent ectopic calcification, which is another pathological feature of muscle pathology in DMD. Yet, in this case it

Figure 1



P2X7 purinoceptor in the dystrophic pathology. Absence of dystrophin and/or sarcoglycans affects functional development of the myofibers, which become damaged (1). Damaged muscle cells release DAMPs, including large quantities of eATP (2). The indirect (dystrophinopathy) or direct (sarcoglycanopathy) loss of ecto-ATPase activity of α-sarcoglycan leads to reduced eATP hydrolysis (3). High DAMP levels and eATP acting on P2X7 trigger inflammation, with infiltrating cells including macrophages (Mϕ) and cytotoxic T-lymphocytes (Tc) contributing to muscle damage (4). Chronically elevated levels of inflammatory mediators released into the bloodstream and crossing the impaired dystrophic blood-brain barrier can affect brain functions (not shown). eATP also activates P2X7 receptors upregulated on dystrophic muscle cells (5). P2X7 activation can have a trophic effect supporting the dystrophic muscle (6) and release of active MMP-2 cleaving P2X7 (7) is one of the mechanisms preventing P2X7 overactivation. The toxic effect, if it occurs, can further damage dystrophic muscle cells (8). Inflammatory mediators are also required to induce muscle regeneration by activating satellite cells (9), subsequent myoblast proliferation (10), migration to damaged sites and repair (11). However, high eATP levels combined with P2X7 overexpression on dystrophic myoblasts can cause dystrophic myoblast dysfunction and even death and thus reduce muscle regeneration. Therefore, pharmacological blockade of P2X7 with specific antagonists or repurposed drugs such as AZT alleviates the dystrophic pathology by eliminating the chronic inflammation, improving repair of dystrophic muscle by promoting the TReg and M2 pro-regenerative over the M1 macrophages and by improving myogenic cell functions (12).

was P2X7 present on infiltrating inflammatory cells rather than in dystrophic muscle [68,69].

Furthermore, although prolonged activation of P2X7 can contribute to the death of dystrophic myofibers and reduce their repair potential, hyperactivation of P2X7 was found to increase both expression and release of active matrix metalloprotease-2 (MMP-2) in dystrophic muscle cells [55•]. MMP-2 activity was shown to be an important P2X7 regulatory mechanism, functionally inhibiting this purinoceptor by cleaving it [55•]. It is therefore unlikely that all the P2X7-evoked mechanisms found in dystrophic muscle cells *in vitro* are active in the same way in dystrophic muscles *in vivo*. Further studies are needed to identify processes that are contributing to the dysregulated homeostasis in dystrophic muscles, as they could become good therapeutic targets complementing the established beneficial impact of P2X7 blockade.

Finally, in relation to neuropsychological impairment associated with DMD [6], the impact of P2X7 has also been noted. Ablation of P2X7 in *Dmd* ^{mdx}/*P2rx7* $^{-/-}$ mice corrected the cognitive impairment [28••], and this is currently the only clinically-viable treatment for this debilitating DMD abnormality. Further studies illustrated the complex nature of P2X7 involvement in this still poorly understood dystrophic brain defect. P2rx7 expression was not altered in *Dmd* ^{mdx} samples and, in fact, it was found decreased in dystrophin-null brain tissue [70]. Intriguingly, loss of all dystrophins is associated with more severe CNS phenotype compared to classical DMD and *Dmd* ^{mdx}, which only lack the full-length dystrophin [71]. Therefore, the role of P2X7 in dystrophin-null brain appears to be very different to that in dystrophic muscle and may be a function of the damaging impact of inflammatory mediators crossing the blood-brain barrier, which is, in fact, permeable in dystrophic brains [72••]. Understanding this mechanism should help in developing effective treatments for this neuropsychological condition. This is essential given that severe impairment affects one-third of patients, further reducing the quality of life of sufferers and their families and it is not tackled by any of the currently developed DMD treatments.

Thus, P2X7 blockade reduces damaging inflammation while promoting the pro-regenerative arm of the inflammatory response, it concomitantly reduces damage to myofibres, and supports the regenerative potential of dystrophic myoblasts (Figure 1). P2X7 inhibition also alleviates cognitive and behavioural impairments in DMD and even improves the dystrophic bone defect [28••,73]. These wide-ranging therapeutic effects reflect the involvement of P2X7 activation in various disease processes. While the mechanisms continue to be studied, a therapeutic strategy that modifies both muscle and non-muscle symptoms and applies across DMD and LGMD types 2C-F, the two most common and debilitating groups of muscular dystrophies, is unique and clinically-relevant. In view of the multi-prong effects by which this purinoceptor impacts these dystrophic pathologies, P2X7 is an attractive therapeutic target with at least one established medicine (AZT) that is ready for re-purposing in the clinical setting.

Conclusions

The dystrophic muscle microenvironment involves elevated levels of eATP leading to P2X7 purinoceptor activity, triggering chronic inflammation that exacerbates dystrophic muscle pathology. Studies in mouse models of dystrophino- and sarcoglycanopathies have demonstrated that P2X7 blockers are good candidates for rapid re-purposing for the treatment of these highly debilitating diseases. Importantly, in both DMD and LGMDs, P2X7 therapy is not constrained by causative mutations and therefore is suitable for all patients. Moreover, it appears effective in alleviating both muscle and non-muscle abnormalities of DMD, which is currently exceptional. Finally, targeting of specific inflammatory/immune functions via P2X7 blockade could not only reduce the dystrophic muscle damage but also prevent immunization by dystrophin re-expressed through molecular therapies.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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