

Prevention of pathology in *mdx* mice by expression of utrophin: analysis using an inducible transgenic expression system

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Duchenne muscular dystrophy results from the absence of dystrophin, a cytoskeletal protein. Previously, we have shown in a transgenic mouse model of the disease (*mdx*) that high levels of expression of the dystrophin-related protein, utrophin can prevent pathology. We developed a new transgenic mouse model where muscle specific utrophin expression was conditioned by addition of tetracycline in water. Transgene expression was turned on at different time points: *in utero*, at birth, 10 and 30 days after birth. We obtained moderate levels of expression, variable from fibre to fibre (mosaicism) but sufficient to induce a correct localization of the dystro-sarcoglycan complex. Histology revealed a reduction of necrotic foci and of the percentage of centronucleated fibres, which remained still largely above the normal level. Isometric force was not improved but the resistance to eccentric contractions was significantly stronger. When utrophin expression was activated 30 days after birth, improvements were marginal, suggesting that the age at which utrophin therapy is initiated could be an important factor. Our results also provide an unexpected insight into the pathogenesis of the dystrophinopathies. We observed a complete normalization of the characteristics of the mechano-sensitive/voltage-independent Ca^{2+} channels (occurrence, open probabilities and Ca^{2+} currents), while the classical markers of dystrophy were still abnormal. These observations question the role of increased Ca^{2+} channel activity in initiating the dystrophic process. The new model shows that utrophin therapy, initiated after birth, can be effective, but the extent of correction of the various symptoms of dystrophinopathy critically depends on the amount of utrophin expressed.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked recessive progressive muscle wasting disorder affecting approximately 1 in 3500 boys. It is well established that the disease is caused by deletions or point mutations in the dystrophin gene (for review see 1). Patients generally go into a wheel chair at 12 years of age and die in their late teens or early twenties. Although there is now accurate diagnosis of the disease, there is no effective treatment. Viral delivery of the dystrophin gene or minigenes is being developed although the delivery to all muscles and the suppression of the immune response to the virus and possibly to the transgene remain major problems (2–4). Other approaches to therapy include the induction of exon skipping to put the

mRNA back in-frame (5,6) and the use of oligonucleotides to repair the mutation (7,8). These approaches have the drawback that they are generally mutation specific. We have shown that the dystrophin-related protein, utrophin, can replace dystrophin in the *mdx* mouse, the mouse model of DMD and prevent the onset of pathology (9–11). We have demonstrated that increased utrophin expression restores muscle function and the dystrophin associated protein complex (DAPC) usually found at the sarcolemma are found associated with the utrophin. Thus the up-regulation of utrophin is a therapeutic possibility for the treatment of DMD. We have also shown that the expression of higher levels of utrophin under the control of ubiquitin C is not toxic suggesting that utrophin up-regulation in a tissue specific manner may not be necessary (12).

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If increased expression of utrophin is to be used for the therapy of DMD, it is important to determine whether increased expression somatically can prevent the onset of the pathology as well as *in utero* which was the case in previous transgenic experiments. Our previous studies using viral delivery suggested that delivery at birth is effective (13). However, it is also important to determine whether utrophin can be effective at later stages of the disease. This is particularly relevant to DMD when unforeseen, new mutations are diagnosed after birth. To address these questions, we have generated *mdx* mice which have a utrophin transgene whose expression is inducible by the presence or absence of tetracycline in the drinking water.

RESULTS

Characterisation of the inducible utrophin expression

The system used here is the tet-off system, that is, when tetracycline (tet) is administered, the expression of the utrophin transgene is switched off. This tet regulatable expression is reversible, that is, when the tetracycline is removed, the expression of the transgene is switched on again. This inducible expression of the transgene is facilitated by the use of the tetracycline responsive-transactivating system originally described by Gossen *et al.* (14). Figure 1 shows a schematic representation of the tet-off inducible expression system. Both the transgenes, MCK-tTA/*mdx* and full-length mouse utrophin/*mdx* were bred to make a double transgenic line, Utr-TET (see Materials and Methods for details). In the absence of tetracycline, the transgene is switched on and in the presence of tetracycline the transgene is switched off. The repression of the transgene expression is brought about by the tetracycline inhibiting the binding of the tTA transactivator to the tet-operator motif flanking the utrophin transgene.

The localization of utrophin at the sarcolemma in the Utr-TET transgenic line is illustrated in Figure 2A. The anti-FLAG antibody did not give clear results so we have used a specific utrophin antibody for all the analyses. In panel (a) strong staining with the utrophin antibody is observed compared with the very low expression of utrophin in the non-transgenic littermate *mdx* control (b). The restoration of the dystrophin associated complex is demonstrated by the localization of β -sarcoglycan at the membrane (c). The muscle fibres are not uniformly labelled suggesting mosaic expression of the transgene which is a typical feature of transgenes driven by the MCK promoter (15–17). The increased amount of utrophin is shown in the western blot in Figure 2B. Clearly, this transgenic line does not express utrophin at levels high enough to completely prevent the pathology as it does not express the protein at the same levels as the Fiona transgenic line where a full length utrophin transgene was expressed under the control of a human skeletal actin promoter. Previous measurements showed that this line expressed 3–4 times more than the *mdx* non-transgenic lines (11), but more recent data point to high levels (33). The increased expression levels in the TET transgenic line described here is high enough to have a significant effect on some typical structural and functional disorders of mouse dystrophinopathy (see later).

The kinetics of the repression and induction of the utrophin gene expression by the administration of tetracycline in the drinking water was monitored by immunocytochemistry. In Figure 3A expression of the Utr-TET transgene was maintained until the mice were 6 weeks old and then repressed by the administration of tetracycline. The analysis of expression was undertaken after 1, 2 and 3 weeks (a, b and c, respectively). After one week of repression (a), the TA and diaphragm both appear to have strong expression of utrophin at the sarcolemma. After 2 weeks (b) there is a decrease in expression in tibialis anterior (TA) muscle but levels remain the same in the diaphragm (DIA). When the Utr-TET transgene has been repressed for 3 weeks (c), expression levels in TA muscle have decreased further whereas diaphragm levels remain constant. Therefore utrophin is still retained at the sarcolemma even 3 weeks after the repression of expression of the transgene, although expression is higher in the diaphragm than in TA muscle. However, more variable muscle fibre sizes are found in sections suggesting some level of muscle regeneration. Figure 3B shows utrophin localization after induction of expression when the animals were 6 weeks old. Analyses were carried out after 1, 2 and 4 weeks (a, b and c, respectively). Only low levels of expression of the transgene were observed after 1 week (a) in TA muscle but the diaphragm showed significantly higher expression. After 2 weeks of induction of expression (b), both TA and diaphragm showed similar levels to the 1 week samples, but by 4 weeks (c) an increase in utrophin can be clearly seen in both TA and diaphragm. The high level of the transgene product at the sarcolemma may indicate high enough expression is achieved to obtain functional improvements after 4 weeks. In our experiments, the transgene was turned on or off at different times but the analyses were all carried out at 3–4 months. The data in Figure 3 suggest that at this time point full expression of utrophin would have been achieved at the sarcolemma if the transgene had been turned on. Conversely, the utrophin protein would have disappeared from the sarcolemma after the turning off of the transgene by administration of tetracycline.

Timing of utrophin expression and prevention of dystrophy—experimental protocols and definitions

Through the different protocols described later, utrophin expression was activated at various time points of the life of the animals. They are summarized in Table 1. For clarity in reporting the results, an acronym (left hand side of the Table) referring to each protocol will be used throughout the paper: UTR will be used as an abbreviated form to designate the double transgenic *mdx* mice, generally denoted Utr-TET (see above). -ON will designate the situation where the utrophin transgene was activated i.e. in the absence of tetracycline, -OFF when it was inhibited by the presence of tetracycline. Five different groups were studied [i] UTR-ON ∞ : tetracycline was never given to the animals; [ii–iv] UTR-ON₀, UTR-ON₁₀ and UTR-ON₃₀: tetracycline was given to the mother during pregnancy, but was withdrawn either at birth (-ON₀), 10 days after birth (-ON₁₀) or 30 days after birth (-ON₃₀) and [v] UTR-OFF: tetracycline was given to the animal from conception onwards.

Each group had its corresponding age-matched *mdx* controls where tetracycline was given for the same period. All mice

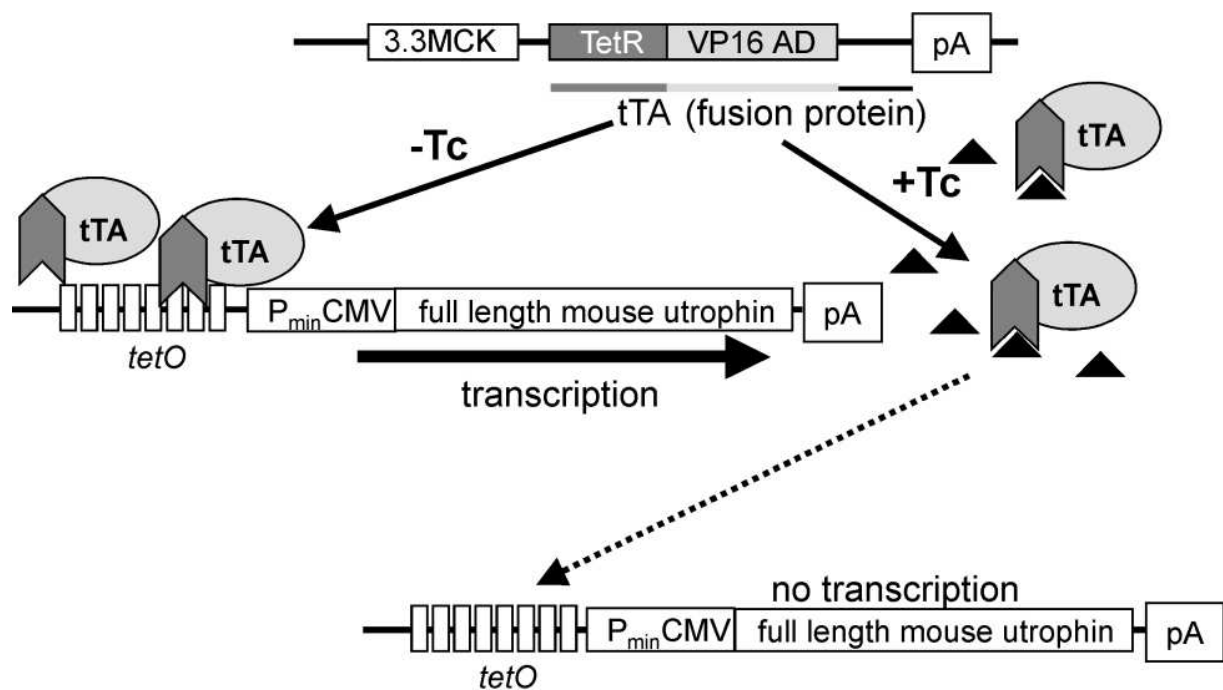


Figure 1. A schematic representation of tetracycline responsive system for muscle specific expression and temporal expression of utrophin. Further details of the transgene constructions can be found in the Materials and Methods section.

Table 1. Protocols of timing of utrophin expression

Acronyms	Tetracycline in water = Utrophin transgene inactivated	Tetracycline withdrawn = Utrophin transgene activated
UTR ON _∞	Never	
UTR ON ₀	From conception	At birth (day 0)
UTR ON ₁₀	From conception	10 days after birth
UTR ON ₃₀	From conception	30 days after birth
UTR OFF	From conception	Never

Analysis

100 d 10 d 1 mth 3 mth 9 mth

UTR ON_∞
UTR ON₀
UTR ON₁₀
UTR ON₃₀
UTR OFF

— Tet
— UTR+

were analysed when they were 3–4 months old. For the UTR-ON animals, the average time without tetracycline (i.e. when the utrophin transgene was turned ON) before analysis was 126, 116 and 107 days for the UTR-ON₀, -ON₁₀ and -ON₃₀, respectively. The corresponding *mdx* controls were studied at exactly the same age. The UTR-OFF group and its controls were studied at the age of 106 days.

In a separate series of experiments designed to study the time course of utrophin expression when the gene was turned on, tetracycline was withdrawn from 6 week old UTR-OFF animals, conversely, the time course of utrophin degradation was studied on 6 week old UTR-ON animals who were given tetracycline. TA and diaphragm muscles were removed at various times after the tetracycline switch.

Body weight

Mdx mice display a typical pseudohypertrophy as do DMD patients (18) and the body weight of 3–4 month old animals is usually about 10–12% heavier than normal. UTR-ON_∞, UTR-ON₀ and UTR-ON₁₀ mice displayed a systematic 9–10% reduction of weight, but this was statistically significant for UTR-ON₁₀ only where the mean weight was reduced from 34.3 to 31 g (*P* < 0.05). No difference with control *mdx* was observed for UTR-ON₃₀ and UTR-OFF.

CPK activity

Plasma creatine phosphokinase (CPK) activity is a classical, though non-specific, index of active dystrophy and is elevated

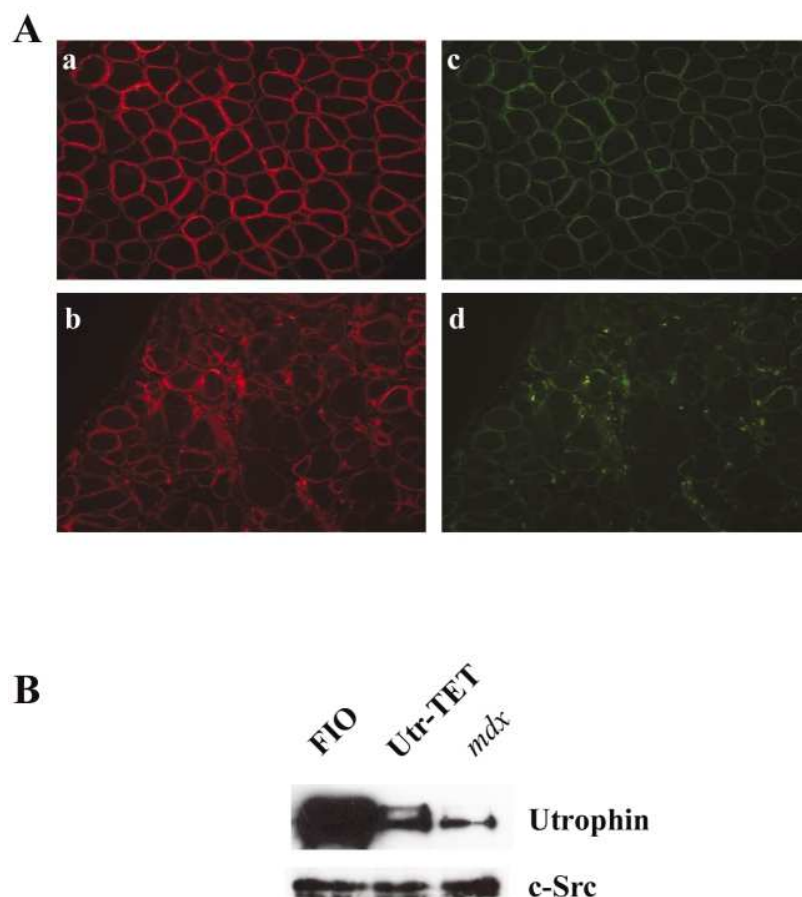


Figure 2. Induction of expression of the Utr-TET transgene. (A) Immunocytochemistry of diaphragm muscle stained with a utrophin antibody (UtroA) showing sarcolemmal localization in the Utr-TET mice (a) while the control *mdx* littermate has endogenous utrophin only (b). Positive staining with β -sarcoglycan suggests restoration of the dystrophin associated protein complex in the Utr-TET (c) compared to the *mdx* (d) mice. (B) Western blot analysis showing the increased levels of expression of utrophin in the transgenic line.

in *mdx* mice and DMD patients. It was monitored in all mice studied here. In all cases, CPK level remained elevated in UTR-ON's mice ($n = 17$) and was not different from the UTR-OFF's ($n = 4$) and *mdx* controls ($n = 21$): results were scattered within the 1000–10 000 IU/l range, typical of *mdx* mice (19) (while normal levels are around 500 IU/l). As judged from this systemic parameter, the dystrophic process was still active in all our UTR-ON's mice.

Histology

Figure 4A shows the histological analysis of the diaphragm which is the most affected muscle in the *mdx* mouse model and therefore is a useful guide to the degree of prevention of the dystrophic process afforded by the expression of the transgene. The haematoxylin and eosin (H & E) staining clearly shows that the expression of the transgene from early embryogenesis results in a significant reduction in the number of centrally nucleated fibres and a reduction in the degree of necrosis.

Figure 4B shows immunocytochemical analysis of the diaphragm muscle stained with the utrophin antibody. Row (a) shows samples where the transgene has been activated at

different timepoints. When the transgene has been activated at birth or before, high levels of expression are observed. Reduced levels of expression are seen when the transgene is switched on 10 days after birth and repression of the Utr-TET transgene expression does not alter the *mdx* phenotype (UTR-OFF). All time points have age matched littermate control *mdx* animals which underwent parallel tetracycline treatment (row (b)). Figure 4C shows the percentage of centrally nucleated fibres (CNF) in the diaphragm, extensor digitorum longus (EDL) and TA muscles (a, b and c, respectively) from these animals. Using an unpaired *t*-test, the numbers of CNF in the TA muscle was found to be significantly different from the *mdx* controls when utrophin was expressed at all times, but not when it was switched on later. This would perhaps be expected since less rescue in TA muscle has always been observed. The EDL muscle and diaphragm were significantly different at all timepoints with the exception of UTR-OFF as expected. Clearly, earlier expression of the transgene results in greater prevention of the *mdx* pathology. Higher numbers of CNFs were seen in TA muscle compared with the diaphragm consistent with the data shown in Figure 3B which shows that in the TA muscle the transgene is slower to appear at the sarcolemma.

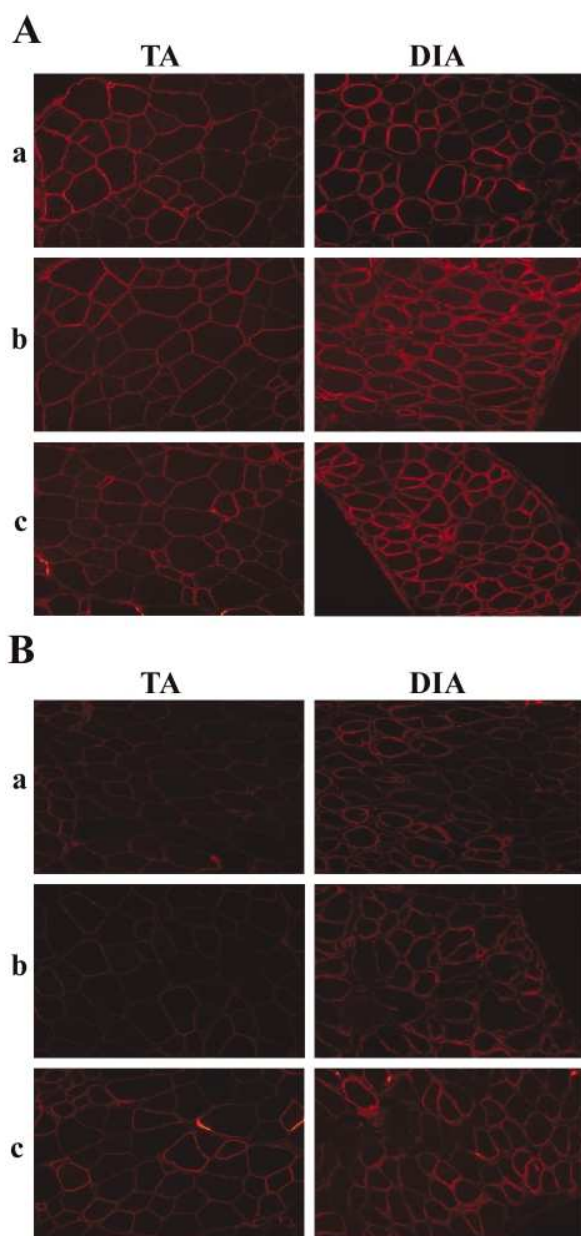


Figure 3. Maintenance and induction of expression of the Utr-TET transgene at the sarcolemma (A) Utr-TET transgene expressed until 6 weeks of age and then repressed for 1 (a), 2 (b) and 3 (c) weeks. (B) Utr-TET transgene turned on at 6 weeks and then analysed at 1 (a), 2 (b) and 4 (c) weeks. Immunocytochemistry was carried out with Utr-A antibody on 8 μ m sections of TA and DIA.

Mechanical responses

Force developed in vivo—escape test. It was shown that the burst of force developed by mice trying to escape in response to a gentle pinch of the tail was about 40% lower for *mdx* mice than for normal ones (20). We previously developed this non-invasive test to evaluate the functional recovery obtained by overexpression of utrophin (truncated and full-length) (10,11). Here we could not detect any significant improvement of the force of the UTR-ON mice in comparison with *mdx* controls.

Mechanical responses of isolated muscles. Studies were made on EDL muscle, as fast-twitch muscles tend to be more affected in the absence of dystrophin (21). It is well documented that dystrophin-lacking muscles show pseudohypertrophy and the EDL weight from *mdx* mouse is typically 30–60% heavier than a normal one. A similar and significant reduction to a normal weight was observed in EDL from UTR-ON $_{\infty}$, -ON $_0$ and -ON $_{10}$ mice where the average weight passed from 15.5 ± 0.4 (*mdx*) to 11.0 ± 0.3 mg (SEM, $n = 15$, $P < 0.01$). No significant reduction was observed for EDL from UTR-ON $_{30}$ and UTR-OFF.

Isometric force developed during tetanic contraction is usually higher in *mdx* muscle, but when this is expressed as normalized force or tension (mN/mm^{-2}), i.e. taking into account the cross-sectional area of the muscle, *mdx* muscles develop about 20% less tension than normal ones. For unknown reasons tension results were very variable both for UTR-ON and UTR-OFF mice as well as for *mdx* controls and no clear conclusion could be drawn from these measurements.

The mechanical parameter most dramatically and specifically affected in dystrophin-lacking muscle is the ability to sustain the high mechanical stress imposed during a series of contractions with forced lengthenings (eccentric contractions; 22,23). It has been proposed that susceptibility to eccentric contractions is responsible for the activity-induced damage so conspicuous in dystrophin-lacking muscles. Typically, the force drop after a series of 5 eccentric contractions (as defined in the Materials and Methods section) can reach 60–85% in *mdx* EDL, while it is of the order of 10–12% in normal muscle. This force drop results from local membrane damage and is accompanied by a proportional penetration of extracellular dye into the muscle fibres (24). Study of the resistance to eccentric contraction is thus the most exacting test to evaluate the recovery obtained by any therapeutic approach and we previously showed that a 100% recovery was obtained by large overexpression of utrophin (11). As seen in Figure 5, the force drop after a series of five eccentric contraction was systematically and significantly reduced for the EDL from UTR-ON $_{\infty}$, -ON $_0$ and -ON $_{10}$ mice, while no recovery was observed for UTR-ON $_{30}$ and UTR-OFF. To correctly assess the importance of the recovery of a given parameter, we previously proposed to give it a recovery score, defined as the ratio (treated *mdx* – untreated *mdx*)/(normal – untreated *mdx*) $\times 100$. Here, for UTR-ON $_{\infty}$, -ON $_0$ and -ON $_{10}$ EDL, the recovery score is about 40%. This reflects a significant improvement of the mechanical resistance of the fibre membrane. However, given the mosaic expression of utrophin among the various fibres (Fig. 1), it is possible that recovery scores of individual fibres (which cannot be measured) were higher or lower than the average 40% value and depended on the amount of utrophin expressed in each fibre.

Density and activity of Ca^{2+} channels

Early measurements had shown that a chronic increase of cytosolic $[\text{Ca}^{2+}]$ was present in *mdx* fibres (25,26), however this observation was not confirmed by several groups (27–30) and the question of an alteration of the intracellular Ca^{2+} homeostasis in *mdx* muscle fibres remains controversial. Instead, there is a general agreement that, both in myotubes and in differentiated

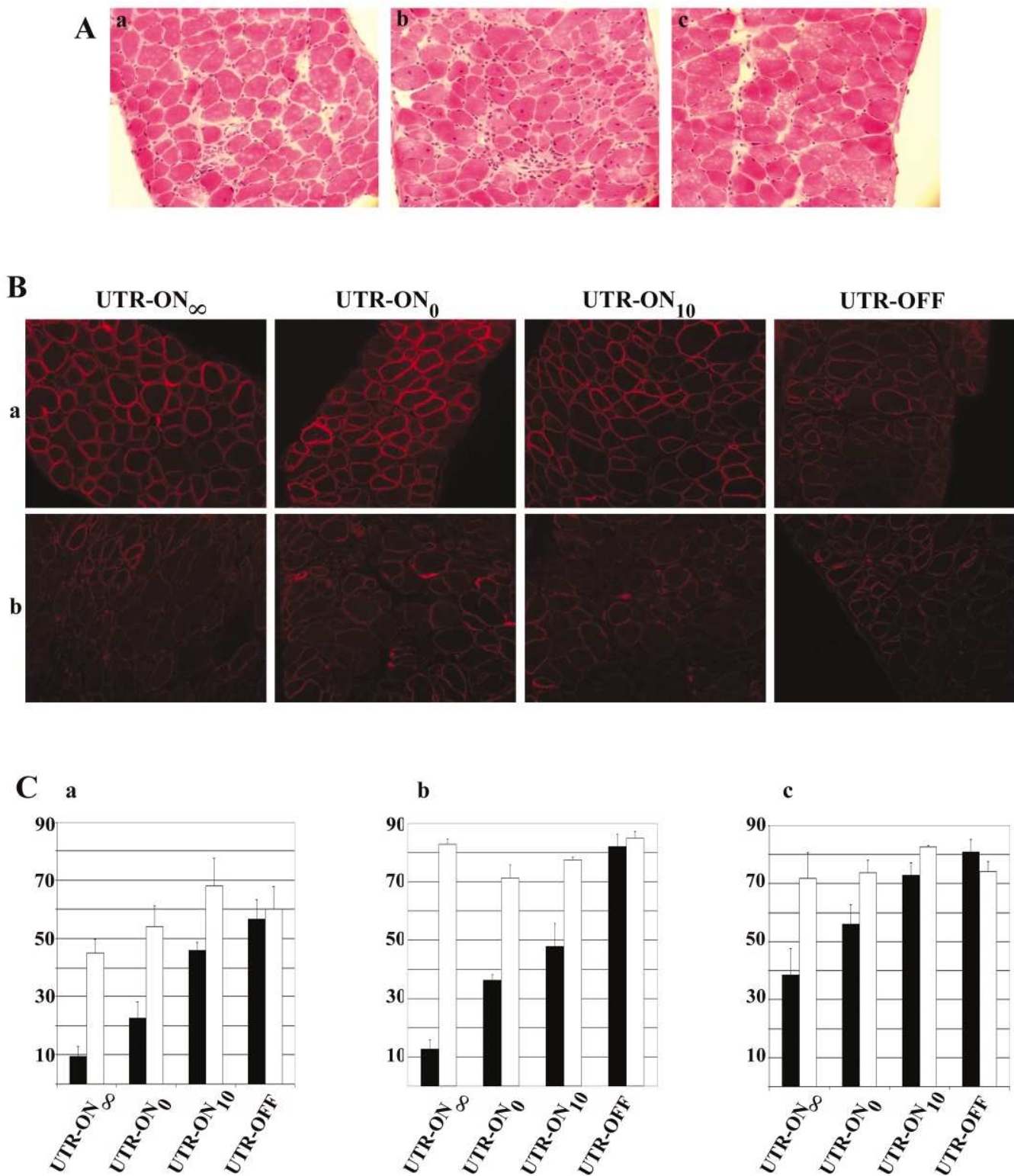


Figure 4. Analysis of the timing of expression of the Utr-TET transgene relative to the prevention of pathology. (A) H & E staining of the diaphragm. Expression of the Utr-TET transgene results in a reduction of centrally nucleated fibres (CNFs) when compared to control mdx littermates (images a and b, respectively). Image c is a healthy pattern in C57/B 110 mice. (B) Immunocytochemical analysis of diaphragm muscle stained with the Utr A antibody. Row (a) shows mice where the transgene has been activated at different timepoints: continuous expression (UTR-ON_∞), switched on at birth (UTR-ON₀), age 10 days (UTR-ON₁₀) and off at all times until analysis age 3–4 months (UTR-OFF). Row (b) shows littermate control mdx animals. (C) Percentage of centrally nucleated fibres (CNF) in diaphragm, EDL and TA muscles (a, b and c, respectively). Expression of Utr-TET is shown in black and mdx in white. Data represents a mean of five samples $\pm$ SEM.

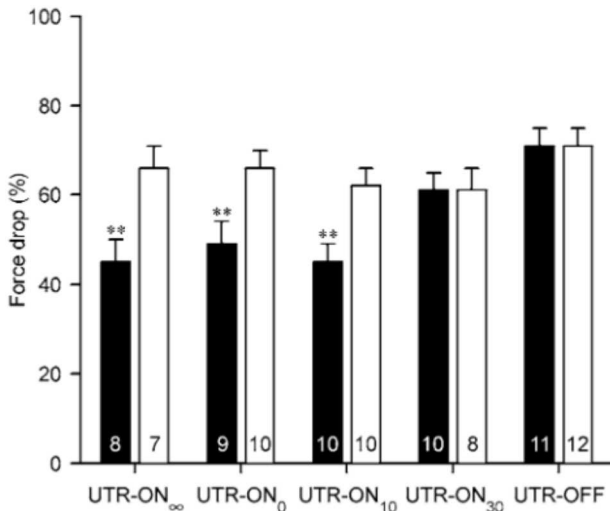


Figure 5. Force drop (%) after a series of eccentric contractions (see text) for the various UTR-transgenic mice (black columns) and their corresponding mdx controls (white columns). Number in each column denotes the number of mice analysed.

fibres, there is a class of Ca^{2+} channels the activity of which is increased in dystrophin-lacking fibres. In cultured myotubes obtained from transgenic mdx mice overexpressing dystrophin, the behaviour of these channels is fully normalized (31). These channels are present in normal fibres and show a marked (though not absolute) selectivity for Ca^{2+} ions; they are spontaneously active at normal membrane potential, moreover, their activity can be changed in response to mechanical stress. We thus studied how the density and activity of these mechanosensitive/voltage independent Ca^{2+} channels were affected in adult fibres from our UTR-ON's mice. Quite recently, we reported a detail study of the characteristics these Ca^{2+} channels in isolated mdx and normal fibres maintained in culture for six days (32). These results will serve here as references.

Ca^{2+} channel density

As explained in the Materials and Methods section, Ca^{2+} channel density was estimated by the fraction of fibres studied that showed the typical spontaneous activity at -60 mV. In our recent study, we observed that soon after collagenase isolation of the fibres, the occurrence of these responding fibres was about the same in mdx and normal fibres (around 80%). However, this occurrence was down-regulated and 3 days after isolation, it was reduced to 45% or less in normal fibres while remaining around 60% or above in mdx fibres. The occurrence of Ca^{2+} channel in our UTR-ON's mice was thus studied at day 3 after isolation when the difference between normal and mdx fibres is well characterized. As seen in Figure 6A, the occurrence of Ca^{2+} channel was significantly reduced to or even below normal level in UTR-ON_∞, -ON₀, -ON₁₀ fibres and to intermediate values in UTR-ON₃₀. There is a clear trend that the longer the time of utrophin expression, the larger the reduction of occurrence. No reduction occurred in UTR-OFF. These studies were made on the toe flexor digitorum brevis (FDB) muscle and it was essential to check if this down

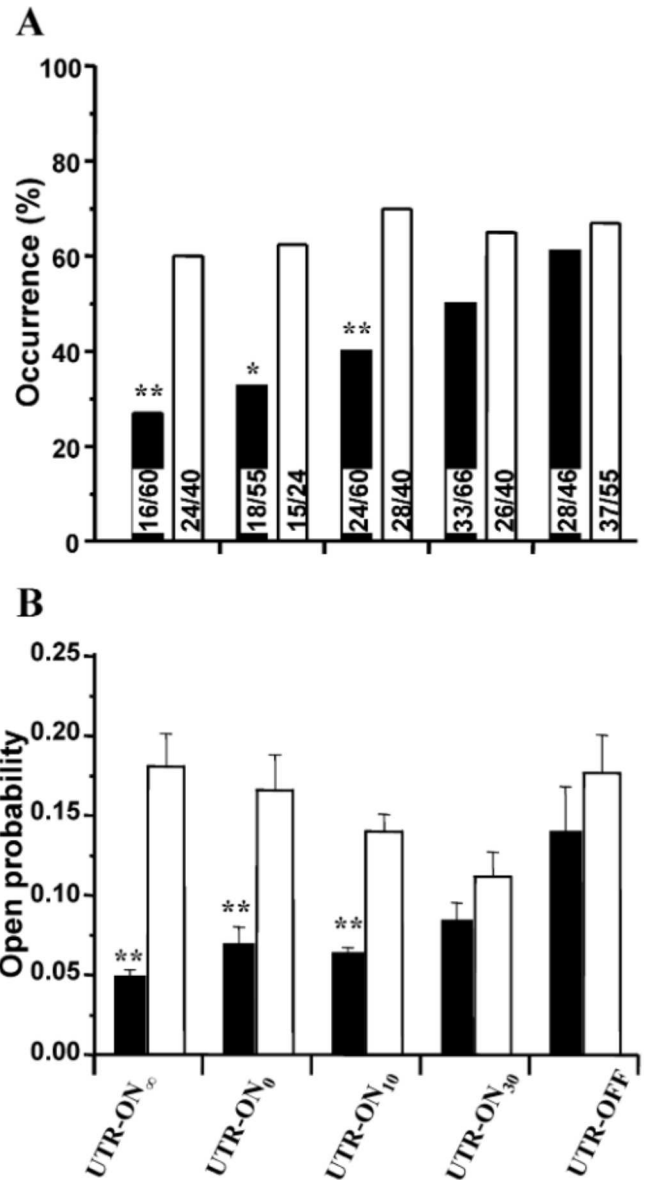


Figure 6. (A) Occurrence of mechanosensitive/voltage independent Ca^{2+} channels in collagenase-isolated single fibres from FDB muscles from the various UTR-transgenic mice (black columns) and the corresponding mdx controls (white columns). Each ratio gives the number of fibres where the Ca^{2+} channel activity was detected over the total number of fibres tested. Fibres were kept in culture for 3 days before being tested. (B) Open probability of the same Ca^{2+} channels studied in A.

regulation of Ca^{2+} channel in UTR-ON fibres was also observed in fibres from a limb muscle. Collagenase-isolated fibres from EDL of UTR-ON₁₀ were studied at day 3. Ca^{2+} channel were detected in 23% (4/17) of the UTR-ON₁₀ fibres and in 58% (7/12) of the mdx fibres.

Ca^{2+} channel activity

An essential measurement to characterize channel activity is the open probability i.e. the fraction of the time of observation

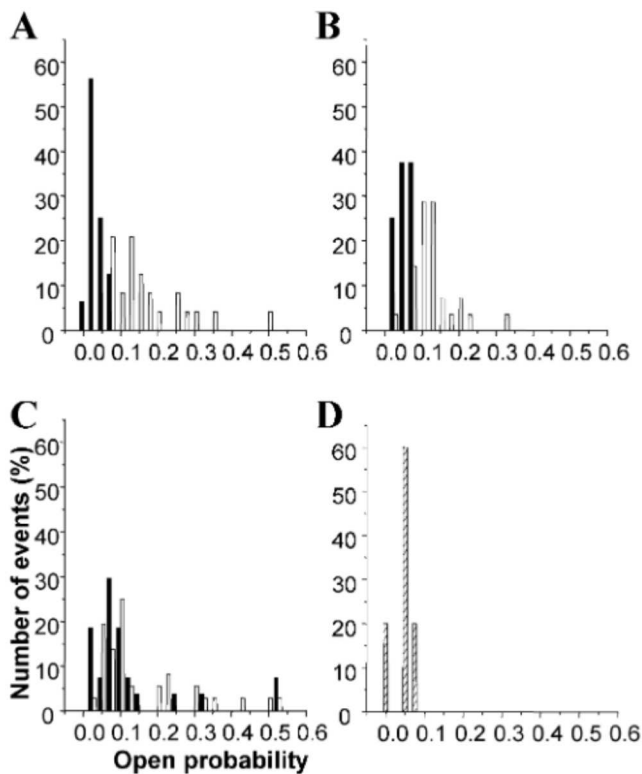


Figure 7. Frequency distribution of the open probabilities of the same Ca^{2+} channels studied in Figure 6B. Black columns: fibres from UTR-transgenic mice, white columns: fibres from the corresponding mdx controls. (A) UTR- ON_{∞} ; (B) UTR- ON_{10} ; (C) UTR-OFF; (D) Data from normal fibres, redrawn from De Backer et al. (2002) (Ref. 32).

when a given channel is open. After collecting the value obtained from different fibres (numbers at the foot of each column of Fig. 6A give the number of fibres studied), the mean open probability could be calculated. This is illustrated in Figure 6B. A sharp and significant reduction of the mean open probabilities was observed in UTR- ON_{∞} , - ON_0 and - ON_{10} fibres compared with the corresponding mdx controls. The difference was smaller and no longer significant for UTR- ON_{30} and UTR-OFF fibres. From the recorded electrical activity during the time of observation (here 2 min), the quantity of charges, here of Ca^{2+} ions, transported through the channel can be calculated (we showed previously (32) that the unitary conductance of these channels was unaffected by the lack of dystrophin). For UTR- ON_{∞} , - ON_0 and - ON_{10} , the average calculated quantity of charges were 2.6, 4.0 and 3.0 pA.s, while it was 6.4 pA.s for UTR- ON_{30} and between 8 and 21 pA.s for UTR-OFF and mdx controls. We reported elsewhere that, in exactly the same experimental conditions, the quantity of charges was around 2.5 pA.s for normal fibres.

The effect of utrophin expression on the Ca^{2+} activity is also well emphasized when the fibre-to-fibre distributions of the open probability are considered. Figure 7 illustrates how the open probability distribution was narrowed within the 0 to 0.1 limits for UTR- ON_{∞} , and - ON_{10} fibres as for the normal fibres (the latter taken from ref. 32), while the values were scattered over the 0–0.55 range for UTR-OFF and control mdx fibres.

Altogether, the density and activity of the mechanosensitive/voltage independent Ca^{2+} channels of adult fibres from UTR- ON_{∞} , - ON_0 and ON_{10} mice presented a return to the values observed in normal fibres, so that the passive Ca^{2+} influx occurring through them was completely normalized. The situation was partially corrected for fibres from UTR- ON_{30} mice, and unchanged in UTR-OFF or control mdx. It is worth recalling that control mdx mice were given tetracycline as were the Utr-TET transgenic mice so that the normalization of Ca^{2+} channel behaviour presented above cannot result from a tetracycline effect.

DISCUSSION

Although the amount of utrophin produced by the utrophin transgene, under the MCK promotor and the tetracycline control, was definitely higher than the spontaneous overexpression in mdx mice, it was still considerably lower than in the transgenic Fiona line we described previously where utrophin was under the control of the HSA promotor (Figs 1 and 4 and ref. 11). In this case, utrophin expression might exceed by 10 times the spontaneous expression in mdx muscles, according to the precise measurement of protein levels (33). In muscles from the Fiona line, all functional parameters studied were normalized, while here we observed a mixed situation: some aspects of the disorder were corrected (dystrophin associated protein complex (DAG) localization, muscle size, Ca^{2+} channel activity), some were partially corrected (resistance to eccentric contractions, percentage of CNF), while others were not (CPK level, muscle force). It could be proposed that CPK remained elevated because dystrophy correction varied greatly from muscle to muscle and that some/many muscles showed no improvement at all. Even if we were not able to examine the dystrophy status of every possible muscle, our results on diaphragm, TA, EDL and FDB were consistent and do not suggest the above interpretation. It seems more likely that the persistence of a certain level of dystrophy was the consequence of an insufficient amount of utrophin produced and not a genuine property of the MCK-Tet-controlled transgenic model. Indeed, we already reported that in some transgenic lines under the HSA promotor, utrophin expression was relatively low and in that case, dystrophy was not fully corrected (11). In addition, it is possible that the FLAG-tagged utrophin gene product is not as functional as the normal full length utrophin. From our time-course analysis of utrophin production and degradation (Fig. 3), we can conclude that the rate of utrophin synthesis is rather slow, though it seems to exceed that of degradation. One would then expect a slow but progressive enrichment of the utrophin content of the membrane. Possibly our standard procedure of evaluating recovery on a 3 month old animal was applied too early. To address this question we later studied a group of 7 month old UTR- ON_{∞} mice for the susceptibility of EDL to eccentric contractions (force drop). In these mice ($n=12$) where utrophin was expressed over an additional 4 month period, the recovery score reached 70%, a definitely better score than at 3 months (40%).

Recovery from the dystrophic process by overexpression of utrophin is thus clearly a dose-dependent phenomenon (10,11). Consequently, two different models of recovery could occur: parallel recovery or sequential recovery. In the first instance, the

extent of recovery of the various structural and functional disorders would progress in parallel and as a function of the amount of utrophin produced. In the second case, correction of the various types of disorders would be obtained for different amounts of utrophin expressed. For low levels of expression, only certain disorders would be corrected; as the level of expression increases, more and more disorders would be corrected until complete correction of all disorders would be obtained for high levels of utrophin (as this was the case for the Fiona transgenic line (11)). Altogether the results presented here and those previously obtained with transgenic mdx mice expressing truncated (10) or full-length utrophin (11) clearly favour the sequential recovery model. From the information available so far, it can be proposed that the disorders resulting from the absence of dystrophin would be corrected in the following sequence as utrophin expression increases: Ca^{2+} channel activity, muscle hypertrophy $\leq$ membrane localization of DAPC $<$ susceptibility to eccentric contractions, centronucleation $<$ isometric force and CPK activity level. Completing and refining this tentative sequence is an urgent task for a better understanding of the role of utrophin as a surrogate for dystrophin.

One of the major interests in developing the Tet-conditioned model was to test the ability of utrophin to alleviate dystrophinopathy when it was expressed after birth. The present results demonstrate that this is the case, at least for the UTR-ON₀ and -ON₁₀ mice. For most UTR-ON₃₀ mice, recovery from dystrophy seemed insignificant; high CNF counts correlated well with several other indices of dystrophy (increased body and muscle weight, reduced force and resistance to eccentric contraction) that showed little or no improvements. Most likely this was due to the low content of utrophin at the time of the evaluation (107 days after tetracycline removal) though it could not be excluded that recovery was genuinely better in young, immature animals, at the time of active muscle growth. In the Tet-inducible dystrophin model (34), no change in CNF was observed, when the dystrophin transgene was turned on after 4–5 weeks of age, or later. The puzzling fact that the central position of the nuclei present an extremely long persistence in fully differentiated, adult mdx fibres (see discussion in ref. 24) makes uncertain the interpretation of this observation, in the absence of the study of more functional parameters.

Our results provide also an unexpected insight into the pathogenesis of the dystrophinopathy. It has been proposed that the increased and sustained activity of some Ca^{2+} channels was the initial disorder triggering a cascade of pathological events leading to necrosis of mdx fibres, through loss of intracellular Ca^{2+} homeostasis and Ca^{2+} -activated proteolysis (35,36). We think that the results reported here suggest re-evaluation of this view. We present an experimental situation where the activity of the Ca^{2+} channels recovered a completely normal behaviour while the classical markers of the Duchenne dystrophic process increased CPK activity (for fibre degeneration) and increased centronucleation (for fibre regeneration) and susceptibility to high mechanical stress were still very far from normal values. These observations question the role of increased Ca^{2+} channel activity in initiating the dystrophic process.

Besides its recognized importance in membrane structural integrity, dystrophin seems to play some—yet ill-defined—role

in transduction of mechanical signals as its presence is required for the fine tuning of a well-defined class of mechanosensitive Ca^{2+} channel, the activity of which is abnormally elevated in its absence (32,37). Indeed, in myotubes obtained from transgenic mdx mice overexpressing dystrophin, Ca^{2+} -leak channels (the developmental equivalent of the Ca^{2+} channels studied here) recover a normal activity (31). Here we showed that overexpression of utrophin in dystrophin-deficient muscles brought back to normal the functional characteristics of these Ca^{2+} channels in adult fibres. This suggests that utrophin could replace dystrophin in its role in the transduction of mechanical signals.

MATERIALS AND METHODS

Transgene construction

The tet-inducer transgenic mouse line was a kind gift from Dr R. Hooft, Glaxo-Wellcome, Geneva, Switzerland. These mice contain the muscle-specific creatine kinase (MCK) promoter driving the tetracycline-regulated transactivator (tTA). For the construction of the MCK-tTA transgene see reference 38. The transgene was bred onto the dystrophin deficient mdx background to produce MCK-tTA/mdx lines to facilitate the future breeding of double transgenic mice onto a dystrophin null background.

A tetracycline regulated utrophin transgenic mouse was made using the murine full-length utrophin cDNA with a FLAG epitope tag at the 5'-end (kind gift from Dr W.X. Guo, Department of Molecular Biology and Pharmacology, School of Medicine, St. Louis, USA) (39). The epitope tagged utrophin cDNA was then cloned into the tetracycline regulated CMV_{min} promoter construct to generate the UTR transgene construct (see Fig. 1). One transgenic line was generated. This was bred onto the mdx background to produce UTR/mdx mice and then crossed with the mdx transgenic line containing the MCK-tTA construct. The double transgenic line, was denoted Utr-TET. For repression of expression of utrophin, the tetracycline was administered in the drinking water at the concentration of 1 mg/ml.

Genotyping

Mice were genotyped by PCR from tail tip DNA. Primer sequences to detect the MCK-tTA/mdx line were as follows: 5' GGAATCGAAGGTTTAACAACCCG 3', and 5' CGAAT-AAGAAGGCTGGCTCTGC 3 and primers for the CMV-full length utrophin/mdx mice were 5' AGTGGACATGGATTG-GACA 3' and 5' GTTTTCATCCACAATCACCA 3'.

Western blotting

Muscle protein extracts were prepared from snap frozen tissue, homogenized in 1 ml extraction buffer (75 mM Tris pH 6.8, 3.8% SDS, 4 M urea, 20% glycerol). Protein was quantified in duplicate using the Biorad DC protein assay kit and samples diluted to an equal volume with extraction buffer/5% β -mercaptoethanol. Generally, 30 μ g protein was run on 8% polyacrylamide gels and transferred to nitrocellulose. The

transgene was detected using a 1/50 dilution of NaHCO₃ and visualized using anti-mouse HRP (Jackson) and chemiluminescence (Roche). C-Src antibody (Santa Cruz) was used to confirm equal loading of the protein. It is a ubiquitously expressed tyrosine kinase which plays a critical role in various transduction pathways.

Histology

Tissues for sectioning were dissected, immersed in OCT compound (BDH) and frozen on liquid nitrogen cooled isopentane. Unfixed 8 µm cryosections were cut and stored at -80°C. For centrally nucleated fibre counts, sections were stained with haematoxylin and eosin. Immunohistochemistry was carried out by blocking sections in 5% fetal calf serum in 50 mM Tris, 150 mM NaCl pH 7.5 (TBS). Primary antibody Utr A (Andrew Weir, unpublished results) was used at a dilution of 1/10 to detect utrophin A with Cy3 conjugated rabbit secondary (Jackson). β-sarcoglycan was detected using a goat primary (kind gift from Dr K. Campbell, Iowa, USA) and FITC conjugated anti-goat secondary (Jackson Laboratories). The anti FLAG antibody was not used as it did not give clear results in our hands.

Creatine phosphokinase activity

After deep anaesthesia (see below), blood was collected by orbital sinus bleeding using heparinized hematocrite tubes. After centrifugation, plasma CPK activity was determined by spectrophotometry making use of the NADPH fluorescence (Roche, 1087533). If activity measurements were not carried out immediately, tubes were stored at -80°C until use. CPK activities, expressed in IU per litre, were calibrated using a purified CPK preparation as reference. Some measurements were made in duplicate.

Escape test

This method for assessing muscle force *in vivo* has been described elsewhere (20,21). Briefly, mice were placed in front of a tube and a cuff was wrapped around the tail and connected to a fixed force transducer. In response to gentle pinching of the tail, mice tried to escape into the tube; this was prevented by the attachment of the tail to the force transducer and a short peak of force was recorded. The procedure was repeated at short intervals, for 2.5 min. The mean of the 5 highest peaks recorded was calculated for each animal and divided by its body weight.

Mechanical responses

Preparation of isolated muscles Animals were deeply anaesthetized with a solution (10 ml/kg, *i.p.*) containing ketamine (10 mg/mE) and xylazine (1 mg/ml) in order to preserve muscle perfusion during dissection of tibialis anterior (TA), extensor digitorum longus (EDL) and flexor digitorum brevis (FDB). Depth of anaesthesia was assessed by the abolition of eyelid and pedal reflexes. After dissection, the animals were killed by rapid neck dislocation. This protocol has been approved by the Animal Ethic Committee of the Catholic University of Louvain, Brussels. TA were used for histology and immuno-

fluorescence studies, EDL were used for mechanical experiments and FDB for the study of Ca²⁺ channel. Diaphragms were removed from other animals from the same litter.

Stimulation of isolated EDL After dissection, EDL muscles were bathed in a continuous flow of oxygenated Krebs solution (O₂ 95%/CO₂ 5%) containing (in mM): NaCl 118, NaHCO₃ 25, KCl 5, KH₂PO₄ 1, CaCl₂ 2.5, MgSO₄ 1, glucose 5, maintained at a temperature of 20°C. One end of the muscle was tied to a force transducer and the other end to an electromagnetic puller. In this setup the muscle length could be changed by a predetermined value and at a selected speed while being monitored (40). Stimulation was delivered through platinum electrodes running parallel to the muscles. Electrical stimuli and isometric force were sampled through a RTI-8 15 AD converter and digitalized. A series of twitches and 0.3 s tetani were applied, in order to determine the length at which isometric tetanus force was maximal. After the recording of representative isometric twitch and tetanus, a 5 min rest was imposed, followed by the eccentric contraction protocol. This consisted in a series of 6 isometric tetani, at 5 min intervals, in which a 7% lengthening of the muscle was applied, at a rate of 1 fibre length/s, when maximal force had been developed (41). Dystrophin-deficient EDL muscles are extremely susceptible to this protocol, which produced a loss of isometric force as eccentric contractions were repeated (22). This loss was presented as the 'force drop' *i.e.* the decrease of the isometric force measured at the sixth eccentric contraction (expressed as a percentage of the initial force).

Density and activity of Ca²⁺ channels

Preparation of isolated muscle fibres. FDB muscles were dissected out and placed in an oxygenated Krebs solution containing 0.2% type IV collagenase (Sigma C-5138), for 45 min, at 37°C. After 2 washes, the muscle was gently dissociated by several passages through a series of Pasteur pipettes of progressively decreasing diameter. Isolated fibres were plated on circular glass coverslips, covered with the Extracellular Matrix Basement Membrane (Harbor Bio-Products) which produced fibre attachment within 2 hours.

Study of Ca²⁺ channels by the patch-clamp technique. This has been described in detail elsewhere (32). In brief, single-channel activity was recorded from cell-attached patches using the technique described (42). Patch electrodes were filled with 110 mM CaCl₂ with 10 mM Hepes and 0.01 mM DIDS (4,4'-diisothiocyanate stilbene-2,2'-disulfonic acid, to block any chloride conductance). The bathing solution was an isotonic potassium aspartate solution containing (mM): 150 K-aspartate, 5 MgCl₂, 10 EGTA and 10 Hepes. The pH was adjusted to 7.4 with NaOH and the osmolarity adjusted to 320–330 mosmol/l with glucose. The potassium aspartate bathing solution was used to zero the cell's resting potential so that the patch potential would be equal to the applied voltage command. Individual fibres were sampled only once, as pipette withdrawal could produce local lesions that might affect channel activity (43). All recordings were made from cell-attached patches at a constant holding potential of -60 mV, at room temperature.

Statistical analysis

Results were expressed as means $\pm$ SEM. For each experimental condition studied, comparison between Utr-TET mice (UTR-ON's or UTR-OFF) and their corresponding mdx mice were made by unpaired t-tests. In the case of the frequency of occurrence of Ca^{2+} channels, differences between Utr-TET and mdx controls were submitted to a χ^2 test. Significance level: * $P \leq 0.05$, ** $P \leq 0.01$.

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