Mild Muscular Features in Tenascin-X Knockout Mice, A Model of Ehlers–Danlos Syndrome


To link to this article: https://doi.org/10.3109/03008207.2010.551616

Published online: 15 Mar 2011.

Submit your article to this journal

Article views: 294

View related articles

Citing articles: 5 View citing articles
Mild Muscular Features in Tenascin-X Knockout Mice, A Model of Ehlers–Danlos Syndrome

N. C. Voermans,1 K. Verrijp,2 L. Eshuis,2 M. C. M. Balemans,3 D. Egging,4 E. Sterrenburg,5 I. A. L. M. van Rooij,6 J. A. W. M. van der Laak,2 J. Schalkwijk,4 S. M. van der Maarel,5 M. Lammens,1,2 B. G. van Engelen1

1Department of Neurology, Donders Institute for Brain, Cognition and Behaviour, Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands, 2Department of Pathology, Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands, 3Department of Human Genetics, Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands, 4Department of Dermatology, Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands, 5Department of Human Genetics, Leiden University Medical Centre, Leiden, the Netherlands, 6Department of Epidemiology, Biostatistics and Health Technology Assessment, Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands

Abstract

Introduction: Tenascin-X (TNX) is an extracellular matrix (ECM) glycoprotein, the absence of which in humans leads to a recessive form of Ehlers–Danlos syndrome (EDS), a group of inherited connective tissue disorders characterized by joint hypermobility, skin hyperextensibility, and tissue fragility. A mouse model of TNX-deficient type EDS has been used to characterize the dermatological, orthopedic, and obstetrical features. The growing insight in the clinical overlap between myopathies and inherited connective tissue disorders asks for a study of the muscular characteristics of inherited connective tissue diseases. Therefore, this study aims to define the muscular phenotype of TNX knockout (KO) mice. Materials and methods: We performed a comprehensive study on the muscular phenotype of these TNX KO mice, consisting of standardized clinical assessment, muscle histology, and gene expression profiling of muscle tissue. Furthermore, peripheral nerve composition was studied by histology and electron microscopy. Results: The main findings are the presence of mild muscle weakness, mild myopathic features on histology, and functional upregulation of genes encoding proteins involved in ECM degradation and synthesis. Additionally, sciatic nerve samples showed mildly reduced collagen fibril density of endoneurium. Discussion: The muscular phenotype of TNX KO mice consists of mild muscle weakness with histological signs of myopathy and of increased turnover of the ECM in muscle. Furthermore, mildly reduced diameter of myelinated fibers and reduction of collagen fibril density of endoneurium may correspond with polyneuropathy in TNX-deficient EDS patients. This comprehensive assessment can serve as a starting point for further investigations on neuromuscular function in TNX KO mice.

Keywords: Ehlers–Danlos syndrome, inherited connective tissue disorder, muscle, peripheral nerve, tenascin-X

INTRODUCTION

Tenascin-X (TNX) is an extracellular matrix (ECM) glycoprotein, the absence of which in humans leads to a recessive form of Ehlers–Danlos syndrome (EDS), a group of related inherited connective tissue disorders [1,2]. TNX-deficient type EDS has a phenotype similar to the classic type EDS with hypermobile joints, hyperextensible skin, and easy bruising, but without atrophic scarring [1–3]. Haploinsufficiency of tenascin-XB (TNXB) is associated with joint hypermobility in females [4].

TNX knockout (KO) mice have been used for detailed study of the dermatological phenotype and to increase insight in the dermatological features of TNX-deficient type EDS [5–8]. Additionally, articular and obstetrical aspects were investigated, the results of which showed that this mouse model only partly reflects the multisystem involvement of TNX-deficient type EDS in human [9,10]. Protein expression studies in TNX KO mice have further suggested a role of TNX in developing and adult muscles; TNX was found to be abundantly expressed in various tissues during embryonic development, among which are perimysium of skeletal muscle and tendons [11,12]. In adulthood, TNX is predominantly expressed in skeletal and cardiac muscle. Therefore, TNX was initially referred to as “the muscle tenascin” [12]. In addition,
in both patients and mice, abnormalities of elastic fibers were observed in skin [13]. TNX was indeed found to be highly expressed in the peripheral nervous system, more specifically in perineurium and endoneurium [14,15]. However, myelin sheath thickness, axonal size, and ultrastructure of the sciatic nerve of TNX KO mice were reported to be normal [15]. Hence, it was suggested that TNX has only a subtle function in peripheral nerve macromolecular organization [15].

The growing insight in the clinical overlap of myopathies and inherited connective tissue disorders such as collagen VI myopathies, EDS, and Marfan syndrome [16–18] calls for a study of the muscular phenotype of inherited connective tissue disorders. In the initial dermatological study, TNX KO mice were reported to have no perinatal abnormalities, exhibit normal growth, and develop progressive skin hyperextensibility, similarly as in EDS patients [5]. Therefore, TNX KO mice probably offer a suitable animal model to study the muscular phenotype of TNX-deficient EDS in more detail. Despite the above-mentioned histological findings, the muscular phenotype of TNX KO mice has not been investigated in detail so far.

This study aims to assess the muscular phenotype of TNX KO mice in a broad approach, consisting of standardized clinical assessment, muscle histology, gene expression profiling on muscle tissue, and peripheral nerve histology and electron microscopy. Such detailed observation will enable a comprehensive assessment of the muscular phenotype of TNX-deficient EDS in more detail. Additionally, it may increase insight in the neuromuscular symptoms in TNX-deficient EDS patients and strengthen the connection between the ECM and neuromuscular features [16].

MATERIALS AND METHODS

Mice

TNX KO mice were obtained from Bristow et al. [3]. These mice have a heterogeneous genetic background. Therefore, mice backcrossed to a C57/BL6 background, as described previously [5,9], were used in these studies. Briefly, → offspring from heterozygous parents was obtained by the backcrossing of TNX KO mice with six generations of C57/BL6 mice. Wild-type (WT) C57/BL6 mice were used as controls. Long-term motor activity assessment was performed in eight female TNX KO mice and eight female WT mice, both at 6 and 16 months. For functional strength measurements, eight female TNX KO mice and eight female WT mice were tested at the ages of 3, 6, and 16 months. Body weight was measured at 16 months of age. Muscle biopsies of the quadriceps muscles for histochemical studies were obtained in six male TNX KO and WT mice of 8 months of age. Gene expression studies were performed on biopsies of the quadriceps muscle of the same male TNX KO and WT mice of 8 months of age. As we detected the presence of axonal sensomotor polyneuropathy in TNX-deficient EDS patients [19], we made a histological and electron microscopic analysis of the sciatic nerve of one TNX KO and one WT mouse of 16 months of age, both females. The experimental design was approved by the animal use committee of Radboud University Nijmegen Medical Centre.

Long-Term Motor Activity Assessment

Straightforward observation of motor behavior of TNX KO mice and WT mice revealed no differences [5]. We therefore assessed long-term motor activity in a home cage-like environment (Phenotypy) as previously reported [20,21]. TNX KO and WT mice (n = 8 in each group; female; 6 and 16 months) were randomly placed in 16 cages with versatile video-based observation system for 1 week (Noldus PhenoTyper, Wageningen, the Netherlands). Distance moved and duration of immobility, mobility, and strong mobility were measured using the mobility detection parameter of the Ethovision video-tracking software (Noldus; immobility threshold setting of 20% and strong mobility threshold of 60%). The level of activity was continuously measured and expressed as mean hourly distance moved (cm) at 6 and 16 months of age. Additionally, we measured mean duration of immobility (s), total duration of mobility (s), and total duration of strong mobility (s) per hour at 16 months of age. Data were recorded digitally and quantified automatically as previously described [20,21].

Functional Muscle Strength Measurement

Functional muscle strength was tested with use of a paw-fall-through test and hang-time test [22]. Mice (n = 8 in each group; female; 3, 6, and 16 months) were placed on a 60 × 40 cm² piece of 1.5-cm mesh hardware cloth, approximately 35 cm above their cage filled with sawdust. During a 1-min observation period, the number of times individual limbs fell through the wire was counted (number of paw-fall-through events in 1 min—“PFT 1 min”). The network was then inverted and the time for the mouse to fall off was recorded during a maximum of 2 min before being placed back into its cage [hang-time duration—“HT dur” (ms)].

Quadriceps Muscle Samples

At 8 months of age, six male TNX KO mice and six male WT littermates were killed by cervical dislocation. The quadriceps femoris muscle samples were obtained bilaterally immediately after death. The material was snap-frozen in chilled isopentane at −140°C. Sections of 10 μm underwent hematoxylin-phloxine staining and were examined under a light microscope. Of each mouse, both left and right sections were studied. Presence of fibrosis was evaluated qualitatively. The number of cells with internal nuclei was measured by evaluating a minimal of 300 fibers of each biopsy and calculating
the percentage of cells with internal nuclei. Up to 3–5% of cells with internal nuclei can be considered normal. A higher percentage of internal nuclei is indicative for myopathy. Fiber diameters were measured in both WT and TNX KO mice using KS400 image analysis software (Zeiss GMBH, Jena, Germany). An interactive digital region growing procedure with possibility for interactive correction was applied to recognize individual muscle fibers. Subsequently, the software calculated the diameter of each muscle fiber automatically. Diameters were converted from pixels to micrometers [23].

Subsequently, immunohistochemical staining with antibodies to collagen I, III, V, VI, TNX, elastin, and laminin α2 was performed. Collagen I, III, and V were selected because they are known to be deficient in the classic or vascular-type EDS, collagen VI because it is deficient in two myopathies (Ullrich congenital muscular dystrophy and Bethlem myopathy) with clinical features overlapping those of EDS [24], and elastin because it is involved in cutis laxa, another inherited connective tissue disorder with muscle weakness. Laminin α2 antibodies were included as a control staining. The following antibodies were used: polyclonal goat anti-collagen I antibodies (2 μ/mL), polyclonal goat anti-collagen III antibodies (2 μ/mL), and polyclonal goat anti-collagen V antibodies (2 μ/mL) were obtained from Southern Biotech (Birmingham, AL, USA); monoclonal mouse anti-human collagen VI antibodies from Chemicon International (Temecula, CA, USA); polyclonal goat anti-rat elastin antibodies from Elastin Products Company (Owensville, MO, USA); and polyclonal rabbit antialaminin a2 antibodies (2 μ/mL) and polyclonal rabbit anti-TNX antibodies from DAKO Cytomation (Glostrup, Denmark) [9]. Frozen sections were fixated in 2% glutaraldehyde in 0.1M Na-cacodylate, postfixed in 1% K-hexacyanoferrat(II).3H2O in 1% osmium tetroxide, and stained with 4% uranylacetate for 30 min and lead citrate for 10 min. For each mouse, images of 10 representative electron microscopic fields of view were recorded using a JEM-1200EX II (Jeol Europe B.V., Nieuw-vennep, the Netherlands) microscope at low-magnification setting (1.2–1.5 k). Image acquisition was performed using digital imaging plate technology for TEM through the Dibis Micron Vario (Ditabis, Pforzheim, Germany). This system uses reusable image plates that are exposed in the TEM identical to classical photo negatives. Plates were read out digitally in a separate system (read out pixel size = 17.5 × 17.5 μm²). Resulting images (size = 4910 × 4340 pixels) were stored as uncompressed tiff files. An image of a carbon replica specimen (2160 lines/mm line replica; EMS, Hatfield, UK) was recorded before acquisition of images for each mouse. Images were analyzed using KS400 image analysis software (Zeiss GMBH). This software uses an adaptive digital region growing algorithm to automatically recognize individual myelinated axons. The inner and outer diameters of each myelinated axon were measured as described previously for the muscle fibers; this implies measurement of the minor axis of the ellipse as the diameter [23].

Statistics
The behavioral data (long-term motor assessment and functional muscle strength measurement) were recorded in a SPSS database (SPSS version 16.0, SPSS Inc., Chicago, IL, USA). Statistical analyses were performed using a Student’s t-test for normally distributed continuous variables and a Mann–Whitney U test for skewed continuous variables.

Differences in density of staining of the muscle biopsies with the various antibodies were calculated with the Mann–Whitney U test. For the analysis of muscle fiber diameters, histograms were produced. The 5th and 95th percentiles of muscle fiber diameter in WT mice were calculated. Subsequently, the percentages of fibers below this 5th and exceeding the 95th percentiles were determined for each mouse biopsy. Percentages in TNX KO mice were compared with WT mice (Mann–Whitney U test).
Histograms of myelinated fiber diameters were produced. The significance of observed differences between distributions was calculated using the non-parametric two-sample Kolmogorov–Smirnov test and the Mann–Whitney U test (central tendency). To find differentially expressed genes, ANOVA analyses were performed on the gene expression data in Rosetta Resolver v4 ($p < 0.001$, Bonferroni correction). Differentially expressed genes were exported and functional annotation was determined using WebGestalt software (Vanderbilt University, Nashville, TN, USA) [26]. A hypergeometric test was performed with the whole gene list as a reference ($p < 0.01$) to find functional groups that were overrepresented in this gene list. Biosemantic analysis was performed using Anni 2.0 (Leiden University Medical Centre and Erasmus Medical Centre, Leiden and Rotterdam, The Netherlands) to find other disorders in which similar genes were shown to be involved in the disease mechanism as in the TNX KO [27].

RESULTS

Long-Term Motor Activity Assessment
As expected, long-term assessment of motor activity showed the longest distance moved during the first night and day and an obvious day–night rhythm for all parameters. Although the TNX KO mice tended to move more at nighttime, no statistically significant differences between the WT and TNX KO mice in hourly walking distance, duration of immobility, duration of mobility, or duration of strong mobility were found at 6 or 16 months of age (Figure 1).

Figure 1. Results of long-term assessment of motor activity showed no statistically significant differences between the WT and TNX KO mice in hourly walking distance, duration of immobility, duration of mobility, or duration of strong mobility at 6 or 16 months of age. (A and B) Graphic representations of hourly distance moved at 6 and 16 months of age (in centimeters), respectively, with standard errors indicated with error bars. (C–E) Graphic representations of hourly duration of immobility, mobility, and strong mobility, respectively, at 16 months of age (in seconds), with standard errors indicated with error bars. No statistical significant differences between TNX KO and WT mice were found.
Functional Muscle Strength Measurement

TNX KO mice showed statistically significantly more paw-fall-through events than the WT mice at 3, 6, and 16 months of age. Furthermore, hang-time duration was shorter in TNX KO mice than in WT mice at all ages; but these differences were not statistically significant (Table 1).

Observation during these tests showed that four of the eight TNX KO mice at 16 months of age hardly moved spontaneously during both trials of the paw-fall-through test. They only slowly moved around in a square of approximately $5 \times 5 \text{ cm}^2$, whereas the WT and other TNX KO mice moved around the whole mesh hardware cloth ($60 \times 40 \text{ cm}^2$). These four KO mice had less than four paw-fall-through events in both trials. Hence, this lack of spontaneous movement on the screen in TNX KO mice might have negatively influenced the results of the paw-fall-through test. Furthermore, results of the hang-time test might have been negatively influenced by the heavier weight of WT mice: at 16 months of age, the mean weight was 30.3 g (SD = 4.8) in WT mice and 23.1 g (SD = 1.5) in the TNX KO mice ($p = 0.03$). The two WT mice with a weight >32 g indeed had a hang-time duration <120 s.

Quadriceps Muscle Samples

Histological analysis of quadriceps muscle biopsies of the TNX KO mice revealed myopathic changes consisting of increase of fiber size variation and increase of internal nuclei (Figure 2A). The number of muscle cells with internal nuclei was higher in TNX KO mice than in WT mice (mean percentage of muscle cells with internal nuclei: 0.76 versus 5.5; $p < 0.001$) (Figure 2B). Furthermore, Mann–Whitney U test in fiber size diameter revealed that the percentage of muscle fibers in the TNX KO biopsies with a diameter below the 5th percentile of WT mice did not differ from the percentage in WT mice and that the percentage of muscle fibers with a diameter above 95th percentile of WT mice was higher in TNX KO mice than in WT mice ($p = 0.012$) (Figure 2C).

For each antibody, the sections showed a consistent pattern of staining intensity. Staining of the collagens I, III, and V, elastin, and laminin α2 did not differ between the two groups. Staining of collagen VI was less in TNX KO mice than in WT mice for perimysial staining ($p = 0.003$); and endomysial collagen VI staining tended to be less ($p = 0.051$). The sections of the TNX KO mice stained negatively for the TNX-antibody as expected, whereas the WT group showed TNX staining of endo- and perimysium. In general, endomysial staining was less than perimysial staining for collagens I, III, V, and VI in both groups, and for TNX in the WT mice. In contrast, laminin α2 staining was more pronounced in the endomysium than in the perimysium (semiquantative data; Table 2), compatible with the transmembrane localization of the two major laminin α2 receptors: the dystrophin–glycoprotein complex and integrins [staining with anti-TNX and

**Table 1. Results of hang-time and paw-fall-through tests**

<table>
<thead>
<tr>
<th></th>
<th>3 months</th>
<th>6 months</th>
<th>16 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFT 1 min: mean (SD)</td>
<td>W T mice n = 8</td>
<td>TNX KO mice n = 8</td>
<td>W T mice n = 8</td>
</tr>
<tr>
<td></td>
<td>2.3 (1.2)</td>
<td>2.3 (0.7)</td>
<td>3.5 (1.0)</td>
</tr>
<tr>
<td></td>
<td>$p = 0.004$</td>
<td>$p = 0.004$</td>
<td>$p = 0.004$</td>
</tr>
<tr>
<td>HT dur (ms): median (range)</td>
<td>W T mice n = 8</td>
<td>TNX KO mice n = 8</td>
<td>W T mice n = 8</td>
</tr>
<tr>
<td></td>
<td>41 (1.0)</td>
<td>41 (1.0)</td>
<td>59 (30–120)</td>
</tr>
<tr>
<td></td>
<td>$p = 0.003$</td>
<td>$p = 0.003$</td>
<td>$p = 0.003$</td>
</tr>
<tr>
<td></td>
<td>120 (75–120)</td>
<td>120 (75–120)</td>
<td>93 (34–120)</td>
</tr>
<tr>
<td></td>
<td>NS $^b$</td>
<td>NS $^b$</td>
<td>NS $^b$</td>
</tr>
</tbody>
</table>

*Student’s t-test.
†Mann–Whitney U test.
Figure 2. Histological analysis of quadriceps muscle biopsies of the TNX KO mice revealed myopathic changes consisting of increase of fiber size variation and increase of internal nuclei (n = 6 in both groups; biopsies of each mouse muscle were taken bilaterally). (A) HE staining of biopsy of one of the TNX KO mice (male, 8 months), showing myopathic features consisting of increased number of internal nuclei and increased variance of fiber diameter (bar = 0.05 mm = 50 μm). Normally, nuclei in muscle cells are located peripherally and immediately below the sarcolemma. Internal nuclei are nuclei that are located anywhere else; the arrow points to one of the internal nuclei. This is a selection of an area in which these myopathic features were most pronounced (lower image). In comparison, a normal biopsy of a WT mouse (male, 8 months) is presented above. (B) Percentage of muscle cells with internal nuclei in WT and TNX KO mice. The outliers are indicated as * and °. The Mann–Whitney U test was used to test the differences, which showed that internal nuclei were more frequent in TNX KO mice (p < 0.001). (C) Mann–Whitney U test in fiber size diameter revealed that the percentage of muscle fibers in the TNX KO biopsies with a diameter below the 5th percentile of WT mice did not differ from the percentage in WT mice, and that the percentage of muscle fibers with a diameter above 95th percentile of WT mice was higher in TNX KO mice than in WT mice (p = 0.012). The vertical lines indicate the 5th and 95th percentiles of the diameters in WT mice.

Table 2. Semiquantitative evaluation of immunohistochemical staining of ECM molecules in muscle

<table>
<thead>
<tr>
<th></th>
<th>Collagen I</th>
<th>Collagen III</th>
<th>Collagen V</th>
<th>Collagen VI</th>
<th>Laminin</th>
<th>Elastin</th>
<th>Tenascin-X</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E</td>
<td>P</td>
<td>E</td>
<td>P</td>
<td>E</td>
<td>P</td>
<td>E</td>
</tr>
<tr>
<td>WT overall score</td>
<td>0.9</td>
<td>2.6</td>
<td>1.6</td>
<td>2.8</td>
<td>2.0</td>
<td>2.6</td>
<td>2.2</td>
</tr>
<tr>
<td>TNX KO overall score</td>
<td>1.0</td>
<td>2.7</td>
<td>1.8</td>
<td>2.9</td>
<td>2.3</td>
<td>2.6</td>
<td>2.2</td>
</tr>
<tr>
<td>Differences in staining *</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.003</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>P</td>
<td>E</td>
<td>P</td>
<td>E</td>
<td>P</td>
<td>E</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>2.4</td>
<td>0.8</td>
<td>2.2</td>
<td>0.7</td>
<td>2.2</td>
<td>1.7</td>
</tr>
</tbody>
</table>

Immunohistochemical staining of the collagens, laminin, and elastin did not differ between the two groups, except collagen VI staining of the perimysium. The sections of the TNX KO mice stained negative for the TNX-antibody as expected, whereas the WT group showed TNX staining of endo- and perimysium. Endomysial staining was generally less than perimysial staining for collagens I, III, V, and VI, and elastin in both groups, and for TNX in the WT mice. In contrast, laminin staining was more pronounced in the endomysium than in the perimysium. Mean score of density of staining, based on semiquantitative evaluation: 0, absence; 1, limited staining; 2, moderate staining; 3, considerable staining; 4, strong staining. TNX, tenascin-XE; KO, knockout; WT, wild type; E, endomysium; P, perimysium.

* Mann–Whitney U test.

NS, not statistically significant.
Gene Expression Profiling

The muscle function of TNX KO mice was further studied with genome-wide transcriptome analysis. Total RNA was isolated from quadriceps muscle of 8-month-old WT (n = 6) and TNX KO mice (n = 6) and hybridized to Illumina Mouse Sentrix-6 BeadChips. ANOVA statistical analysis of the microarray data revealed 266 genes differentially expressed (p < 0.001, Bonferroni corrected). Of these, 74 were down-regulated and 192 were up-regulated. [Supplemental Table S1 available online at http://informahealthcare.com/doi/suppl/ (10.3109/03008207.2010.551616)]. Annotation of the genes with the Webgestalt software enabled the different genes to be classified according to function [Supplemental Table S2 available online at http://informahealthcare.com/doi/suppl/ (10.3109/03008207.2010.551616)]. Table 3 shows a summary of the functional groups that are overrepresented in the list of differentially expressed genes in TNX KO mice (cellular components: lysosome, ECM, and cell surface; molecular function: carbohydrate binding, peptidase activity, and structural molecule activity; biological process: inflammatory response, proteolysis, and vascular development). Striking is that the genes in these functional groups are mainly upregulated. The increased expression of matrix metalloproteinases (MMPs) was reported before in skin of TNX KO mice [28] and is also observed in the muscles (MMP2, MMP3, Mt-1). The biosemantic analysis showed a list of muscular disorders in which similar genes were shown to be involved in the disease mechanism as in the TNX KO mice. These neuromuscular disorders are amyotrophic lateral sclerosis, nemaline myopathy, minicore myopathy, Charcot-Marie-Tooth disease, Duchenne muscular dystrophy, and spinal muscular atrophy.

Table 3. Functional groups overrepresented in TNX KO mice

<table>
<thead>
<tr>
<th>Functional group overrepresented</th>
<th>Number of genes</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cellular component</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysosome</td>
<td>10</td>
<td>6.72E–06</td>
</tr>
<tr>
<td>Extracellular matrix</td>
<td>15</td>
<td>5.19E–05</td>
</tr>
<tr>
<td>Cell surface</td>
<td>7</td>
<td>2.93E–03</td>
</tr>
<tr>
<td><strong>Molecular function</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbohydrate binding</td>
<td>17</td>
<td>5.96E–08</td>
</tr>
<tr>
<td>Peptidase activity</td>
<td>20</td>
<td>1.42E–04</td>
</tr>
<tr>
<td>Structural molecule activity</td>
<td>14</td>
<td>8.75E–03</td>
</tr>
<tr>
<td><strong>Biological process</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflammatory response</td>
<td>15</td>
<td>2.83E–08</td>
</tr>
<tr>
<td>Proteolysis</td>
<td>24</td>
<td>6.83E–06</td>
</tr>
<tr>
<td>Vascular development</td>
<td>8</td>
<td>1.80E–03</td>
</tr>
</tbody>
</table>

Summary of the functional groups that are overrepresented in the list of differentially expressed genes in TNX KO mice.
Sciatic Nerve Specimen

Electron microscopy of the sciatic nerve revealed many areas with lower density of the connective tissue fibrils in TNX KO mouse in comparison with that in the WT mouse (qualitative evaluation; Figure 4A). Furthermore, a few signs of degeneration and regeneration were seen in the sciatic nerve of the TNX KO mouse. Histometry of the myelinated fibers of the sciatic nerve showed smaller inner and outer diameters in the TNX KO mouse (outer diameters; Figure 4B). In addition, results of the two-sample Kolmogorov–Smirnov test and Mann–Whitney U test showed that the diameter distributions and central tendencies differed significantly between the TNX KO mouse and the WT mouse for both inner and outer diameters (Table 4).

DISCUSSION

The main findings of this study on the muscular phenotype of TNX KO mice are normal long-term

Figure 4. Electron microscopy of the sciatic nerve in a TNX KO mouse revealed zones with lower density of the connective tissue fibrils, signs of degeneration and regeneration of myelinated fibers, and smaller inner and outer diameters of myelinated fibers. (A) Electron microscopy of the sciatic nerve revealed zones with lower density of the connective tissue fibrils in TNX KO mice (lower image) in comparison with the WT mice (upper image). The enlarged box in both images magnifies the lower density of connective tissue fibrils in TNX KO mice. Furthermore, more signs of degeneration (asterisks) and regeneration were seen in the TNX KO biopsy (bar = 1 μm). The inner diameter (ID) and outer diameter (OD) of the myelinated fibers have been indicated, (B) Histogram of outer diameter of myelinated fibers shows a mild shift to the smaller diameters in the TNX KO mouse. Diameters on x-axis represent the outer diameter of myelinated axons, and the density on the y-axis represents the frequency of the axons of a specific diameter, normalized for the surface measured.

Table 4. Diameters of myelinated fibers of sciatic nerve in TNX KO and WT mice

<table>
<thead>
<tr>
<th>Diameter of myelinated fibers</th>
<th>Myelinated fibers in WT mouse (n = 320)</th>
<th>Myelinated fibers in TNX KO mouse (n = 377)</th>
<th>Distribution shape difference*</th>
<th>Central tendency†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inner diameter [median (P25–75) in mm]</td>
<td>2.3 (1.5–3.6)</td>
<td>2.1 (1.3–3.1)</td>
<td>p = 0.01</td>
<td>p = 0.02</td>
</tr>
<tr>
<td>Outer diameter [median (P25–75) in nm]</td>
<td>4.2 (2.8–5.8)</td>
<td>3.8 (2.4–5.3)</td>
<td>p = 0.02</td>
<td>p = 0.005</td>
</tr>
</tbody>
</table>

Results of the two-sample Kolmogorov–Smirnov test and Mann–Whitney U test showed that the diameter distributions and central tendency differed significantly between the TNX KO mouse and the WT mouse for both inner and outer diameters. TNX, tenascin-XE; KO, knockout; WT, wild type.

*Two-sample Kolmogorov–Smirnov test.
†Mann–Whitney U test.
spontaneous locomotor activity, the presence of mild functional muscle weakness, mild myopathic features on histology, and functional upregulation of genes encoding proteins involved in degradation and the synthesis of the ECM in muscle. Additionally, the sciatic nerve specimen showed mildly reduced collagen fibril density of endoneurium between these fibers. Together, these findings point to mild changes in muscle function and composition and possibly to altered endoneurium composition in TNX KO mice. Furthermore, the results of gene expression profiling suggest a possible pathophysiological role of TNX deficiency in myopathy in EDS. These findings are discussed below.

TNX KO mice performed less at the paw-fall-through test, whereas differences in performance on the hang-time test were not statistically significant. These differences may have been influenced by the higher weight and longer distances moved in WT mice; a higher weight may predispose to shorter hang-time duration, and the mice that move most on the mesh are at risk for more paw-fall-through events. If we had corrected for these parameters, differences between TNX KO and WT mice might have been larger. What causes this higher weight in WT mice at 16 months of age has not been investigated further; it might be due to increase of fat, muscle tissue, or body size, or due to water retention or differences in bone mass. Osteoporosis and osteopenia is reported in EDS [2], and it has been suggested that changes found in skin collagen also occur in bone collagen [29]. Furthermore, the TNX KO mice tended to move slightly more during the long-term spontaneous locomotor activity task, but these differences were not statistically significant. Most likely, muscle strength is only mildly reduced in TNX KO mice, and this reduction does not interfere with spontaneous walking of mice held captive.

Histological analysis of the muscle biopsies revealed mild to moderate myopathic features in the majority of mice; this was confirmed by quantitative analysis of percentage of cells with internal nuclei and of variation of fiber size diameter. This was more pronounced than the myopathic features detected in muscle biopsies of TNX-deficient EDS patients [19]. This might be related to relatively old age of these mice (8 months for mice is late adulthood). The presence of even more pronounced myopathic features that we detected in very old TNX KO mice (22 months; data not shown) supports this. A progression of myopathic features with aging is also observed in other myopathies and muscular dystrophies. Furthermore, no differences in collagens I, III, and V, elastin, and laminin \( \alpha 2 \) staining were found; but perimysial collagen VI staining was less in TNX KO mice. This is compatible with previous findings of Minamitani et al. [30,31] and our previous observation in a patient with TNX-deficient type EDS [18].

The results of gene expression profiling show a significant upregulation of genes encoding structural ECM components as well as genes involved in synthesis and degradation of the ECM. This probably results from altered interstitial fibroblast function in muscle, which has been shown to contribute significantly to the deposition of the ECM in skeletal muscle [32]. These genes are, according to the available literature, also differentially expressed in various other myopathies such as nemaline myopathy (\textit{TPM2}) [33] and minicore myopathy (\textit{SEPN1}) [34]. TNX deficiency in perimysium, and to a lesser extent in endomysium, may play a role in the development of myopathic features and functional muscle weakness in TNX KO mice.

The sciatic nerve histology and electron microscopy revealed mildly reduced diameter of myelinated fibers and reduction of collagen fibril density of endoneurium between these fibers. This might correspond with the presence of mild axonal polynuropathy in TNX-deficient EDS patients [19]. Clearly, this observation requires further investigation in a larger number of mice. Our findings are in contrast with the results of Matsumoto et al. [15], who showed that the thickness of myelin sheaths and the size of the individual axons in these mice appeared normal, and that the ultrastructures of the sciatic nerves of TNX KO mice were similar to those of WT mice. However, quantization of the number and size of sciatic nerve axons from WT and TNX KO mice in this study did show a trend toward smaller axons in TNX KO mice [15]. The larger differences between TNX KO and WT mice in our study might be related to the older age of mice.

Tenascin-C (TNC), another member of the tenascin family, is predominantly expressed in tendons and ligaments, peripheral and central nervous system, and the ECM of tumor stroma [35–37]. TNC modulates the adhesion of cells to fibronectin and can be classified as an adhesion-modulating ECM protein. Reduced muscle strength in TNC KO mice has been described, consisting of reduced grip strength and lower latency to fall on the wire hanging test [38]. Another comparison can be made with lysyl hydroxylase-I KO mice, a mice model of the kyphoscoliotic type of EDS [39]. These mice show difficulty in locomotion, most likely due to laxity or dislocation of the joints, enhanced by general weakness of the muscles [39]. Neuromuscular function could also be studied in more detail in this mice model.

The results of this animal study strengthen our recent finding of mild to moderate neuromuscular involvement in patients with various types of EDS [19]. This consisted of axonal sensorimotor polynuropathy in the TNX-deficient type and mixed myopathic–neurogenic or myopathic features on electromyography in all patients. Hence, with their mild muscular features, the TNX KO mice form a good fit to the mild neuromuscular phenotype in EDS patients.

Our study has several limitations. First, the use of animals of an advanced age raises doubt whether the abnormalities are of pathological or of biological interest. Furthermore, the use of animals of different ages and different genders for various investigations may
complicate the interpretation of the results. However, due to its explorative design, these results could serve as a starting point for further physiological studies on muscle function in TNX KO mice.

To summarize, the muscular phenotype of TNX KO mice consists of mild muscle weakness with histological signs of myopathy and of increased turnover of the ECM in muscle. Furthermore, mildly reduced diameter of myelinated fibers and reduction of collagen fibril density of endoneurium may correspond with polyneuropathy in TNX-deficient EDS patients. Together, these results strengthen the clinical overlap of myopathies and inherited connective tissue disorders caused by ECM defects [16,18,24,40], and thus support the concept that a normal composition of the ECM is important for adequate functioning of muscle and maybe also of peripheral nerve. Furthermore, the results show that this mouse model can be used for further investigations on the influence of TNX deficiency on muscle and peripheral nerve function. Quantitative muscle function testing of isolated muscles and muscle groups will enable the direct measurement of the influence of the ECM alterations on muscle function [41]. Eventually, this may lead to studies on treatment approaches such as training or pharmacological interventions, similarly as in a mouse model of collagen VI myopathies [42].

ACKNOWLEDGMENTS

We are grateful to Mr. L. Lubbers and Mrs. I. Van der Zee for their assistance in performing the Phenotyper studies; to Mrs. I.v.d. Brink for her assistance in performing the quadriceps biopsies; and to Mrs. D. Smits, Mr. J. Mooren, and Mrs. H. Janssen-Wagenaar for their assistance in maintaining the population of mice.

N.V. was supported by a grant of the NWO (Netherlands Organization for Scientific Research).

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

REFERENCES


