Myotonic Dystrophy Transgenic Mice Exhibit Pathologic Abnormalities in Diaphragm Neuromuscular Junctions and Phrenic Nerves

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Abstract

Myotonic dystrophy Type 1 (DM-1) is caused by abnormal expansion of a (CTG) repeat located in the DM protein kinase gene. Respiratory problems have long been recognized to be a major feature of this disorder. Because respiratory failure can be associated with dysfunction of phrenic nerves and diaphragm muscle, we examined the diaphragm and respiratory neural network in transgenic mice carrying the human genomic DM-1 region with expanded repeats of more than 300 CTG, a valid model of the human disease. Morphologic and morphometric analyses revealed distal denervation of diaphragm neuromuscular junctions in DM-1 transgenic mice indicated by a decrease in the size and shape complexity of end-plates and a reduction in the concentration of acetyl choline receptors on the postsynaptic membrane. More importantly, there was a significant reduction in numbers of unmyelinated, but not of myelinated, fibers in DM-1 phrenic nerves; no morphologic alterations of the nerves or loss of neuronal cells were detected in medullary respiratory centers or cervical phrenic motor neurons. Because neuromuscular junctions are involved in action potential transmission and the afferent phrenic unmyelinated fibers control the inspiratory activity, our results suggest that the respiratory impairment associated with DM-1 may be partially due to pathologic alterations in neuromuscular junctions and phrenic nerves.

Key Words: Cervical motor neurons, Diaphragm neuromuscular junctions, Medullary respiratory neurons, Morphometric analysis, Myotonic dystrophy, Phrenic nerves, Respiratory failure.

INTRODUCTION

Myotonic dystrophy or dystrophia myotonica (DM-1), also known as Steinert disease, is an autosomal dominant progressive muscle disease characterized by myotonia and multigorgan damage that combines various degrees of muscle weakness, arrhythmia and cardiac conduction disorders, cataract, endocrine damage, sleep disorders, and baldness. It is the most frequent of the adult-onset muscular dystrophies; its prevalence is estimated at 1 per 20000 (1, 2). All DM-1 mutations appear as unstable amplifications of a CTG trinucleotide repeat in the 3'-untranslated region of a protein kinase gene (DMPK) on chromosome 19q. Mildly affected patients have 50 to 150 repeats, “classic” DM patients have 100 to 1,000, and congenital cases can have more than 2,000 repeats. Triplet size correlates significantly with the severity of the disorder, and a greater number of repeats are observed with each new generation and are associated with a more severe phenotype, a phenomenon known as anticipation (3, 4).

DMPK transcripts carrying CUG expansions are retained in the nucleus and have a deleterious effect of alternative splicing of other RNAs. This dominant toxic effect of the expanded transcripts plays a central role in the multisystemic features of DM-1 (5). Several clinical studies have reported that DM-1 patients frequently develop respiratory failure and pneumonia as a result of alveolar hypoventilation, particularly in the later stages of the disease (6–11). Indeed, involvement of the respiratory system is probably the major factor contributing to mortality in adult DM-1 patients (11, 12). Other studies have reported that DM-1 patients are particularly susceptible to respiratory infections and have a high incidence of respiratory distress after general anesthesia (13–15). Moreover, attention has been focused on the fact that respiratory distress constitutes the principal problem in neonatal forms of DM-1 (16, 17).

Anatomic, radiologic, and electromyographic evidence indicate that the causes of this respiratory failure are mainly due to the involvement of respiratory diaphragmatic muscle in the dystrophic and myotonic process (7, 11, 18) and to a decreased response to neurogenic stimuli (19, 20). Radiologic and autopsy examinations have shown unilateral elevation of the diaphragm in DM-1 patients, and autopsies have revealed degenerative changes in diaphragmatic muscles (21–24). Electrophysiologic studies revealed reduced relaxation of the diaphragm and marked irregularity in breathing patterns (1, 25–27). These studies have concluded that in addition to the primary abnormalities of the respiratory muscles themselves, the neural network that controls respiratory drive is probably affected. However, at present, there are no direct histologic studies indicating the type of neuro-pathologic alterations that can cause respiratory failure in DM-1.

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Transgenic mice carrying the human genomic DM-1 region and expressing human DM-1 phenotypes (28, 29) are a valid tool to examine the structural changes in several organs and systems that cannot usually be studied directly on patients. To identify pathologic structural alterations that may be involved in respiratory failure in DM-1, we performed a morphologic and morphometric analysis of the diaphragm-phrenic nerve complex and the cervical motor neurons and brainstem nuclei involved in breathing in DM-1 transgenic mice.

**MATERIALS AND METHODS**

**Generation of DM-1 Transgenic Mice**

Transgenic mice carrying the human genomic DM-1 region with expanded repeats either of more than 300 CTG or with the normal 20 CTG repeats were produced and screened by the group of G. Gourdon, as previously described (28, 29). The homozygous transgenic mice used in this study carried 400 to 550 CTG repeats (DM-1 mice) and displayed abnormalities in skeletal muscles that are similar to those observed in DM-1 patients. Transgenic mice expressing 20 CTG repeats (DM-20) do not develop these changes and were used as an internal control (28, 29).

**Animal Perfusion and Tissue Preparation**

All animal procedures were conducted according to local guidelines for care and manipulation of experimental animals. Thirty-six mice (10 months of age) were used in this study: 12 DM-1 transgenic mice (body weight, 25.3 ± 3.1 g), 12 DM-20 transgenic mice (internal control; body weight, 31.7 ± 3.7 g), and 12 C57BL/6J wild-type mice (genetic background of the DM-1 transgenic mice; body weight, 32.8 ± 2.8 g). The DM-20 and wild-type mice are both called controls. Mice were deeply anesthetized with pentobarbital (75 mg/kg) then transcardially perfused with 0.1 mol/L phosphate-buffered saline (PBS) containing 0.1% heparin and 0.1% procaine, followed by a fixative solution (4% paraformaldehyde in 0.1 mol/L PBS at pH 7.4). The brainstem, cervical spinal cord, right and left phrenic nerves, and diaphragm muscles were carefully dissected and removed from each animal. The tissues were postfixed for 2 hours at 4°C in the same fixative solution and then washed in PBS. The brainstems, cervical spinal cords, and the diaphragm muscles were cryoprotected in 30% sucrose overnight and then frozen in liquid nitrogen and stored at −80°C. Phrenic nerve segments were postfixed in 2% osmium tetroxide in PBS for 3 hours, dehydrated in a series of graded ethanol, and embedded in Epon.

**Diaphragm Muscle Section Preparation for Neuromuscular Junction Analysis**

Serial 20-μm-thick longitudinal sections from diaphragm muscles were cut with a cryostat and mounted on microscope slides. Sections were incubated for 45 minutes in 1 μg/ml of tetramethylrhodamine-conjugated α-bungarotoxin (α-BTX; Molecular Probes, Invitrogen, Burlington, CA) at room temperature to stain acetylcholine receptors (AChRs). Sections were washed in PBS and blocked in 0.1% triton, 10% horse serum in PBS for 30 minutes before incubation overnight, at +4°C with the primary polyclonal antibody (1:200), and directed against the 200-kd neurofilament protein (AB1982; Millipore, Billerica, MA). After washing with PBS, the muscle sections were incubated for 2 hours with a 1:200 dilution of the secondary antibody conjugated to Alexa Fluor 488 (Invitrogen).

Muscle sections were observed under fluorescent microscopy (Zeiss, Axioplan 2) and photographed using an image acquisition program (Zeiss, Axiovision with AxioCam HRC). Images were imported into an image-processing program (ImageJ 1.34; National Institutes of Health, Bethesda, MD) to analyze all end plates (EPs) on the sections systematically, that is, there was no random selection. To avoid counting and measuring the same junction twice, every third section was used. To determine whether the EPs are innervated, the nerve fibers were followed on several sections to determine whether they made contact with the EP. The number of EPs was estimated by counting the number of EPs that lack the axon terminal and the number of EPs that were in clear contact with the nerve terminal. The morphometric parameters of every EP were determined by drawing the highly labeled postsynaptic membrane following the edge of each fold. The shape complexity was estimated using the formula \(P^2/(4π \times A)\) (30–32), which was adapted to the pretzel-like shape of the EPs by multiplying it by the ratio between the perimeter and the maximum Ferret diameter to take into account the folds of the neuromuscular junction (NMJ): \(C = P^2/(4π \times A \times D_f)\), where \(P\) indicates perimeter; \(A\), area; and \(D_f\), Ferret diameter. This formula is dimensionless and is not influenced by the overall size of the objects.

The fluorescence intensity of rhodamine-α-BTX labeling at the EPs was measured using a quantitative fluorescence imaging technique. To calculate the intensity of pixels within the EP region, the background fluorescence was approximated by selecting a boundary region around the junction, and after subtracting it from the original image, the total fluorescence of the pixels inside the EP was measured. More than 1,400 EPs were measured from 8 mice in each mouse line. All analyses and counts were performed by observers blinded to the animal genotype. For histologic muscle fiber examination, 8-μm-thick transverse sections of diaphragm muscle were prepared and stained with Harris hematoxylin and eosin.

**Phrenic Nerve Section Preparation for Morphologic and Morphometric Analysis**

Semithin transverse sections (1 μm) were cut at different levels from phrenic nerve trunks. The sections were stained with toluidine blue for light microscopy and morphometric analysis. For electron microscopy examination, ultrathin sections (i.e. 80 nm) were cut and mounted on slot grids, stained with uranyl acetate and lead citrate, and then viewed in the Zeiss EM 10C electron microscope. The morphometric analysis was performed as described in detail in our previous study (33), and all counting and measurements were performed blinded to experimental conditions. Using the StereoInvestigator program (MBF Bioscience, Williston, VT), the total numbers of myelinated axons were counted on...
FIGURE 1. Cryostat sections of diaphragm muscle stained with rhodamine αbungarotoxin (red) and neurofilament antibody (green). End plates (EPs) and axons are visible in control mice (wild type [A–E]; internal control [F–J]) and in myotonic dystrophy Type 1 (DM-1) mice (K–O). In controls, nearly all EPs are innervated by branches of axons, whereas EPs without any contact to axon terminals are easily identified in DM-1 mice. Representative higher magnification images illustrate a single EP in control mice (wild-type [C–E]; internal control [H–J]) and DM-1 transgenic mice (M–O). In DM-1 mice, most EPs are smaller and have less complex shapes than in the controls.
8 semithin nerve sections taken from DM-1 (n = 8), internal control (n = 8), and wild-type (n = 8) mice. Briefly, using a cursor, each fiber on the whole surface of the nerve section was recorded and counted directly from the histologic section using a Zeiss Universal light microscope equipped with a motorized stage and a Sony 930XP video camera connected to a Dell computer. On the same semithin sections, the outline of each myelinated axon in the whole section was also digitized using Neurolucida software (MBF Bioscience). The diameter of the fibers was calculated using the Neuroexplorer and Excel programs.

Micrographs (×5,800) of ultrathin phrenic nerve sections were captured using electron microscopy and an image acquisition program (Soft Imaging Systems; Olympus Soft Imaging Solutions, Munster, Germany). A micrograph mount was made for the entire surface of each nerve cross section. For each mount (n = 6 for each mouse line), the numbers of unmyelinated and myelinated axons were counted using Neurolucida software (MBF Bioscience).

**Brainstem and Cervical Spinal Cord Section Preparation for Morphologic and Morphometric Analysis**

Serial 40-μm-thick transverse sections from the brainstem and cervical spinal cord were cut using a freezing microtome and stained with toluidine blue. Medullary respiratory neurons in the brainstem and cervical spinal cord motor neurons were carefully examined by light microscopy (objective ×100). Sections were photographed using a Zeiss Axiosplan microscope and an image acquisition program (Zeiss AxioVision with Axiocam HRc) with constant illumination at ×10. The mounted images of the entire sections were analyzed using an image processing program (ImageJ 1.34; National Institutes of Health), and the numbers of all motor neurons in 2-mm-thick segments of cervical spinal cord were counted in both anterior horn areas. Only multipolar cells with granular cytoplasmic staining of Nissl bodies and clear nuclei regardless of size were counted. Cell counts were done blinded to the genotype of the animal.

**Statistical Analysis**

The counted number of myelinated versus unmyelinated axons and the measured sizes, shape complexity, and intensities of NMJ α-BTX staining in DM-1 transgenic mice were compared with internal and wild-type mice control values using a hierarchic analysis of variance (general linear model procedure; SAS statistical package; SAS Institute, Inc., Cary, NC). First, the distribution of values was checked for normality, and transformations were applied

![Figure 2](https://academic.oup.com/jnen/article-abstract/67/8/763/2916979)
RESULTS

Phrenic nerve–diaphragm muscle junctions are of particular interest in studies aimed at understanding the pathologic basis of respiratory failure.

Pathologic Alterations in Diaphragm NMJs in DM-1 Mice

The systematic histologic analysis of adjacent diaphragm muscle sections labeled with rhodamine α-BTX and neurofilament antibody revealed the presence of numerous EPs in the central region of the diaphragm and nerve fibers in DM-1 mice and control mice (internal control and wild-type mice). There was, however, a marked difference noticed in the size, shape complexity, labeling intensity, and innervation of EPs between the DM-1 mice and the controls. In the control mice, most EPs displayed typical pretzel-like shapes with strong fluorescence labeling and direct contact to nerve terminals (Figs. 1A–J). In DM-1 transgenic mice, however, EPs generally had smaller sizes, simpler shapes, and less intense α-BTX labeling (Figs. 1K–O). In addition, a neurogenic abnormality was indicated by 24% of EPs lacking axon terminals. Surprisingly, no excessive axonal sprouting was observed on DM-1 diaphragm muscle sections (Fig. 1). These observations suggest a possible denervation of 24% of EPs in DM-1 mice, which can then lead to structural alterations.

To verify the morphologic observations, morphometric and statistical methods were used. The measurement and statistical analysis of the size and the shape complexity of 1,400 EPs from each mouse line revealed no significant difference between internal control and wild-type mice. In contrast, EPs in DM-1 mice had significantly smaller sizes and less complex shapes compared with control mice. The mean size of EP in DM-1 mice was significantly (p = 0.03) smaller than in controls (Fig. 2A). The estimated complexity of EP shape was also reduced by 17% in DM-1 transgenic mice compared with control mice; this difference was highly
significant (p = 0.004; Fig. 2B). There was also a decrease of 25% (p = 0.02) in the density of AChRs on postsynaptic membranes quantitated by measuring the α-BTX fluorescence labeling intensity (Fig. 2C). The pathologic alterations observed in the diaphragm NMJs in DM-1 mice are likely to be independent of their lower weights because we previously did not detect abnormalities in the hindlimb NMJs in the same mice (33).

The histologic examination of diaphragm muscle sections in DM-1 mice stained with hematoxylin and eosin showed scattered muscle fibers with central nuclei and an increase in interfascicular connective tissue. The morphometric analysis revealed a slight, but not significant, reduction in the diameter of the muscle fibers compared with controls. These results indicate a slight diaphragm muscle dystrophy in DM-1 transgenic mice.

Loss of Unmyelinated but Not Myelinated Fibers in DM-1 Mice Phrenic Nerves

In controls and DM-1 transgenic mice, light microscopic examination of transverse sections of phrenic nerve stained with toluidine blue showed that the nerve consisted of a single fascicle enveloped by layers of flattened cells that constitute the epineurium. Large myelinated nerve fibers were present in the endoneural spaces, and the general cytoarchitecture of DM-1 nerves did not differ noticeably from the control nerves (Fig. 3). Examination of DM-1 nerve sections at higher magnification revealed no clear Wallerian degeneration or inflammatory cell nuclei. The total numbers of myelinated fibers on 8 semithin sections of phrenic nerve trunks from each mouse line (n = 8) were not different (Fig. 4A). Moreover, measurements of the perimeter of myelinated fibers on the same nerve sections revealed no significant difference in the mean diameter of myelinated axons between DM-1 (6.43 ± 0.69 μm) and control mice (internal control, 6.87 ± 0.18 μm; wild type, 6.79 ± 0.18 μm). The histogram (Fig. 4B) demonstrates a comparable size frequency distribution of myelinated axon diameters in the 3 groups; the distribution was unimodal, with higher frequencies between 5.5 and 8.5 μm.

Electron microscopic examination of numerous ultrathin nerve sections demonstrated a lower frequency of unmyelinated fibers in DM-1 mice compared with control (Fig. 5). To address the question of whether there is a loss of unmyelinated axons or perhaps in very thin myelinated fibers that are not visible on semithin sections, the numbers of each (unmyelinated and myelinated) nerve fiber were counted in 6 entire transverse sections of phrenic nerves from each mouse line. The results confirmed normal numbers of myelinated fibers in DM-1 phrenic nerves and, surprisingly, a severe and significant (p < 0.01) reduction (40%) in the numbers of unmyelinated axons in DM-1 (75 ± 10) compared with control (wild type, 128 ± 11; internal control, 126 ± 9) mice (Fig. 6).
There Are No Changes in Cervical Motor Neurons or in Medullary Respiratory Neurons in DM-1 Mice

To determine whether the cervical spinal cord or brainstem respiratory centers were affected in DM-1 mice, we analyzed C1 to C4 spinal cervical motor neurons and the motor medullary respiratory neurons. The careful histologic examination of cervical motor neurons, hypoglossal, spinal trigeminal, inferior olive, vestibular, and ambiguous nuclei revealed no cytoplasmic features of apoptosis in neurons such as nuclear eccentricity, loss of Nissl bodies, cytoplasm granularity, or vacuolization and membrane irregularities (Fig. 7). These morphologic results indicate an absence of neuronopathy in DM-1 mouse spinal motor and medullary respiratory neurons. The absence of neuronopathy in cervical spinal cord was confirmed by motor neuron counting. In 2-mm-thick segments of cervical spinal cord, the mean number of motor neurons in both anterior horn areas in DM-1 mice (5,036 ± 456) was not significantly different (p = 0.37) from numbers in control mice (wild type, 4,898 ± 556; internal control, 5,033 ± 237).

DISCUSSION

Access to DM patient diaphragmatic tissue is usually only possible at the end-stage of the disease. Therefore, the use of valid animal models for understanding the disease pathogenesis is imperative. In this study, we used transgenic mice carrying more than 300 unstable CTG repeats and displaying the DM-1 phenotype. The validity of this animal model has been demonstrated by histologic, molecular, and electromyographic analysis (28, 29). For the first time, we report here the presence of pathologic changes in diaphragm NMJs and phrenic nerves. Careful histologic and morphometric analysis of the diaphragm and the neural network involved in the respiratory drive demonstrated the following in DM-1 transgenic mice: 1) 24% of the EPs lack an axonal terminal; 2) there is a significant reduction in the size and shape complexity of diaphragm EPs; 3) there is a significantly lower density of AChRs on the postsynaptic membrane; 4) there are markedly fewer unmyelinated nerve fibers but not myelinated fibers in phrenic nerve trunks; and 5) there are no detectable pathologic features in cervical motor neurons or medullary respiratory center neurons. Because NMJs are involved in the transmission of action potentials and the afferent phrenic unmyelinated fibers control the inspiratory activity, our results suggest that the respiratory impairment associated with DM-1 diseases may be partially due to pathologic alterations detected in NMJs and phrenic nerves in the DM-1 animal model. Moreover, our results, along with those of other research groups, contribute to the understanding of the mechanisms underlying respiratory failure associated with DM-1.

Myotonic dystrophy Type 1 disorders are characterized by a wide variation of CTG repeats and by multisystem involvement. Respiratory failure has long been recognized to
be a main feature in DM-1 patients and probably the major factor contributing to mortality (1). Despite several clinical studies showing respiratory failure in DM-1 patients, the pathologic mechanisms are not completely known. Some studies have suggested that the causes of respiratory failure may be varied and either due to the involvement of respiratory muscles in the dystrophic and myotonic process or to abnormalities in the nervous system that generate and control the breathing drive (1, 11, 18, 19, 22, 25–27, 34, 35). The studies demonstrating the influence of central or peripheral control on breathing in DM-1 cases are, however, scant and controversial. Whereas some authors have suggested that an underlying neurogenic deficit of central structures can explain the high incidence of respiratory failure in DM-1 subjects (8, 20), others found that the central inspiratory drive is normal (6). These contradictory results are based only on electrophysiologic examination, and, to our knowledge, there is no anatomic evidence of a possible type of alteration.

It is well known that the normal function of the diaphragm during both the inspiratory and expiratory phases of breathing requires neural stimulation transmitted via normal NMJ. Alterations in synapse structure result in failure of diaphragm function. In the present study, careful morphologic and morphometric analysis provided evidence that in DM-1 mice, the diaphragm NMJs displayed pathologic alterations demonstrated by the reduction in size and shape complexity of EPs and by the decrease in the density of AChRs on postsynaptic membranes in DM-1 mice. The changes in NMJ architecture after skeletal muscle denervation are well documented in the literature. Several studies have indicated that the numbers of nerve terminals decrease within a few hours of nerve injury and disappear completely after 24 hours (36–39). Denervation also induces changes in the postsynaptic part of NMJs. Acetylcholine esterase staining and scanning electron microscopy have demonstrated that the denervation of different types of muscles resulted in large changes in the structural integrity of EPs, that is, they become smaller and their folds shorter and poorly defined (36, 38, 40–43). In addition to the morphologic changes, the density of AChRs on postsynaptic membranes falls significantly after muscle denervation. The number of AChRs assessed either by measuring of the amount of 125I-labeled α-BTX bound to muscle EPs or by quantification of α-BTX fluorescence labeling decreased clearly after denervation (37, 44–46). On the basis of these data, we infer that the major modifications in EPs detected in DM-1 transgenic mice are very likely due to diaphragm muscle denervation. Because it has been demonstrated that the efficient transmission of action potentials at neuromuscular junctions requires a certain number of AChRs on the postjunctional membrane (47), the significantly lower AChR density in DM-1 transgenic mice probably induces a reduction in the transmission of action potential that affects the normal function of the diaphragm.

The morphologic denervation of EPs observed in DM-1 mice in this study correlates with the recent findings reported by Wheeler et al (48), who demonstrated high expression of CUG8+5 RNA and sequestration of the muscleblind-like protein 1 in postsynaptic nuclei of NMJs in transgenic mice and DM-1 muscle biopsies. Those authors suggest that these alterations may affect the function or stability of the NMJ. Not all DM-1 muscle EPs displayed abnormalities, however, and some studies have reported that EPs from either finger extensor muscles, hindlimb muscles, or other DM-1 skeletal muscle biopsies did not show abnormalities (33, 49, 50).

Our analysis of serial diaphragm muscle sections in DM-1 mice showed that most EPs are in the central region of the diaphragm (data not shown). Taking into consideration the recent report of Heeroma et al (51), which demonstrates that aberrant diaphragm EP development is characterized by diffuse and peripheral EP localization, our results lead us to believe that the diaphragm muscle denervation observed in DM-1 mice results from postnatal degenerative effects of DM, rather than abnormal development during embryonic life. Balice-Gordon and Lichtman (52) showed that all mouse EPs at birth are polyneuronally innervated, and that changes in EP innervation occur only during early postnatal life when muscle fibers undergo transition from multiple to single innervation. Because more than 70% of the EPs are innervated by a single axon in the DM-1 diaphragm muscle sections, this indicates an initial normal development of EPs.

Because we observed no loss in cervical motor neuron cell bodies or myelinated fibers in phrenic nerve trunks in DM-1 mice, this rules out a motor neuronopathy in the spinal cord or axonal degeneration in phrenic nerves. It suggests that the denervation of EPs in diaphragm DM-1 mice is due to a distal axonal degeneration in phrenic nerves or to intramuscular terminal nerve degeneration. We expect that the denervation of 24% of the EPs and the morphologic changes observed in the diaphragm such as the presence of scattered muscle fibers with central nuclei and an increase in interfascicular connective tissue would induce changes in gene expression in nerve and muscle fibers. This will be the subject of future study.

The second major point in the present study is a significant loss (40%) of unmyelinated axons in phrenic nerves. Several studies have shown that unmyelinated fibers are abundant in phrenic nerves, and, presumably, many of these are afferent (53–55). These thin afferent fibers are believed to be connected to polymodal receptors on the surface of the muscle and within the muscle (55). Free nerve endings in muscle are presumed to represent these receptors. The groups of these fibers are activated principally by chemical, rather than mechanical stimuli (56). The selective stimulation of large or small afferent phrenic fibers affects phrenic motor discharge (57). Another study has shown that the electric stimulation of phrenic nerve afferents induced an increase in ventilation and blood pressure that was mediated by unmyelinated fibers and possibly thin myelinated fibers (58). The authors concluded that both large and small afferent phrenic fibers control the inspiratory activity with a prominent role of small fibers in phrenic motor neuron impulse frequency. The projections of phrenic afferent to the medulla cerebellum and sensorimotor cortex have been reported (59–61). Verification of these projection pathways indicated that phrenic afferents may be involved in shaping the respiratory motor output and providing sensory information.
with respect to perception of the sensation of breathing (62). Taking all of these data into consideration, we infer that if there is a similar loss of 40% of the unmyelinated fibers in DM patients as there is in DM-1 transgenic mice, this could explain, at least in part, the respiratory failure observed in the patients.

It is known that the basic breathing rhythm is generated in the medullary respiratory neurons (63). These neural elements have been identified in adult mice in the medulla oblongata (pre-Bötzing complex) and the pons (64–66). Our analysis of densely distributed neurons in the medullary reticular formation showed an absence of degenerative features in neurons in DM-1 mice. These results are consistent with previous chemical control of breathing data that showed that the respiratory centers are well preserved in DM-1 (6). Together, our results suggest that generation of the breathing rhythm is not affected in DM-1 mice, but rather a deficiency in the transmission of action potentials at the level of the NMJ.

When performing this study, only 1 mouse line carrying more than 300 CTG repeats was available (DM328), and offspring from a second line (DM-1177) were difficult to obtain. We cannot exclude the possibility that the site of integration in the transgene is not involved in the observed neuropathy; myopathy and myotonia have both been reported in these 2 independent lines (29). Molecular analysis to characterize the transgene integration site in detail is ongoing.

Because we did not detect abnormalities in sciatic nerves or in hindlimb NMJs in the same DM-1 mice as previously indicated, loss of unmyelinated axons and the changes in the NMJs seem to be specific to phrenic nerves and the diaphragm. One possible explanation for the differences in muscle involvement is differences in levels of mutated DMPK transcripts that are known to have a central role in causing the manifestations of DM-1. In the DM-1 mice that we studied, the DMPK transgene is expressed with a pattern very similar to the endogenous DMPK gene (67). Furthermore, the level of DMPK transcripts can vary significantly between muscles and is much higher in the diaphragm than in the tibialis anterior (3 times more) or in the soleus (1.5 times more) (unpublished data). Expression of DMPK can also vary between nerves. Progressive somatic mosaicism is the most plausible explanation for these results. Indeed, the expanded CTG repeat is somatically unstable and biased toward further expansion throughout life. Indeed, Jansen et al (68) studied the CTG repeat lengths from a broad range of tissues in single DM-1 individuals and in twins with mild, classic, or congenital DM-1 and demonstrated that in the same individual, although there was some overlap, the distribution of CTG repeat lengths varies among tissues. A high level of somatic instability has also been observed in transgenic mice carrying the DM-1 locus with more than 300 CTG (28), and, as in DM-1 patients, different levels of somatic mosaicism were detected in various tissues.

CONCLUSIONS

We have shown that in transgenic DM-1 mice carrying more than 300 CTG repeats, the diaphragmatic NMJs display reduction in the size of EPs, altered shapes, and a decreased density of AChRs on the postsynaptic membrane. More importantly, there was a marked loss in numbers of unmyelinated, but not myelinated, nerve fibers in DM-1 phrenic nerves, and cervical phrenic motor neurons and medullary respiratory neurons were unaffected. Because afferent phrenic unmyelinated fibers control ventilation and because NMJs are also involved in the transmission of action potentials, our results suggest that the respiratory failure observed in DM-1 mice may be partially due to these pathologic alterations in the structure of diaphragmatic NMJs. Because central neurons are normal, the generation of the breathing rhythm is likely not affected in DM-1 mice; rather, the transmission of NMJ action potentials are deficient. Taken together, these results elucidate the mechanisms underlying respiratory failure in human DM-1.

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