Functional and histopathological identification of the respiratory failure in a DMSXL transgenic mouse model of Myotonic Dystrophy

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Running title: Breathing impairment in DM1 animal model

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Summary (296 words)

Acute and chronic respiratory failure is one of the major and potentially life-threatening features in patients with Myotonic Dystrophy type 1 (DM1). Despite the several clinical demonstrations showing respiratory problems in DM1 patients, the mechanisms are still not completely known. This study was designed to investigate whether the DMSXL transgenic mouse model for DM1 exhibit respiratory disorder, and if so to identify the pathological changes underlying these respiratory problems. Using pressure plethysmography we assessed the breathing function in control mice and DMSXL mice generated after large expansions of CTG repeat in successive generations of DM1 transgenic mice. The statistical analysis of breathing function measurements revealed a significant decrease in the most relevant respiratory parameters in DMSXL mice, indicating impaired respiratory function. The histological and morphometric analysis showed pathological changes in diaphragmatic muscle of DMSXL mice characterized by an increase in the percentage of type I muscle fibers, the presence of central nuclei, partial denervation of end-plates (EPs) and a significant reduction in their size, shape complexity and density of acetylcholine receptors, all of which reflect a possible break down in communication between the diaphragmatic muscles fibers and the nerve terminals. Diaphragm muscle abnormalities were accompanied by an accumulation of mutant DMPK RNA foci in muscle fiber nuclei. Moreover, in DMSXL mice, the unmyelinated phrenic afferents are significantly lower. Also in these mice, significant neuronopathy was not detected in either cervical phrenic motor neurons or brainstem respiratory neurons. Since EPs are involved in the transmission of action potentials and the unmyelinated phrenic afferents exert a modulating influence on the respiratory drive, the pathological alterations affecting these structures may underlie the respiratory impairment detected in DMSXL mice.
Understanding mechanisms of respiratory deficiency should guide pharmaceutical and clinical research towards better therapy for the respiratory deficits associated with DM1.

**Introduction**

Myotonic dystrophy or dystrophia myotonica type 1 (DM1) is an autosomal dominant genetic neuromuscular disorder, that can be accompanied by abnormalities in other organs and systems with a highly variable phenotype. DM1 is caused by an expansion of a CTG repeat located in the 3’ untranslated region of the DM protein kinase gene (DMPK) on chromosome 19q (Fu et al., 1992; Mahadevan et al., 1992). The number of repeats tends to increase from generation to generation, accounting for the genetic anticipation characteristic of this disease (Ashizawa et al., 1992; Mahadevan et al., 1992; Salehi et al., 2007). Healthy individuals have alleles with 5-35 repeats that are stably transmitted, but when this number exceeds 50, instability occurs and leads to disease. The number of CTG repeats is 50-150 in late-onset, mild DM1, 100-1000 in classic DM1, and even more than 2000 in the congenital form. A significant inverse correlation is noted between onset age and repeat number and there is a general correlation between the degree of expansion and the severity of clinical manifestations (Gennarelli et al., 1996; Hunter et al., 1992).

The neuromuscular clinical manifestations of the disease include myotonia and progressive muscle weakness, with dystrophic changes in skeletal muscles including the diaphragm, cranial and limb muscles (Harper, 2001). The pathology of DM1 however, extends to other organs and systems and, as such, can be associated with several significant complications. Respiratory and cardiac problems have long been recognized as the main complications of DM1 patients and are the major factors contributing to mortality. In fact, a study based on a cohort of 115 patients with congenital myotonic dystrophy reported that only about 50% of patients survived to their mid 30s and the death of about 66% of surviving patients is due to respiratory causes (Reardon et al., 1993). Moreover, large cohort studies have also reported
that respiratory problems are the leading cause of death (around 40%) in adult onset DM1 (de Die-Smulders et al., 1998; Groh et al., 2008; Mathieu et al., 1999). The progressive respiratory muscle weakness leads to impairment of airway clearance, inadequate ventilation and respiratory failure (Berry et al., 1996), while no reduction in central airway caliber was found in the patients with the adult form of DM1 (Fodil et al., 2004). DM1 patients frequently develop pneumonia and respiratory failure as a result of alveolar hypoventilation, particularly in the later stages of the disease (Bogaard et al., 1992; Jammes et al., 1986). Despite the many clinical examinations showing respiratory failure in DM1 patients, the pathological mechanisms underlying this failure are still not completely known. Several studies suggested that the causes of respiratory failure are varied and may be due either to the involvement of respiratory muscles in the dystrophic and myotonic process (Begin et al., 1980; Bogaard et al., 1992; Jammes et al., 1985; Serisier et al., 1982; Zifko et al., 1996), or to abnormalities in the nervous system which generates and controls breathing drive (Kilburn et al., 1959; Ono et al., 1996; Takasugi et al., 1995).

In neurodegenerative diseases, in humans access to affected tissue is generally limited to the end-stage of the disease and to some extent biopsies, making animal models extremely useful (Gomes-Pereira et al., 2011; Wansink and Wieringa, 2003). In the present study we used DMSXL transgenic mice. These mice express the \textit{DMPK} gene in a variety of tissues with a pattern similar to the patterns of the murine \textit{Dmpk} gene in mice and the \textit{DMPK} gene in human tissues (Huguet et al, \textit{in press}). To address whether the DMSXL transgenic mice animal model which carry long CTG repeat and display DM1 features (Gomes-Pereira et al., 2011) have respiratory problems, we tested and compared the breathing function in DMSXL and control mice. To identify the pathological changes underlying any respiratory complications we analyzed the diaphragm muscle and the neural network that generates, controls and transmits the respiratory rhythm.
Results

Impaired respiratory function in DMSXL mice

To investigate whether the DMSXL transgenic mice have respiratory alterations, first we measured, in the same conditions, the most relevant respiratory parameters in awake young or adult DMSXL and control (wild type and DM20) mice. The analysis of breathing patterns (Fig. 1) and parameters recorded in young awake mice (2 month-old) revealed a small but significant decrease in tidal volume and volume per minute in DMSXL mice. We noticed that awake mice, especially the DMSXL mice, appeared highly stressed and agitated during recording, and so we assessed the respiratory function in anaesthetized mice. The results of these experiments showed that in both two month-old anaesthetised mice, the respiratory rate and the tidal volume are significantly decreased in DMSXL mice compared to control mice taken from the same litter and DM20 mice which do not express the DM1 phenotype (WT and DM20). Consequently, the mean value for minute volume/body weight (Tidal volume X breathing frequency/body weight) was also reduced in DMSXL mice (table 1). In addition, a significant reduction was detected in arterial blood oxygen saturation (SpO2) in DMSXL mice. Overall, we found that alterations in respiratory function observed in young DMSXL mice do not really change as a function of age. In fact, measurement of respiratory parameters in 6 month-old mice confirmed the decrease in respiratory rate, in tidal volume and minute volume, and in SpO2 in DMSXL mice (table 2). To be sure that the alterations in respiratory pattern observed in DMSXL mice are not related to their small size, we measured the respiratory function in WT mice having similar size and weight (24 ± 0.6 g). The results of these experiments showed no changes in RR and in SpO2 in the small control mice compared to normal control mice (RR: 135.7 ± 8.2 in small control vs 126 ± 15.1 in normal control; SpO2: 98.23 ± 0.21 vs 98.62 ± 0.38). This result ruled out the possibility that the respiratory alterations observed in DMSXL mice are
caused by their small size. Moreover, the comparison and statistical analysis of all our results revealed two points: i) the breathing function in DM20 transgenic mice which do not express the phenotype of DM1 disease was similar to WT mice; ii) anaesthesia caused a reduction in breathing parameters in all mice compared to awake condition.

**Pathological changes in diaphragmatic muscle fibers and neuromuscular junctions**

To assess whether modifications in the diaphragm are responsible for the impairment in respiratory function in DMSXL mice, we first examined diaphragm sections stained for either ATPase activity or with haematoxylin and eosin (HE). In both control (WT and DM20) and DMSXL mice we observed that the majority of fibers were did not show phosphorylase activity, only counterstained with Luxol fast blue (>85 % type II) and a small number of fibers strongly stained (type I) (Fig. 2). However, quantification of muscle fibers showed an increase (14.7%) in the mean percentage of type I fibers in DMSXL mice compared to control mice. Other dystrophic alterations were observed in DMSXL diaphragm sections, such as centrally located nuclei, an increase in interfascicular connective tissue and the presence of infiltrating inflammatory cells (Fig. 2). The abnormality of DMSXL diaphragm muscle was accompanied by an accumulation of DMPK RNA with its expanded CUG repeat, found as foci in the nuclei of muscle fibers (Fig. 3). About 58% of DMSXL diaphragm muscle fibers nuclei contained between 1 and 7 foci with an average of 2.2 foci per affected nucleus. No nuclear RNA foci were detected in the diaphragm muscle fibers of control mice.

The diaphragm neuromuscular junctions (NMJs) have also undergone pathological changes in DMSXL mice. Despite the presence of bundles of axons on the serial muscle sections, about 20% of the endplates were denervated and had no contact with nerve terminals. Moreover, numerous EPs exhibited lengthened shapes and faint labelling compared to controls (Fig. 4). Statistical analysis of the morphometric results confirmed the abnormality
of EPs in DMSXL mice. A significant decrease in mean EP area (11%) and shape complexity (16.3%) was found in DMSXL mice, as well as in the density of AChRs on postsynaptic membranes (reduced by 19.9%, Fig. 4). Combined staining with α-BTX and FISH revealed that several foci were located in sub-synaptic nuclei.

**Severe loss of phrenic nerve unmyelinated axons**

Light microscopy revealed no obvious morphological differences between control (WT and DM20) phrenic nerve transverse sections of DMSXL mice, except for smaller size of DMSXL nerve sections. In all animals, the phrenic nerve consisted of a single fascicle containing myelinated and unmyelinated axons. In electron microscopy analysis apart from some pathological signs (aberrant Schwann cell proliferation, increased number of macrophages) observed on DMSXL thin nerve sections, a severe and significant loss (41%; P < 0.01) in the number of unmyelinated axons was found (Fig. 5). In contrast, the number of myelinated fibers showed a slight but not significant decrease (5%; P = 0.30), although the thickness of myelin sheath was reduced significantly (P < 0.01) in DMSXL mice compared to controls (628 ± 9 nm vs. 770 ± 54 nm). The FISH analysis showed that several Schwann cells that intermingled with axons, contained one or two ribonuclear foci in their nuclei (Fig. 5).

**Absence of significant alterations in phrenic motor or brainstem respiratory neurons in DMSXL mice**

Histological examination of cervical phrenic motor neurons showed rare apoptotic features (nuclear eccentricity, loss of Nissl bodies or cytoplasm granulation). The estimation of the number of neurons by the physical dissector method revealed a slight but not significant decrease (5%, P = 0.13) in the mean number of cervical phrenic motor neurons in DMSXL mice. In addition, only a few mRNA foci were observed in phrenic motor neurons in DMSXL mice.
The histological and morphometric analysis of five brainstem disector pairs containing respiratory neurons located in region that extends from the compact portion of the nucleus ambiguous to just behind the facial motor nucleus (Fig. 6) ruled out the presence of neuronopathy in DMSXL mice respiratory neurons. In fact, the histological examination of respiratory neurons located in the area of the ventrolateral nucleus of the solitary tract and neurons scattered in the vicinity of the nucleus ambiguous and the surrounding reticular formation did not reveal apoptotic features. Moreover, the physical disector estimation of neuron density showed only a non-significant reduction (3%) in the density of DMSXL respiratory neurons as compared to controls.

Discussion

The major result provided by this study is the demonstration that DMSXL mice, a transgenic mouse model of DM1, have respiratory impairment characterized by a significant decrease in RR, TV, MV and SpO₂ compared to control mice. The second important result is the detection of a partial denervation of diaphragmatic EPs and significant pathomorphological changes in diaphragm EPs and muscle fibers in DMSXL mice, which indicate a possible break down in communication between the diaphragmatic muscles fibers and the nerve terminals. Also a severe and significant loss in the number of phrenic unmyelinated afferents was detected in DMSXL mice, while no significant alterations were observed in phrenic motor neurons or brain stem respiratory neurons.

Taking into consideration that the diaphragm muscle is the principal respiratory muscle and that afferent phrenic unmyelinated fibers are involved in the control of the basic respiratory rhythm, we believe that the pathological changes observed in the diaphragm and phrenic nerve afferents in DMSXL mice are responsible of the respiratory failure assessed in these
mice. Because central neurons have not shown significant neuronopathy, the generation of the breathing rhythm is likely not affected in DMSXL mice.

**Impaired respiratory function in DMSXL mice**

For the study of mechanisms of myotonic dystrophy diseases, there is a growing use of various animal models (Gomes-Pereira et al., 2011; Wansink and Wieringa, 2003). Consequently, some of the same techniques used in the clinic to examine patients should be adapted for analysis in animal models. Several studies reported that pressure plethysmography can serve as a valuable respiratory function test in conscious or anaesthetized small animals. This noninvasive method is particularly appropriate for quick and repeatable screening of respiratory function over short and extended periods of time for large numbers of mice and rats. Moreover, the intraindividual variability in respiratory function measured by pressure plethysmography is acceptable (Huang et al., 2011; Studden et al., 2001; Yilmaz et al., 2005). Therefore, in the current study we used pressure plethysmography to assess the respiratory function in DMSXL and control mice. The comparison and the statistical analysis of several breathing measurements demonstrated a significant decrease in most relevant respiratory parameters in anaesthetized DMSXL mice, indicating an impairment in breathing function in young and adult mice. As alterations in the breathing parameters observed in young mice do not become more severe in adult animals, we inferred that respiratory impairment in DMSXL mice does not depend on the age of the animals. We also demonstrated that respiratory changes including RR and SpO2 were not related to the small weight of the DMSXL transgenic mice. In addition, the hypothesis that the respiratory abnormalities observed in these mice are due to over expression of the genes carried by the transgene, is excluded since the DM20 transgenic mice carrying the same transgene but with a normal number of CTG repeats did not show alterations in respiratory function compared to wild type mice.
The statistical analysis of the results of several recordings showed that the mean values of respiratory parameters in anaesthetized mice were reproducible and comparable with values previously reported (Daubenspeck et al., 2008; Hamelmann et al., 1997; Hoymann, 2007; Huang et al., 2011). Several studies reported that the breathing frequency decreases by about 40 - 60% in anaesthetized mice compared to conscious mice (Hamelmann et al., 1997; Takezawa et al., 1980). In our study, the RR and MV were decreased under anaesthesia to about 47% in controls and 34% in DMSXL mice compared to conscious animals, which indicates that DMSXL mice are more sensitive to anaesthesia. In agreement, clinical reports have mentioned that in general, patients with DM1 disease present potential respiratory depression during or after anaesthesia, although the mechanisms underlying the anaesthesia effect on respiratory depression in DM1 patients is not well investigated (Ogawa et al., 1993; Yomosa et al., 1991). It is conceivable that in DMSXL mice anaesthesia acts directly on the neural network that generates, controls and transmits the respiratory rhythm and also on the diaphragm, which is the main respiratory muscle. Another possibility is that the anaesthesia has an effect on several areas of the central nervous system, which in turn exert an influence on the structures involved in respiratory function.

Since DMSXL mice displayed impairment in respiratory function, these mice present a useful tool to investigate the pathological changes underlying respiratory failure associated with DM1 disease, and importantly the evaluation of respiratory parameters by non-invasive pressure plethysmography could be used to evaluate the effect of potential therapies in DMSXL mice.

**Role of pathological changes in the DMSXL diaphragm in respiratory impairment**

Anatomical and electrophysiological data suggest that the basic breathing rhythm of higher mammals is generated and regulated in brainstem respiratory neurons, then the stimuli are
transmitted via cervical phrenic motor neurons and phrenic nerves to the diaphragmatic muscle which is the principal respiratory muscle (Ramirez et al., 2002). Therefore, the impairment in respiratory function observed in DMSXL mice may have a central origin in decreased respiratory drive, or peripheral origin such as a weakness of the diaphragmatic muscles, or both.

Because diaphragmatic muscle is the only effective respiratory muscle pump and its action contributes to more than 66% of the tidal volume (Mead et al., 1995; Singh et al., 2003), our efforts were focused on analyzing diaphragm muscle fibers and NMJs. Several of the alterations observed in DMSXL diaphragm muscle lead us to believe that they may cause, at least partially, the respiratory failure in these mice. In fact the increase in histochemically defined type I muscle fibers, the presence of central nuclei, the gain in interfascicular connective tissue and inflammatory cells, as well as the accumulation of expanded CUG mRNA in muscle fiber nuclei, all these alterations indicate that the diaphragm muscle of DMSXL mice is involved in the dystrophic processes. This assumption can be supported on the one hand by the presence of similar histopathological changes in biopsies of DM1 patients (Harper, 1989; Romeo, 2012); on the other hand by the changes in fiber type composition of the diaphragm muscles in an animal model of Duchenne muscular dystrophy which is characterized by severe muscle degeneration, (Guido et al., 2010; Petrof et al., 1993). In this report the authors hypothesized that the progressive changes in fiber types probably permit better adaptation of the muscle to its function during progression of the disease. It is possible that the increased number of type I diaphragm fibers (slow-twitch) in DMSXL, due to a selective degeneration of type II fibers (fast-twitch), probably causes alterations in diaphragmatic muscle function in these mice.

The detection of accumulation of CUG expanded RNA as foci in diaphragm muscle fiber nuclei, is another indication of abnormality of the DMSXL diaphragm. The presence of foci and the sequestration of muscleblind protein in the nuclei diaphragm NMJs has also
been reported in DM1 muscle biopsies and in another DM1 transgenic mouse model (Wheeler et al., 2007). It is well known that the main pathogenic process at the basis of DM1 is a toxic RNA-gain of function effect of mutant DMPK transcripts which are retained in distinct ribonuclear foci within the cells nuclei. Taking into consideration this hypothesis, inferring that these nuclear foci sequester essential proteins and disrupt their normal function in the cell (Kuyumcu-Martinez and Cooper, 2006; Nykamp and Swanson, 2004; Querido et al., 2011), we can hypothesize that nuclear retention of mutant DMPK mRNA in diaphragmatic muscle fibers alters the function of the diaphragm which may lead to respiratory failure.

In DMSXL mice the diaphragmatic NMJs have also undergone pathological changes. About 20% of diaphragm EPs were denervated and the area and the shape complexity of EPs were significantly reduced. Moreover, the AChRs density on postsynaptic membranes was significantly lower. It should be noted that in previous experiments in DM1 transgenic mice carrying only 500 CTG repeats we detected similar abnormalities in the structure of diaphragmatic EPs (Panaite et al., 2008). The fact that the statistical analysis revealed that the pathological changes in diaphragmatic EPs found in DMSXL mice, carrying about 1300 CTG repeats, do not vary significantly from those observed in DM1 mice, indicates that there is no relationship between the severity of EPs alterations and length of the CTG repeat. This is consistent with clinical studies reporting that some DM1 features such as cataract, sleep disorders, gastrointestinal, respiratory insufficiency and cardiac abnormalities do not correlate with the size of the unstable base CTG triplet (Jaspert et al., 1995; Marchini et al., 2000).

It is quite possible that the denervation and abnormalities seen in EPs structure are induced by the loss of 5% of phrenic myelinated fibers and also by the alterations affecting muscle fibers. A recent study reported that one of the mechanisms underlying the impairment of neuromuscular connections observed in DM1 tissue cultures is the misexpression of two
members of the *SLITRK* family genes which are involved in neurite outgrowth, neuritogenesis and synaptogenesis (Marteyn et al., 2011).

Since the normal function of the diaphragm in both inspiratory and expiratory phases of breathing requires neural stimulation via normal NMJs, the damage and alterations to the phrenic nerve affect the diaphragmatic DMSXL NMJs and probably results in failure of action potential transmission (Andreose et al., 1995; Kumai et al., 2005). Numerous clinical data reported that patients with signs of muscular weakness and myotonia have also been shown to have respiratory impairment (Begin et al., 1997; Jammes et al., 1985; Serisier et al., 1982; Zifko et al., 1996), therefore, the pathological changes observed in DMSXL diaphragm muscle are probably sufficient to affect the normal function of the diaphragm during breathing in these mice.

**Role of alterations in phrenic afferents in breathing impairment in DMSXL mice**

In addition to the motor function, the phrenic nerve is also rich (43%) in afferent axons composed mainly of unmyelinated axons. The motor component of the phrenic nerve is mainly myelinated while the sensory one is mainly unmyelinated (Langford and Schmidt, 1983). The thin afferent fibers are believed to be connected to polymodal receptors on the surface of the muscle and within the muscle. Free nerve endings located in muscle are presumed to represent these receptors (Brown and Fyffe, 1978; Duron and Marlot, 1980; Goshgarian and Roubal, 1986; Kumazawa and Mizumura, 1977). Groups of these fibers are activated principally by chemical stimuli rather than mechanical stimuli (Kumazawa and Mizumura, 1977).

The tracer combination of the anterogradely transported fast blue and the retrogradely transported nuclear yellow allowed the demonstration that some phrenic nerve afferents terminate at C4 and C5 spinal cord in rats (Revelette et al., 1988), other afferents project to the brain stem nuclei including dorsal respiratory group neurons (DRG) and ventral
respiratory group neurons (VRG) (Larnicol et al., 1985; Macron et al., 1985; Marlot and Duron, 1981). The neurons in the DRG and VRG in the brain stem are primarily responsible for the generation of the rhythmic pattern of respiration. However, there is growing evidence that diaphragmatic afferent stimuli exert a modulating influence on the basic respiratory rhythm, although they do not play a critical primary role in generating or maintenance of the rhythm.

Electrical stimulation of phrenic nerve afferents during inspiration produced a premature transition from the inspiratory to expiratory phase, and authors concluded that phrenic afferents exert an inhibitory influence on inspiratory motor drive (Jammes et al., 1986; Marlot et al., 1988). However, other studies reported that the activation of small-fiber afferents in the diaphragm of anaesthetized dogs induced a marked excitatory effect on phrenic motor neurons and brain stem respiratory neurons (Revelette et al., 1988; Speck and Revelette, 1987). These studies concluded that the phrenic nerve afferent populations are capable of producing at least two distinct effects: a net excitation of inspiratory activity at the pre-motor level and a strong inhibitory effect on motor output at the spinal level.

In our current study, the statistical analysis of morphometric results showed a severe loss of unmyelinated phrenic afferents (> 40%), whereas phrenic myelinated fibers undergo a slight and non significant reduction (5%). Furthermore, the phrenic motor neurons and the brainstem respiratory neurons do not suffer from significant neuronopathy since only a small non-significant loss in the number of neurons was found. With reference to the data reported in the studies mentioned above, we can deduce that in DMSXL mice, the afferent feedback is reduced and the regulation of respiratory drive is altered, while the generation of breathing rhythm is probably not affected.

In summary, despite the large cohort studies showing that respiratory problems are the leading cause of death, especially in the surviving patients with the congenital DM1 form (Reardon and al., 1993), and to a lesser degree in adult onset DM1 form (de Die-Smulders
et al., 1998; Groh et al., 2008; Mathieu et al., 1999), the pathological mechanisms underlying respiratory failure are still not completely known.

Our study in a transgenic mouse model of DM1 sheds light on possible mechanisms of respiratory failure, as the respiratory impairment seen in these mice are caused by pathological changes affecting mainly the diaphragm respiratory pump and phrenic unmyelinated afferents. Since cervical phrenic motor neurons and brainstem respiratory neurons do not show significant neuronopathy, the generation of the breathing rhythm is likely not to be affected in DMSXL mice.

**Methods**

All experiments were carried out in accordance with the local veterinary guidelines for care and use of experimental animals and all analyses and counts were performed blinded to the animal genotype.

**Generation of the DM animal model**

The generation of transgenic mice carrying the human genomic DM1 region with expanded repeats of either approximately 500 CTG (DM1 mice, displaying mild DM1 phenotype) or 20 CTG (normal, do not develop DM1 phenotype) has been described previously (Gantelet et al., 2007; Seznec et al., 2001). DMSXL mice carrying more than 1,300 CTG were obtained from DM1 mice, after large expansions of the CTG repeat over successive generations (Gomes-Pereira et al., 2007; Panaite et al., 2011). Expression of expanded CUG leads to the formation of numerous foci that co-localize with muscleblind-like protein 1 and 2 (MBNL1 and MBNL2). Mild missplicing of target RNA is observed in muscles and heart tissues. These molecular features of DM1-associated RNA toxicity were associated with high mortality, growth retardation and muscle defects (abnormal
histopathology, reduced muscle strength and lower motor performances) (Huguet et al, in press).

Heterozygous mice, homozygous and wild type mice are obtained by breeding from the same litter. Only homozygous transgenic mice were used in our study since the heterozygous mice expressing a low level of DMPK transcripts do not have an obvious phenotype. Thirty mice were used: group 1, consisted of 5 two month-old DMSXL mice and 5 control mice taken from the same litter, (body weight 20.2 ± 1.1 g vs 27.6 ± 0.8 g respectively); group 2, consisted of 5 two month-old DM20 mice and 5 controls mice (28.0 ± 0.6 g vs 28.5 ± 0.8 g); and group 3, consisted of 5 six month-old DMSXL mice and 5 control mice (22.0 ± 2.0 g vs 33.8 ± 3.6 g).

**Respiratory function analysis (Pressure plethysmography protocol)**

To investigate whether the DMSXL mice have respiratory failure, we measured the breathing function in DMSXL and control mice using pressure plethysmography (Respiromax system, Columbus Instruments, Columbus, OH, USA), a precise method adapted for both awake and anaesthetized small animals (Stunden et al., 2001; Yilmaz et al., 2005). Individual mice were firstly weighed then placed in a respiromax cylindrical chamber. A tail rod was moved forward until it secured the animal preventing it from backing up and maintaining a prone position. The head of the animal emerged through an inflatable latex cuff into a head chamber and the neck cuff was pressurized to seal the body chamber. Each mouse was allowed to acclimatize to the plethysmography chamber for approximately 5 min before tests began. A sensitive transducer measured the changes in pressure in the body chamber caused by the animal’s respiration. Signals from the pressure transducer were amplified and digitized by a respiratory function software (Columbus Instruments), displayed and stored in a computer for graphical and statistical analysis. In all experiments the temperature of the body chamber was continuously monitored using a
thermal probe. The following parameters were evaluated: Tidal Volume (TV, ml), Respiratory Rate (RR, breaths per minutes), Minute Volume (MV, tidal volume multiplied by respiratory rate, ml/min). The respiratory function of each mouse was tested several times over a period of 2 weeks.

In another series of experiments, the respiratory function was measured in mice anaesthetized with isoflurane using a Matrix Quantiflex low flow V.M.C. anaesthesia system (Midmark, Versailles, Ohio, USA). The animals were placed individually in an induction chamber, and anaesthesia was induced with 5% isoflurane in a gas mix of O₂/N₂O₂ (30%/70%). The animals were then quickly placed inside the Respiromax system, with a 1.5% isoflurane flow in the head chamber in the same O₂/N₂O₂ gas mix. A MouseOx pulse oximeter (Midmark, Versailles, Ohio, USA) was placed on the upper, right hindleg of each mouse to measure the oxygen saturation of arterial haemoglobin (SpO₂) and heart rate; the body chamber was closed and the neck cuff swollen. Animals were allowed to settle for 5 minutes before the first set of measurements.

Animal perfusion and tissue preparation

After testing the respiratory function, mice were deeply anaesthetized and the brainstem, cervical spinal cord, right and left phrenic nerves and diaphragm muscles were carefully removed from each animal as previously described (Panaite et al., 2008; Panaite et al., 2011).

Analysis of diaphragm muscle sections

To examine diaphragm muscle fibers, 10 µm thick transverse cryostat sections were prepared and myofibrillar adenosine triphosphatase (ATPase) activity was assessed after preincubation at pH 4.2 (Sheehan and Hrapchak, 1980) followed by counterstaining with Luxol fast blue. Other sections were stained with hematoxylin and eosin (HE). The number of each fiber type was assessed using an image-processing program (ImageJ 1.40, National
Institutes of Health, Bethesda, MD, USA). To analyze the neuromuscular junctions (NMJs), serial longitudinal cryostat sections (20 µm thick) of diaphragm muscle were double labelled with tetramethylrhodamine-conjugated α-bungarotoxin (α-BTX, Invitrogen, Life Technologies, Carlsbad, CA, USA) and with a primary polyclonal antibody directed against the 200-KDa neurofilament protein (AB1982, Millipore, Billerica, MA, USA).

Muscle sections were observed under a Zeiss AxioPlan 2 microscope (Zeiss; Oberkochen, Germany), photographed using an image-acquisition program (Zeiss AxioVision with Axiocam HRc) and images were systematically analyzed in the ImageJ 1.40 program. The percentage of denervated endplates (EPs) was estimated and the morphometric parameters (area, shape complexity and fluorescence intensity of rhodamine-α-BTX labelling) of each EP were measured and calculated as described in our previous studies (Panaite et al., 2008; Panaite et al., 2011). More than 1500 EPs were measured from each mouse line (n = 7 DMSXL and control mice).

**Detection of nuclear RNA foci**

Fluorescence in situ hybridization (FISH) was performed on 7 µm thick frozen sections as described by Guiraud-Dogan et al (Guiraud-Dogan et al., 2007). In short, sections of diaphragm muscle, phrenic nerve, cervical spinal cord and brain stem were dried, fixed then incubated in 30% (v/v) formamide and 2x standard sodium citrate (SSC) for 10 min, hybridized with the PNA probe Cy3-OO-CAGCAGCAGCAGCAG (1 ng/µl) (Eurogentec, Liège, Belgium) for 2 h at 37°C in buffer (30% (v/v) formamide, SSC2x, 0.02% (w/v), BSA, 66 µg/ml yeast tRNA (Invitrogen), 2 mM vanadyl complex (Sigma-Aldrich, St-Louis, MO, USA). Sections were then washed for 30 min in 30% (v/v) formamide/SSC2x at 50 °C and then for 30 min in SSC1x at 20 °C. Nuclei were stained by incubating with DAPI for 15 min at room temperature. The cervical spinal cord, phrenic nerves and brain
stem sections were additionally labelled with polyclonal anti Choline-Acetyltransferase antibody (ChAT, 1:50; AB144P, Millipore) as previously described (Panaite et al., 2011).

**Analysis of phrenic nerve sections**

For light microscopy analysis, semi-thin transverse sections (1 µm) were cut at different levels from phrenic nerve trunks and stained with toluidine blue. For electron microscopy examination, ultra-thin sections (80 nm) were cut and contrasted with uranyl acetate and lead citrate. Sections were viewed in the Zeiss EM 10C electron microscope and morphometric analysis was performed as previously described (Gantelet et al., 2007; Panaite et al., 2008). The number of myelinated and unmyelinated axons was counted, and the thickness of the myelin sheath calculated (n = 7 for each DMSXL and controls).

**Analysis of cervical phrenic motor neurons and brainstem respiratory neurons**

Serial transverse cryostat sections (20 µm) were prepared from cervical spinal cords (C3-C5) and from brainstems (caudal and rostral to the obex). Sections were either immunolabelled with polyclonal anti-ChAT antibody which labelles motor neurons, or stained with toluidine blue. The physical disector method was used to estimate the number of motor neurons in 2 mm segments of either cervical spinal cord or the medullary reticular formation. Identification of respiratory neurons on brainstem sections was achieved according to their localization as described in several studies (Alheid et al., 2002; Kuwana et al., 2006; Stornetta, 2008). The physical disector method is based on sampling sections, called disector pairs. About 4-5 disector pairs were used from each animal to estimate the density of neurons, as previously described (Panaite et al., 2011).

**Data analysis and statistics**

Each respiratory parameter for each mouse (TV, RR, MV, MV/g, heart rate, oxygen saturation) and the data obtained from the diaphragm, phrenic nerves, cervical spinal cord...
and brainstem examinations were analyzed and compared. ANOVA was used to determine significant difference between the groups, followed by Bonferonni-Holm post-hoc test, and pair-groups were compared by student’s t-test. In all cases, plethysmograph data were normalized to the weight of the animal. Values are reported as mean ± SD. P < 0.05 was considered as a significant difference. All statistical analyses and histograms were performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA).

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**Author contributions**

I.B.W. conceived and designed the experiments; G.G. generated and provided the DM1 animal model; P.A.P. and T.K. performed the respiratory tests; P.A.P. performed animal perfusion, tissue preparation, immunohistochemistry and fluorescence in situ hybridization experiments; P.A.P., J.A.L. and I.B.W. performed the experiments of histopathological analysis of the diaphragm muscle; P.A.P; T.K. and I.B.W. performed the histological analysis of brainstem respiratory neurons, spinal motor neurons, phrenic nerve etc.; P.A.P. performed statistical analysis. I.B.W. wrote the paper; P.A.P, G.G, J.A.L, T.K and I.B.W have read the manuscript and approved it.

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Translational impact box

Clinical Issue

Myotonic dystrophy type1 (DM1) or Steinert disease is a multisystemic autosomal dominant disorder that is the most common form of adult onset muscular dystrophy. DM1 is caused by abnormal expansion of CTG trinucleotide repeat in the region of DM protein kinase gene. Respiratory failure has long been recognized as the main complication of patients with DM1, and is probably one of the major factors contributing to mortality. Despite the several clinical demonstrations showing respiratory impairment in DM1 patients, the pathological mechanisms are still not completely known. While some studies reported that respiratory problems associated with DM1 result only from the involvement of the respiratory diaphragm muscle in progressive dystrophic and myotonic processes, others suggested that the neuronal network which generates and regulates the respiratory rhythm is also involved. Because the histological examination of nervous system in DM1 patients is only possible by autopsy, the use of animal model has become indispensable to study the mechanisms of DM1 disease.

Results

The advantage of our study is the use of a valid animal model for DM1 disease (DMSXL transgenic mice) that has a known length of CTG repeat. The DMSXL mice allow to test in the same animal the respiratory function and to analyze the structures involved in this function. The statistical analysis of breathing function measurements revealed a significant decrease in the most relevant respiratory parameters in DMSXL mice, indicating impaired respiratory function. The histological and morphometric analysis showed pathological changes in diaphragmatic NMJs and muscle fibers of DMSXL mice and a significant
decrease in the number of unmyelinated phrenic afferents, while no significant neuronopathy was detected in either cervical phrenic motor neurons or brainstem respiratory neurons.

**Implications and Future Directions**

These findings obtained in a transgenic mouse model of DM1 shed light on possible mechanisms of respiratory failure. The denervation and the pathological changes of diaphragmatic EPs may indicated a break down in communication between the diaphragmatic muscle fibers and the nerve endings which may be the main cause of respiratory failure observed in DMSXL mice. In addition, the severe loss in phrenic unmyelinated afferents which alters the regulation of breathing drive may also play a role. The DMSXL transgenic mice which are generated after large expansions of CTG repeat over successive generations of DM1 transgenic mice could be a valuable model for congenital DM1 form. Therefore, the results of our study, may contribute towards the understanding of cellular mechanisms of respiratory failure which cause the death of more than 66% of surviving patients with congenital DM1, and importantly the evaluation of respiratory parameters by non-invasive pressure plethysmography could be used to evaluate the effect of potential therapies in DMSXL mice.

In the future, the analysis of gene expression in DMSXL mice could allow us to identify the genes involved in respiratory impairment and to understand the molecular mechanisms. In summary, our study represents a crucial step for pharmaceutical and clinical research towards the development of efficient treatment of respiratory problems associated with DM1.
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**Figure legends:**

Fig.1. Representative recording showing a typical example of breathing patterns over a 4 sec snapshot in six month-old awake (A and B) and anaesthetized (C and D) wild type (A and C) and DMSXL mice (B and D), (bar = 0.5 s)

Fig.2. Cross diaphragm muscle sections of two month-old wild type (A and D), DM20 (B and E) and DMSXL (C and F) mice labelled either for myofibrillar ATPase activity after pre-incubation at pH 4.2, or with hematoxylin and eosin (HE).

On ATPase stained sections, two main fiber types can be observed in control and DMSXL mice. In wild type and DM20 control mice, there are 90% ATPase unstained fibers (fast-twitch type II fibers), only stained with Luxol fast blue, and 10% are ATPase darkly stained fibers (slow-twitch type I fibers) (A and B respectively). In DMSXL mice, a slight increase (11.5%) in the number of fast-twitch type I fibers was counted (C).

Diaphragm muscle sections taken from wild type and DM20 control mice (D and E) and DMSXL mice (F) stained with HE. The presence of interfascicular connective tissue, inflammatory cells and central nucleated fibers (arrowheads) can be easily observed on DMSXL sections (F). (scale bars = 50 μm)

Fig.3. Detection of nuclear foci by FISH on frozen sections of diaphragm muscle prepared from two month-old DMSXL mice. Using the Cy3-labelled CAG-repeats PNA-probe, the RNA inclusions are labelled in red within the nuclei of muscle fibers stained in blue with DAPI. Low magnification (A) show numerous nuclei stained in blue surrounding the diaphragm muscle fibers (bar = 20 μm). At higher magnification the presence of one or more foci inside the nuclei is easily observed (B and C, bar = 5 μm).
Fig. 4. Representative micrographs of diaphragm muscle cryostat sections stained with rhodamine α-BTX (red) and neurofilament antibody (green). In wild type control mice (A – C) practically all the EPs are innervated by branches of axons, while in DMSXL mice (D – F) EPs with no contact to axon terminals are easily identified. (C and F) illustrate a single EP at higher magnification. In DMSXL mice (D – F), the EPs have smaller size and less complex shape than in control mice (A – C). The mean surface area of EPs, the shape complexity and the density of acetylcholine receptors on post synaptic membranes labelled with rhodamine α-BTX are represented in the three histograms. More than 1500 EPs were measured from each mouse line (n = 7). Statistical analysis of the results reveals that all 3 parameters are significantly (P < 0.01) smaller in DMSXL mice compared to wild type control mice. (scale bars: A, D 100 μm; B, E 50 μm; C, F 20 μm)

Fig. 5. Representative electron micrographs of ultrathin transverse phrenic nerve sections taken from six month-old wild type (A) and DMSXL transgenic mice (B). On sections from control mice, numerous unmyelinated axons surrounded by a Schwann cell can be observed (A), while on the DMSXL mice section (B) the presence of unmyelinated axons is less frequent than in control mice. (scale bar = 1 μm)

On frozen sections prepared from DMSXL phrenic nerve (C, D), the FISH combined with neurofilament immunostaining demonstrates the presence of CUG RNA foci (red) in Schwann cell nuclei (blue) which are intermingled with the axons (green). (scale bars: C 10 μm; D 2 μm)

Histograms showing the mean number of unmyelinated fibers (E) and myelinated fibers (F) counted on ultrathin phrenic nerve sections taken from 7 wild type mice and 7 DMSXL mice. Asterisks indicate significant difference (P < 0.01).

Fig. 6. Left: caudal and rostral disector pairs sections. The density of respiratory neurons located in a region extending from the nucleus ambiguous (caudal disector pair sections)
and facial motor nucleus (rostral disector pair sections) was estimated by physical disector method. Five disector pairs were analyzed from each animal (n = 5). (scale bar = 500 µm)

Right: The two sections of a disector pair were overlaid one on top of the other using Adobe Photoshop CS5 (Adobe Systems, San Jose, CA, USA) and carefully aligned using common landmarks (general contour, brain stem nuclei, blood vessels, etc); then the neurons present in the front (C) and behind (D) sections were marked with two different types of symbols. The set of symbols from the behind section were superimposed on the ones from the front section (E) and the neurons present only in the front but not in the behind section were counted and used to estimate the density of neurons. (scale bar = 25 µm)

AN: Ambiguus Nucleus; SN: Solitary tract Nucleus; STN: Spinal tract of the Trigeminal nerve Nucleus, FN: Facial Nucleus.
Table 1.

Medium values of respiratory parameters in 2 month-old anaesthetized wild type and DM transgenic mice (n = 5)

<table>
<thead>
<tr>
<th>Respiratory parameter*</th>
<th>Wild-type mice</th>
<th>DM20 mice</th>
<th>DMSXL mice</th>
<th>P1#</th>
<th>P2#</th>
<th>P3#</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW = 28.5±0.6 g</td>
<td></td>
<td>BW = 28.0±0.8 g</td>
<td>BW = 20.2±0.8 g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RR</td>
<td>126.2±15.1</td>
<td>124.5±18.6</td>
<td>86.2±7.6</td>
<td>ns</td>
<td>&lt;0.01</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>TV (ml)</td>
<td>0.312±0.024</td>
<td>0.281±0.046</td>
<td>0.226±0.034</td>
<td>ns</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>MV (ml)</td>
<td>39.61±7.82</td>
<td>35.05±5.76</td>
<td>19.43±2.68</td>
<td>ns</td>
<td>&lt;0.01</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>TV/BW (ml/g)</td>
<td>0.011±0.001</td>
<td>0.010±0.001</td>
<td>0.011±0.001</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>MV/BW (ml/g)</td>
<td>1.43±0.23</td>
<td>1.25±0.19</td>
<td>0.96±0.14</td>
<td>ns</td>
<td>&lt;0.01</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>SpO₂</td>
<td>98.62%±0.38%</td>
<td>97.85%±1.13%</td>
<td>94.23%±1.47%</td>
<td>ns</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HR</td>
<td>574±48</td>
<td>630±64</td>
<td>619±40</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

* RR = respiratory rate; TV = tidal volume; MV = minute volume; SpO₂ = arterial blood oxygen saturation; HR = heart rate, BW = body weight. # P1: comparisons between WT and DM20 mice; P2: comparisons between WT and young DMSXL mice; P3: comparisons between DM20 and young DMSXL mice; ns = non significant.
Table 2.
Mean values of respiratory parameters in 6 month-old anaesthetized wild type and DM transgenic mice (n = 5)

<table>
<thead>
<tr>
<th>Respiratory parameter*</th>
<th>Wild-type mice</th>
<th>DMSXL mice</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW = 33.8±3.6 g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RR</td>
<td>125.7±14.4</td>
<td>87.8±5.3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TV (ml)</td>
<td>0.381±0.041</td>
<td>0.228±0.013</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>MV (ml)</td>
<td>47.72±11.01</td>
<td>20.48±1.33</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TV/BW (ml/g)</td>
<td>0.011±0.001</td>
<td>0.010±0.001</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>MV/BW (ml/g)</td>
<td>1.4±0.17</td>
<td>0.94±0.09</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

*RR = respiratory rate; TV = tidal volume; MV = minute volume; BW = body weight. # ns = non significant.