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Sensorimotor control of breathing in the \textit{mdx} mouse model of Duchenne muscular dystrophy

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Short title: \textit{Hypoventilation in young \textit{mdx} mice}

Abbreviations: AUC, area under the curve; CSA, cross-sectional area; CSN, carotid sinus nerve; CT, contraction time; DMD, Duchenne muscular dystrophy; EMG, electromyogram; $f_R$, respiratory frequency; $F_iO_2$, fractional inspired oxygen concentration; Hb, haemoglobin; $HCO_3^-$, bicarbonate; $L_o$, optimum length; MFO, mean frequency oscillation; $PCO_2$, partial pressure of $CO_2$; $PO_2$, partial pressure of $O_2$; $P_t$, isometric twitch force; $SpO_2$, arterial $O_2$ percent saturation; $TCO_2$, total $CO_2$; $V_e$, minute ventilation; $V_e/VCO_2$, ventilatory equivalent for $CO_2$; $V_e/VO_2$, ventilatory equivalent for $O_2$; $VCO_2$, carbon dioxide production; $VO_2$, oxygen consumption; $V_t$, tidal volume; $\frac{1}{2}RT$, half-relaxation time.

Key points

- Respiratory failure is a leading cause of mortality in Duchenne muscular dystrophy (DMD), but little is known about the control of breathing in DMD and animal models.
We show that young (8 weeks of age) mdx mice hypoventilate during basal breathing due to reduced tidal volume. Basal CO₂ production is equivalent in wild-type and mdx mice.

We show that carotid bodies from mdx mice have blunted responses to hyperoxia, revealing hypoactivity in normoxia. However, carotid body, ventilatory and metabolic responses to hypoxia are equivalent in wild-type and mdx mice.

Our study revealed profound muscle weakness and muscle fibre remodelling in young mdx diaphragm suggesting severe mechanical disadvantage in mdx mice at an early age.

Our study reveals a potentiated neural motor drive to breathe in mdx mice during maximal chemoactivation, suggesting compensatory neuroplasticity enhancing respiratory motor output to the diaphragm and likely other accessory muscles.

Abstract

Patients with Duchenne muscular dystrophy (DMD) hypoventilate with consequential arterial blood gas derangement relevant to disease progression. Whereas deficits in DMD diaphragm are recognized, there is a paucity of knowledge in respect of the neural control of breathing in dystrophinopathies. We sought to perform an analysis of respiratory control in a model of DMD, the mdx mouse. In eight week old male wild-type and mdx mice, ventilation and metabolism, carotid body afferent activity, and diaphragm muscle force-generating capacity, and muscle fibre size, distribution and centronucleation were determined. Diaphragm EMG activity and responsiveness to chemostimulation was determined. During normoxia, mdx mice hypoventilated, owing to a reduction in tidal volume. Basal CO₂ production was not different between wild-type and mdx mice. Carotid sinus nerve responses to hyperoxia were blunted in mdx suggesting hypoactivity. However, carotid body, ventilatory and metabolic responses to hypoxia were equivalent in wild-type and mdx mice. Diaphragm force was severely depressed in mdx mice, with evidence of fibre remodelling and damage. Diaphragm EMG responses to chemoactivation were enhanced in mdx mice. We conclude that there is evidence of chronic hypoventilation in young mdx mice. Diaphragm dysfunction confers mechanical deficiency in mdx resulting in impaired capacity to generate normal tidal volume at rest and decreased absolute ventilation during chemoactivation. Enhanced mdx diaphragm EMG responsiveness suggests compensatory neuroplasticity facilitating respiratory motor output, which may extend to accessory muscles of breathing. Our results may have relevance to emerging treatments for human DMD aiming to preserve ventilatory capacity.

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Keywords: Duchenne muscular dystrophy, mdx, hypoventilation, carotid body, diaphragm, EMG

Abstract word count= 245

1. Introduction

Duchenne muscular dystrophy (DMD) is a fatal X-linked neuromuscular disease characterized by dystrophin deficiency. Deficits in this structural protein lead to aberrant structural remodelling and damage in skeletal and other muscles (Muntoni et al., 2003; Deconinck & Dan, 2007; Ervasti, 2007; Mosqueira et al., 2013b), with consequential profound muscle weakness extending to the striated muscles of breathing—the final effectors in the respiratory control network. Respiratory insufficiency is a hallmark of DMD (Baydur et al., 1990; Bersanini et al., 2012). Whereas diaphragm dysfunction is a recognized primary morbid feature in DMD (De Bruin et al., 1997; Beck et al., 2006; Khirani et al., 2014), there is a paucity of knowledge in respect of the neural control of breathing in the human dystrophinopathies. Of note, DMD patients hypoventilate with resultant hypoxaemia (Smith et al., 1989a, b; Melacini et al., 1996), a symptom which may have particular relevance to muscle pathology and disease progression, in the light of data from rodent models revealing hypoxia-induced respiratory muscle weakness (McMorrow et al., 2011; Skelly et al., 2012; Shortt et al., 2014) due to altered redox signalling and overt oxidative stress (Lewis et al., 2015; Lewis et al., 2016), which are features of DMD.

Respiratory failure in DMD results in premature death. Yet, the early impact of dystrophin deficiency on the respiratory control network is unclear. This knowledge gap is significant and has potential relevance to the treatment of DMD. It is not known if dystrophin deficiency has consequences for sensorimotor control of breathing. There is ample evidence in support of remarkable capacity for plasticity within the respiratory control network governing arterial blood gas and pH homeostasis in health and disease (Mitchell & Johnson, 2003; Kumar & Prabhakar, 2012; Fuller & Mitchell, 2017). Sensory and motor plasticity is context-dependent, with a capacity for adaptive or maladaptive outcomes. Compensatory neuroplasticity at one or more sites of the respiratory control network could ameliorate respiratory muscle deficits in early stages of DMD. Conversely, sensorimotor deficits could exacerbate aberrant respiratory control in DMD and contribute to disease progression. Dystrophin is present in the carotid body (Mosqueira et al., 2013a), the primary blood oxygen sensor, but it is unclear if dystrophin deficiency affects chemoafferent discharge and as such, the control of breathing. Dystrophin is also present in neurons (Lidov, 1996), but it is not known if central respiratory motor drive is affected by dystrophinopathies, potentiating or ameliorating...
impaired respiratory mechanics due to respiratory muscle weakness. A greater understanding of the control of breathing in DMD is likely to prove important to therapeutic strategies, and might offer novel targets in interventional therapies particularly early in disease onset before the establishment of overt respiratory pathology.

We sought to perform an assessment of respiratory control in a young murine model of DMD—the mdx mouse. Whereas, diaphragm dysfunction faithfully recapitulating the human condition has been established in the model, assessment of respiratory control, especially in young animals, is lacking. We hypothesized that there should be evidence of neuroplasticity in the respiratory control network of the mdx mouse.

2. Methods

2.1 Ethical approval

Procedures concerning live animals were performed under licence in accordance with Irish and European directive 2010/63/EU following approval by University College Cork animal research ethics committee. Carotid body recordings were performed in accordance with The Canadian Council on Animal Care Guidelines and were approved locally by the Animal Care Committee of the Cumming School of Medicine, University of Calgary, Canada.

2.2 Experimental Animals

Male and female wild-type (C57BL/10ScSnJ) and mdx (C57BL/10ScSn-Dmd<sup>mdx</sup>/J) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and bred at University College Cork’s animal housing facility. Eight week old male wild-type (n=53) and mdx (n=52) mice were studied for respiratory and metabolism measurements, ex vivo muscle function tests, tissue harvesting for immunohistochemistry, in vivo EMG recordings and arterial blood gas analysis. For carotid body-carotid sinus nerve preparations, male wild-type (n=6) and mdx (n=6) mice were purchased from the Jackson Laboratory and transferred to the University of Calgary for subsequent experiments at eight weeks of age. Animals were housed conventionally in temperature- and humidity-controlled facilities, operating on a 12 h light: 12 h dark cycle with food and water available ad libitum.

2.3 Respiratory recordings

Respiratory flow recordings were performed using whole-body plethysmography in unrestrained, unanaesthetized mice during quiet rest. Wild-type (n=13) and mdx (n=12) mice were introduced into plethysmograph chambers (Model PLY4211; volume=0.6L, Buxco Research Systems, Wilmington, NC,
USA) and allowed a 60-90 minute acclimation period until sufficiently settled, with room air passing through each chamber (1 L/min). Recordings were typically performed in parallel, with contemporaneous assessment of breathing in one wild-type mouse and one mdx mouse using a two chamber set-up. For successive recording sessions, mice were assigned to chambers based on genotype in an alternating fashion to avoid any potential bias associated with a given chamber. Post hoc analysis of breathing within each genotype confirmed no difference in parameters recorded comparing data derived from the two chambers. **Experimental protocol:** Following the acclimation period, a 20-30 minute baseline recording was performed in normoxia. This was followed by a 20 minute hypoxic challenge (F\(_{\text{O}_2}\)=0.1; balance N\(_2\)). Following a 60 minute recovery period in normoxia, a 20-30 minute normoxic baseline period was recorded. This was followed by a graded hypoxic challenge in which animals were challenged with decreasing levels of inspired oxygen: F\(_{\text{O}_2}\)=0.15, 0.12, 0.10, and 0.08 (balance N\(_2\)) consecutively for 5 minutes each. Respiratory parameters including respiratory frequency (f\(_R\)), tidal volume (V\(_T\)) and minute ventilation (V\(_E\)) were recorded on a breath-by-breath basis for analysis offline. **Data analysis:** To maximize the recording period for the assessment of ventilatory parameters in normoxia, both normoxic bouts were pooled to generate one set of baseline (normoxia) data; there was no significant difference for ventilatory parameters between the two normoxic periods. Based on the time constant for the plethysmograph chambers and that gases were thoroughly mixed before entry to the plethysmograph chambers, we assumed complete gas mixing during gas challenges by ~3 minutes of exposure. For the sustained (20 minute) hypoxic challenge, data are shown after 5 minutes of exposure and are presented on a minute-by-minute basis thereafter. For the graded hypoxic challenge, measurements were taken during the 5\(^{th}\) minute of exposure to the given F\(_{\text{O}_2}\) challenge. For each of the hypoxic challenges (sustained and graded), data during hypoxic exposure were compared with the preceding 5 minutes of baseline (F\(_{\text{O}_2}\): 0.21). In a separate historical cohort of wild-type (n=10) and mdx (n=11) mice (Burns et al., 2015), we assessed the ventilatory response to a 10 minute hypercapnic gas challenge (5% CO\(_2\), balance O\(_2\)). V\(_T\) and V\(_E\) were normalized for body mass (g).

### 2.4 Metabolism measurements

O\(_2\) consumption (VO\(_2\)) and CO\(_2\) production (VCO\(_2\)) were measured in wild-type (n=13) and mdx (n=12) mice undergoing the whole-body plethysmography protocol. Airflow through the chamber was maintained at 1 L/min. Fractional concentrations of O\(_2\) and CO\(_2\) were measured in air entering and exiting the plethysmograph (O\(_2\) and CO\(_2\) analyzer; ADInstruments, Colorado Springs, CO, USA) similar to that previously described (Bavis et al., 2010; Bavis et al., 2014). **Data analysis:** calculation of VO\(_2\) and VCO\(_2\) was performed as previously described (Haouzi et al., 2009). For the sustained (20
minute) hypoxic challenge data are shown after 5 minutes of exposure and are presented on a minute-by-minute basis thereafter. For the graded hypoxic challenge, measurements were taken during the 5th minute of exposure to the given FIO2 challenge. For each of the hypoxic challenges (sustained and graded), data during hypoxic exposure were compared with the preceding 5 minutes of baseline (FIO2: 0.21). VO2 and VCO2 were normalized for body mass (g).

2.5 Blood gas analysis

Wild-type (n=7) and mdx (n=7) mice were anaesthetized with 5% isoflurane in air. A laparotomy was performed and cardiac puncture was performed by advancing a 25G needle through the diaphragm and into the apex of the heart for blood sampling for analysis. 0.2ml of blood was collected and used to measure pH, the partial pressure of O2 (PO2) and CO2 (PCO2), total CO2 (TCO2), bicarbonate concentration [HCO3-], arterial saturation (SaO2), sodium ion concentration [Na+], potassium ion concentration [K+], haematocrit and haemoglobin concentration [Hb] using a blood gas analyzer (i-Stat; Heska, Fort Collins, CO, USA). Euthanasia was confirmed with cervical dislocation.

2.6 Arterially perfused ex vivo carotid body-carotid sinus nerve preparation

At the University of Calgary, wild-type (n=6) and mdx (n=6) mice were heavily anaesthetized with isoflurane and then decapitated (lower cervical level). The carotid bifurcation, including the carotid body-carotid sinus nerve-superior cervical ganglion, was quickly isolated en bloc for in vitro perfusion as described previously (Roy et al., 2012). The carotid bifurcation was then transferred to a dissection dish containing physiological saline (in mM: 1 MgSO4, 1.25 NaH2PO4, 4 KCl, 24 NaHCO3, 115 NaCl, 10 glucose, 12 sucrose, and 2 CaCl2) and equilibrated with hyperoxia (95% O2/5% CO2). After 15-20 min, the isolated tissue was transferred to a recording chamber with a built-in water-fed heating circuit and the common carotid artery was immediately cannulated for luminal perfusion with physiological saline equilibrated with 100 mmHg PO2 and 36 mmHg PCO2 (balance N2). The carotid sinus nerve was then carefully desheathed and the carotid sinus region was bisected. The occipital, internal, and external arteries were ligated, and small incisions were made on the internal and external carotid arteries to allow perfusate to exit. A peristaltic pump was used to set the perfusion rate at ~10 ml/min, which was sufficient to maintain a constant pressure of 90-100 mmHg at the tip of the cannula. The perfusate was equilibrated with computer-controlled gas mixtures monitored using CO2 and O2 gas analyzers (models CA-2A and PA1B, respectively, Sable Systems, Las Vegas, NV, USA); a gas mixture of 100 mmHg PO2 and 36 mmHg PCO2 (balance N2) was used to start the experiments (yielding pH ~7.4). Before reaching the cannula, the perfusate was passed through a bubble trap and heat exchanger. The temperature of the perfusate, measured continuously as it
departed the preparation, was maintained at 37±0.5°C. The effluent from the chamber was recirculated.

Chemosensory discharge was recorded extracellularly from the whole desheathed carotid sinus nerve, which was placed on a platinum electrode and lifted into a thin film of paraffin oil. A reference electrode was placed close to the bifurcation. Carotid sinus nerve activity was monitored using a differential AC amplifier (model 1700, AM Systems) and a secondary amplifier (model AMS02, Tektronix, Beaverton, OR). The neural activity was amplified, filtered (300-Hz low cutoff, 5-kHz high cutoff), displayed on an oscilloscope, rectified, integrated (200-ms time constant), and stored on a computer using an analogue-to-digital board (Digidata 1322A, Axon Instruments) and data acquisition software (Axoscope 9.0). Preparations were left undisturbed for 45 min to stabilize before the experimental protocol began. Experimental protocol: The following protocol was used for all experiments: 1) the carotid body was perfused for 5 min with normoxia (100 mmHg PO₂ and 36 mmHg PCO₂; balance N₂) to determine baseline carotid sinus nerve activity; 2) neural responses were obtained by challenging the carotid body for 5 min with mild, moderate and severe hypoxia (80, 60 & 40 mmHg, respectively) interspersed with normoxia; 3) finally a hyperoxic (500 mmHg PO₂ and 36 mmHg PCO₂; balance N₂) challenge was given for 5 min to examine the sensitivity of the carotid body (Dejours test). Data analysis: Data were analyzed offline using custom software (written by R.J.A. Wilson). Carotid sinus nerve activity was divided into 2s time bins, and activity in each bin was rectified and summed (expressed as integrated neural discharge). Data are shown as absolute carotid sinus nerve discharge frequencies in each of the different conditions in the protocol. Neural responses to PO₂ challenge were determined by comparing absolute discharge frequencies and also responses normalized to the hyperoxic condition, and separately to the normoxic condition.

2.7 Ex vivo muscle function tests

Diaphragm muscle function was examined ex vivo under isometric conditions using a standardized protocol as previously described (Shortt et al., 2014). Wild-type (n=7) and mdx (n=7) mice were anaesthetized with 5% isoflurane by inhalation in air and euthanized by cervical dislocation. The diaphragm muscle was excised immediately with rib and central tendon attached. Longitudinally arranged bundles were prepared for assessment of contractile function and were suspended vertically between two platinum plate electrodes. The rib was attached to a fixed hook at one end and the central tendon was attached to a force transducer with non-elastic string at the other end. Muscle baths contained Krebs solution (in mM: 120 NaCl, 5 KCl, 2.5 Ca²⁺, 1.2 MgSO₄, 1.2 NaH₂PO₄, 25 NaHCO₃, 11.5 glucose) and α-tubocurarine (25μM) and were equilibrated under hyperoxic conditions.
The optimum length (L_o) was determined by adjusting the position of the force transducer, and hence the length of the muscle preparations, by use of a micro-positioner between intermittent twitch contractions (Burns & O'Halloran, 2016; Burns et al., 2017). L_o was taken as the muscle length associated with maximal isometric twitch force in response to single isometric twitch stimulation (supramaximal stimulation, 1ms duration). Once L_o was determined, the muscle was held at this length for the duration of the protocol. **Experimental protocol:** A single isometric twitch was measured. Peak isometric twitch force (P_t), contraction time (CT; time to peak force) and half-relaxation time (½ RT; time for peak force to decay by 50%) were determined. The force-frequency relationship was determined by sequentially stimulating the muscle at 10, 20, 40, 60, 80, 100, 120, 140, 160 Hz (300ms train duration), allowing a 1 min interval between stimulation. **Data analysis:** Specific force was calculated in N/cm² of muscle cross-sectional area (CSA). The CSA of each strip was determined by dividing the muscle mass (weight in grams) by the product of muscle L_o (cm) and muscle density (assumed to be 1.06 g/cm³). The CT and ½ RT were measured as indices of isometric twitch kinetics. Normalization of diaphragm forces to CSA was principally a means of standardizing the ex vivo muscle preparations. It should be noted that tissue, and not muscle fibre CSA was estimated with this approach and our data do not provide information on the specific force of muscle fibres per se, which might also require consideration of revised muscle density values in mdx. Absolute muscle forces (N) were also assessed and compared in our study.

### 2.8 Muscle immunohistochemistry

Wild-type (n=8) and mdx (n=8) mice were anaesthetized with 5% isoflurane by inhalation in air and euthanized by cervical dislocation. Diaphragm muscle was excised immediately and a section of the hemi-diaphragm was mounted on a block of liver. Tissue samples were embedded in optimum cutting temperature (OCT; VWR International, Dublin, Ireland) embedding medium, frozen in isopentane (Sigma Aldrich, Wicklow, Ireland) cooled in liquid nitrogen and stored at -80°C for subsequent structural analysis. Serial transverse muscle sections (10µm) were cryosectioned (Leica CM3050; Leica Microsystems, Nussloch, Germany) at -22°C and mounted on polylysine-coated glass slides (VWR International, Dublin, Ireland). Slides were immersed in PBS (0.01 M) containing 1% bovine serum albumin (BSA) for 15 minutes. After 3x5 minute PBS washes, slides were immersed in PBS containing 5% normal goat serum (Sigma Aldrich, Wicklow, Ireland) for 30 minutes. Slides then underwent a further 3x5 minute PBS washes prior to application of the primary antibody (rabbit anti-laminin, 1:500; Sigma Aldrich, Wicklow, Ireland), diluted in PBS and 1% BSA. Slides were incubated overnight at 4°C in a humidity chamber. After the incubation period, slides were washed with PBS for 3x5 minutes before the secondary antibody (FITC-conjugated goat anti-rabbit; 1.250,
Sigma Aldrich, Wicklow, Ireland), diluted in PBS and 1% BSA, was applied. Slides were incubated for 1 hour in the dark at room temperature. To identify myonuclei, the nuclear stain Hoechst (Sigma Aldrich, Wicklow, Ireland) was diluted in PBS (1:4) and applied to a subset of muscle sections for 10 minutes. Slides were rinsed with PBS for 5 minutes, cover-slipped with polyvinyl alcohol mounting medium with DABCO® anti-fade (Sigma Aldrich, Wicklow, Ireland). Data analysis: Muscle sections were viewed at x10 magnification and images captured using an Olympus BX51 microscope and an Olympus DP71 camera. For each animal, 3-4 images were captured for analysis from multiple muscle sections. For measurements, a square test frame of (600 x 600 μm), with inclusion and exclusion boundaries, was placed randomly over each image (Shortt et al., 2014). To determine the size distribution of muscle fibres within the diaphragm the individual fibre boundaries were determined using Imaris software. From this, the fibre cross-sectional area as well as Feret’s minimal diameter were determined ( Dubach-Powell, 2008). The coefficient of variation of muscle fibre minimal Feret’s diameter, was also constructed for wild-type and mdx. In a subset of animals (n=5 per genotype) centrally nucleated muscle fibres were identified using ImageJ software on merged laminin and hoescht stained images. The proportion of centrally nucleated muscle fibres was expressed relative to the total number of myofibres analyzed per image. Data generated from multiple images was averaged per animal before computing group means.

2.9 Diaphragm EMG activity and responsiveness

Anaesthesia was induced with 5% isoflurane in 60% O₂ (balance N₂) followed by urethane (1.8 g/kg i.p.). Wild-type (n=8) and mdx (n=7) mice were then placed in the supine position, gradually weaned off the isoflurane and body temperature was maintained at 37°C via a rectal probe and thermostatically-controlled heating blanket (Harvard Apparatus, Holliston, MA, USA). Supplemental anaesthetic was administered if necessary to maintain a surgical plane of anaesthesia, which was assessed by assessment of pedal withdrawal reflex to noxious pinch. A pulse oximeter clip (MouseOx™, Starr Life Sciences Corporation, Oakmount, PA, USA) was placed on the thigh of each mouse for the measurement of arterial O₂ saturation. A mid-cervical tracheotomy was performed to avoid upper airway obstruction. All animals were maintained with a bias flow of supplemental O₂ (FiO₂ = 0.60) unless otherwise stated. Concentric needle electrodes (26G; Natus Manufacturing Ltd, Ireland) were inserted into the costal diaphragm for the continuous measurement of diaphragm EMG activity which was amplified (x5,000), filtered (500Hz low cut-off to 5,000Hz high cut-off) and integrated (50ms time constant; Neurolog system, Digitimer Ltd, UK). All signals were passed through an analogue-to-digital converter (r8/30; ADInstruments, Colorado Springs, CO, USA) and were acquired using LabChart 7 (ADInstruments, Colorado Springs, CO, USA). Experimental protocol:
Spontaneously breathing animals were vagotomized and allowed to stabilize for a minimum of 5 minutes before baseline parameters were measured. It is established that chemostimulation in spontaneously breathing mice elicits a significantly greater phrenic motor response following vagotomy compared with vagi intact (Kline et al., 2002). Next, animals were sequentially challenged with hypercapnia (5% CO$_2$ and 10% CO$_2$; 2min each), hypoxia (15% O$_2$; 1min), and asphyxia (15% O$_2$/5% CO$_2$; 1min) to examine the effects of chemostimulation on diaphragm EMG activity. Following completion of diaphragm EMG recordings, animals were euthanized via cervical dislocation. Data analysis: Amplitude and area under the curve (AUC) of integrated respiratory EMG activity was analyzed and averaged under steady-state basal conditions, and for 1 minute of baseline immediately prior to chemostimulation challenges. Amplitude and AUC of integrated respiratory EMG activity was analyzed and averaged for the final 15 breaths (maximal response) of the chemostimulation challenges. Baseline data were reported in absolute units. Responses to chemostimulation were expressed as percent change from the preceding baseline value. This portrayal of the data was considered appropriate given that baseline EMG activity was found to be equivalent in wild-type and mdx mice. As such, percent change from baseline is in effect equivalent to ΔEMG activity and importantly, a difference in the percent change between groups corresponds to a true difference in total EMG activity, important in the context of a transduction of neuromuscular-to-mechanical activity. Because we tested the hypothesis that diaphragm EMG activity would be altered in mdx mice compared with wild-type mice we did not normalize EMG data to a maximum reference value within each preparation (e.g. augmented breath or swallow), because such a maximum value could itself be changed in mdx mice resulting from neuroplasticity. Our principal focus was the level of EMG activity per se. In 1 mdx and 2 wild-type mice, the EMG response to gas challenges was characterized by tachypnoea and reduced EMG amplitude. Since our aim was to compare the magnitude of the increase in EMG amplitude (motor recruitment) during chemostimulation, we established a priori that trials characterized by frequency-only responses to gas challenge (tachypnoea) would be excluded from group analysis of the effects of chemostimulation on EMG amplitude and area under the curve in wild-type and mdx mice.

2.10 Statistical Analysis

Values are expressed as mean±SD or as box and whiskers plot (median, 25-75% centiles and minimum and maximum values). Data were statistically analyzed by Prism 6.0 (Graphpad Software, San Diego, CA, USA). For measures of baseline ventilation and metabolism, basal carotid sinus nerve activity during normoxia and hyperoxia, basal diaphragm EMG activity, diaphragm muscle twitch force and contractile kinetics, diaphragm muscle fibres, and arterial blood gas analysis, all data for wild-type and mdx groups were tested for normal distribution and equal variances and were
statistically compared using unpaired two-tailed Student t tests, with Welch’s correction for unequal variances used as appropriate. Ventilatory and metabolic responsiveness to hypoxia (separate sustained and graded challenges), carotid sinus nerve activity response to hypoxic challenge, diaphragm muscle force-frequency relationship, and diaphragm EMG responses to chemostimulation in wild-type and mdx groups were statistically compared by repeated measures two-way ANOVA (gas x gene) with Bonferroni post hoc test. Data for absolute carotid sinus nerve activity were non-parametric and therefore were log transformed. \( P < 0.05 \) was considered statistically significant in all tests.

3. Results

3.1 Baseline ventilation and metabolism

Representative respiratory flow traces for wild-type and mdx mice during baseline (normoxic) ventilation are shown in Fig. 1A. Minute ventilation during baseline conditions (combined normoxic bouts) was significantly reduced in mdx compared with wild-type mice \( (P = 0.0001; \text{unpaired Student } t \text{ test}; \text{Fig. 1D}) \). This reduction in normoxic \( V_t \) in mdx mice was the result of a lower \( V_e \) \( (P = 0.0003; \text{Fig. 1C}) \); \( f_e \) did not differ significantly between groups \( (P = 0.2612; \text{Fig. 1B}) \). Performing this analysis on absolute volume data (not normalized for body mass) yielded similar effects for both \( V_e \) and \( V_t \) (data not shown). Respiratory and metabolic parameters are shown in Table 1. Assessment of O\(_2\) consumption (VO\(_2\)) and CO\(_2\) production (VCO\(_2\)) revealed minimal differences between wild-type and mdx mice. VO\(_2\) (normalized to body mass) was significantly reduced for mdx \( (P = 0.0043) \) compared with wild-type, but not when expressed in absolute terms of oxygen consumed \( (P = 0.6985) \). No difference was noted for VCO\(_2\) between groups when analysis was performed on absolute and normalized values. Carbon dioxide production, chosen \textit{a priori} as the preferred index of metabolism, was effectively unchanged between wild-type and mdx mice. The ventilatory equivalent for CO\(_2\) \( (V_t/\text{VCO}_2) \) was significantly reduced for mdx \( (P = 0.0243) \) compared with wild-type mice, indicative of hypoventilation in mdx mice. The ratio of VCO\(_2\) to VO\(_2\) (respiratory exchange ratio) was not different between groups.

3.2 Blood gas analysis

Intra-cardiac blood gas data for wild-type and mdx mice are shown in Table 2. Haematocrit was significantly lower in mdx \( (P = 0.0005; \text{unpaired Student } t \text{ test}) \) compared with wild-type, but haemoglobin concentration was equivalent between the two groups. Significant increases in blood values for \([\text{HCO}_3^-]\) \( (P = 0.0496) \), TCO\(_2\) \( (P = 0.00382) \), \([\text{K}^+]\) \( (P = 0.0078) \), and \([\text{Na}^+]\) \( (P = 0.0442) \) were observed in mdx compared with wild-type.
3.3 Carotid sinus nerve discharge

Figure 2 shows representative traces from wild-type (A) and mdx (B) carotid body-carotid sinus nerve preparations ex vivo. Group data for carotid sinus nerve activity (normalized to hyperoxia) for wild-type and mdx preparations during normoxia (100 mmHg) and in response to a graded hypoxic challenge (80, 60 and 40 mmHg) are shown in Fig. 2C. Of note during normoxia, carotid sinus nerve activity was ~30% less in mdx compared with wild-type preparations (P = 0.0064; unpaired Student t test), revealing a relative hypoactivity of carotid body afferent discharge in normoxia. Both wild-type and mdx preparations responded to decreasing levels of O₂ by corresponding increases in carotid sinus nerve activity (P < 0.0001; two-way ANOVA); however, no significant difference in hypoxic responsiveness was noted between wild-type and mdx (gas x gene P = 0.6452). Statistical judgment of the data was equivalent whether normalized to hyperoxia or normoxia. Data for absolute carotid sinus nerve discharge frequencies in each condition are shown in Table 3. Consistent with the normalized data, there was no statistical difference in hypoxic responsiveness between wild-type and mdx preparations (Table 3). The carotid sinus nerve discharge frequency response to hyperoxia (Dejours test) was significantly blunted in mdx compared with wild-type (-18.7±7.2 vs. -7.7±3.1 impulses per min, unpaired Student t test, P = 0.011 for wild-type (n=6) vs. mdx (n=6) mice), further suggestive of a relative hypoactivity during normoxia in mdx carotid body.

3.4 Ventilatory responsiveness to hypoxia and hypercapnia

Figure 3 shows ventilatory and metabolic data during normoxia and in response to graded hypoxia. Minute ventilation was lower in mdx (P = 0.0054; two-way ANOVA) compared with wild-type, attributed to a lower tidal volume in mdx mice. VCO₂ decreased in response to hypoxia in wild-type and mdx mice (P < 0.0001). V̇E/VCO₂ increased for both wild-type and mdx mice with decreasing levels of inspired O₂ (P < 0.0001). No significant difference in V̇E/VCO₂ was observed between wild-type and mdx mice in response to graded hypoxia. Fig. 4 shows the time course of minute ventilation to a 20 minute sustained hypoxic challenge (ḞO₂ = 0.1; balance N₂). Ventilation increased rapidly in wild-type and mdx mice at the onset of hypoxia (P < 0.0001) and then declined towards baseline values (Fig. 4). A significantly lower V̇E in mdx compared with wild-type mice was observed during gas challenge (P = 0.03), owing to reduced V̇E in mdx mice (Fig. 4). The peak hypoxic ventilatory response was not different between strains (delta V̇E was +1.44±0.80 vs. +0.90±0.70 ml/min/g, unpaired Student t test, P = 0.07 for wild-type (n=13) vs. mdx (n=12); % change from baseline was +131.8±52.1% vs. +111.7±69.6%, P = 0.4185). Figure 5 shows data for V̇E during baseline and hypercapnic gas challenge. Minute ventilation was increased significantly both in wild-type and mdx mice during CO₂ exposure. Minute ventilation remained significantly lower in mdx compared with...
wild-type mice during hypercapnic breathing, but the ventilatory response to hypercapnia was not different between wild-type and mdx mice (delta VE was +1.9±0.8 vs. +1.7±0.6 ml/min/g, unpaired Student t test, P = 0.5768 for wild-type (n=10) vs. mdx mice (n=11); % change from baseline was +131±47% vs. +178±80%, P = 0.1194).

3.5 Diaphragm muscle function

Representative original traces for wild-type and mdx diaphragm twitch contraction (A) and force-frequency relationship (B) are shown in Fig. 6. Isometric twitch force and contractile kinetics for wild-type and mdx mice are shown in Table 4. Twitch contraction time was significantly prolonged in mdx compared with wild-type diaphragms (P = 0.0155; unpaired Student t test). Twitch force was significantly reduced in mdx diaphragm muscle preparations (P = 0.0292). For force-frequency relationship, diaphragm specific force was significantly depressed in mdx compared with wild-type preparations (P = 0.0002, repeated measures two-way ANOVA). Post-hoc analysis revealed significant differences between wild-type and mdx diaphragm force generation across a broad stimulus range (40-160 Hz). Absolute measurements of diaphragm force (N), prior to normalization to CSA, were significantly depressed in mdx compared with wild-type.

3.6 Diaphragm muscle fibre size and distribution

Representative immunofluorescence images from wild-type and mdx diaphragm muscle are shown in figure 7. Dystrophin deficiency in mdx diaphragm resulted in a significant increase in the coefficient of variation of muscle fibre size (P < 0.0001; unpaired Student t test), as measured by minimal Feret’s diameter (Fig. 7C). There was a significantly increased proportion of centralized myonuclei, indicative of muscle damage in mdx compared with wild-type (P = 0.0005; Fig. 7D). A leftward shift in the frequency distribution of muscle fibre size was evident in mdx diaphragm, based on minimal Feret’s diameter (Fig. 7E) or CSA (Fig. 7F).

3.7 Diaphragm EMG responsiveness

Representative original diaphragm EMG traces during baseline and in response to chemostimulation are shown in figure 8A. Diaphragm EMG activity was examined in wild-type and mdx mice during baseline (60% O₂) and in response to chemostimulation challenges. Basal diaphragm EMG amplitude and AUC was not different between wild-type and mdx mice (Fig. 8B, inset). Gas challenges typically increased EMG responsiveness for both wild-type and mdx for amplitude (P = 0.0202; repeated measures two-way ANOVA; Fig. 8B) and AUC (P = 0.0531; Fig. 8C). For AUC, a significant genotype difference was noted (P = 0.0452) and post-hoc analysis revealed EMG responsiveness to maximal

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chemostimulation (asphyxia) was significantly enhanced in mdx compared with wild-type mice \((P < 0.05;\) two-way ANOVA with Bonferroni post-hoc test).

4. Discussion

The main findings of our study are: 1) young (eight week old) mdx mice hypoventilate during basal breathing, owing to reduced tidal volume; 2) intra-cardiac blood total CO\(_2\) and [HCO\(_3^-\)] are elevated in young mdx mice suggesting compensated respiratory acidosis; 3) the carotid body response to hyperoxia is depressed in young mdx mice; 4) chemosensory, ventilatory, and metabolic responses to hypoxia are unaffected in young mdx mice; 5) there is profound diaphragm muscle weakness, fibre remodelling and damage in young mdx mice; 6) diaphragm EMG responsiveness to chemostimulation is enhanced in young mdx mice suggesting compensatory neuroplasticity.

Overall, our study revealed that basal CO\(_2\) production was unaffected in mdx mice, but mdx mice had a substantial reduction in tidal volume that was not compensated for by increased breathing frequency resulting in reduced minute ventilation. Thus, mdx mice hypoventilate by 8 weeks of age i.e. inadequate ventilation to meet metabolic demand, which is suggested by the data for reduced ventilatory equivalent for carbon dioxide \((V_e/VCO_2)\) in mdx mice. Cardiac puncture for blood gas analysis under isoflurane anesthesia (breathing air) revealed a trend towards decreased PO\(_2\) and increased PCO\(_2\) in mdx, but the changes were modest in comparison to the ventilatory data derived by plethysmography. There are at least three factors to consider. First, the blood was sampled from anaesthetized animals, and anaesthesia has pronounced inhibitory effects on ventilation, though this might be expected to potentiate respiratory depression in mdx mice. Second, there may be cardio-pulmonary adjustments that compensate for mechanical deficits in young mdx mice. Third, the sample size may have been too small to reveal statistical significance in some key parameters (PO\(_2\) and PCO\(_2\)) that did change in a manner consistent with hypoventilation. Nonetheless, total CO\(_2\) and [HCO\(_3^-\)] were significantly elevated in mdx mice suggesting the development of a compensated respiratory acidosis secondary to chronic hypoventilation. Persistent hypoventilation and resultant hypoxaemia, which have been described in mdx mice at 6 months of age (Mosqueira et al., 2013a), would be expected to cause increased haematocrit and haemoglobin concentration. Of interest, we noted significantly enlarged, darkened spleens in mdx mice, which may have implications for the control of circulating red blood cells. It would be interesting to determine if mdx mice are chronically hypoxic in early life, as hypoxic stress has the capacity to drive plasticity at multiple sites within the respiratory control network.
Sensory inputs from the peripheral chemoreceptors are generally regarded as providing a tonic drive to “eupnoeic” breathing. Moreover, while they primarily detect hypoxia, their sensitivity is strongly modulated by PaCO₂ and they have powerful non-linear interactions with central (brainstem) chemoreceptors that also detect CO₂ concentration (Wilson & Teppema, 2016). Therefore, the relative hypoactivity of mdx compared with wild-type carotid bodies might have contributed to the resting hypoventilation of mdx mice. Carotid body responses to hypoxia (mild, moderate and severe), whether assessed as absolute or normalized data, were equivalent in mdx and wild-type mice with no statistical genotype or genotype x gas effect. Of note, carotid sinus nerve afferent discharge was less at all PO₂ levels in mdx preparations, such that chemoafferent drive to the respiratory centres was lower in mdx compared with wild-type representing a potentially physiologically relevant sensory deficit in the control of breathing. Interestingly, ventilatory responsiveness to sustained and graded hypoxia was equivalent in mdx and wild-type mice with no genotype effect. Of note, however, ventilation was reduced in mdx due to significant reductions in tidal volume at all levels of hypoxic ventilation, which may relate to sensory deficit as well as mechanical disadvantage in mdx over a range of ventilations compared with wild-type. Metabolism and metabolic responses to hypoxia were generally equivalent between wild-type and mdx mice.

While our report is the first to characterize respiratory and metabolic parameters in young mdx mice and describe that a significant respiratory phenotype presents as early as 8 weeks of age, our data are generally consistent with reports of hypoventilation in older (6-12 months of age) mdx mice (Huang et al., 2011; Mosqueira et al., 2013a). When viewed together, the implication of these findings is that respiratory insufficiency presents early in the mdx model, and thus likely impacts on the progression and manifestation of respiratory morbidity typically reported in older animals.

Our study confirmed profound diaphragm weakness in mdx mice. Coirault et al. (1999) have previously reported reduced strength in mdx diaphragm which was associated with a reduction in the number of cross bridges generating contractile force and in the elementary force generated per actomyosin interaction. These functional changes were associated with changes in myosin isoform composition (shift from myosin heavy chain type IIX to IIA). Interestingly, diaphragm mechanical dysfunction is present at a young age (6 weeks) when muscle fibre necrosis and/or fibrosis remain limited (Coirault et al., 2003). Diaphragm force-generating capacity in the present study was depressed across a broad range of stimulation frequencies including the range relevant to basal breathing. Respiratory nerve activity in mice displays medium frequency oscillations (MFOs) in the range of 20-50Hz (O'Neal et al., 2005; ElMallah et al., 2016), which are believed to reflect the underlying motor neuron discharge rates (Christakos et al., 1991). The severe weakness in mdx

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diaphragm at this early age appears primarily responsible for the mechanical disadvantage manifest in reduced tidal volumes in freely-behaving mice. Force increases as a function of stimulation frequency in mdx mice, which provides capacity to increase breathing in response to increased neural drive. However, the intrinsic weakness in mdx diaphragm is impressive even at this early stage, presumably placing a limit on ventilatory capacity. Moreover, force-generating capacity remains severely compromised at higher stimulation frequencies.

There are few studies examining the hypoxic ventilatory response in mdx (Mosqueira et al., 2013a; Burns et al., 2015). The hypometabolic response to hypoxia in mice is such that ventilatory responses to hypoxia are modest. Indeed, tidal volume and ventilation do not increase much above baseline in response to graded hypoxia, and the ventilatory response to sustained hypoxia is primarily driven by increased respiratory frequency. Therefore, it is difficult to discern from ventilatory responses to hypoxia if mdx mice are capable of transducing increased diaphragm force-generating capacity resulting from motor recruitment to increase ventilation. To examine this further, we assessed hypercapnic ventilatory responses in wild-type and mdx mice. Ventilation increased in response to hypercapnic challenge, due to increases in respiratory frequency and tidal volume, and the response was equivalent in the two groups. The data reveal that mdx mice are capable of increasing tidal volume during chemostimulation. Although, ventilation remains significantly lower in mdx compared with wild-type mice during hypercapnic breathing, the ventilatory response to hypercapnia is not different between the two groups of mice. This reveals an interesting feature of respiratory control in the young mdx mouse, namely that a considerable reserve in ventilatory capacity prevails, which presumably extends to accessory muscles of breathing given the profound weakness noted in the diaphragm muscle. Analysis of fibre size and distribution in diaphragm muscle revealed considerable fibre remodelling and evidence of centronucleation, which is a hallmark of fibre necrosis. Diaphragm muscle structure of mdx mice has been well described with muscle fibres undergoing inflammatory cell infiltration, fibrosis and necrosis (Gayraud et al., 2007; Ishizaki et al., 2008; Huang et al., 2011). In addition to structural abnormalities in mdx resulting in loss of function, there is growing evidence implicating oxidative stress in dystrophic diaphragm pathology (Kim & Lawler, 2012; Kim et al., 2013).

In anaesthetized mice, we examined respiratory neural drive to the diaphragm under baseline conditions and in response to chemostimulation with hypoxia, hypercapnia, and asphyxia. Baseline diaphragm EMG activity was equivalent in wild-type and mdx mice (although respiratory frequency was higher in mdx mice), but since anaesthetized animals breathed 60% oxygen under baseline conditions, it is likely that carotid body chemoafferent input was depressed in our studies such that

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putative differences in peripheral control of breathing between mdx and wild-type (i.e., sensory deficit suggested by carotid body preparations) would not have contributed to the EMG findings. We further acknowledge the recognized limitations in respect of comparisons of EMG activity between animals. Whether dystrophin deficiency affects central respiratory motor outflow in normoxia was not established in our studies; however, given the significance of this observation, it is worthy of future investigation. Chemoactivation of diaphragm EMG activity was enhanced in mdx, with EMG activity considerably potentiated under maximal chemostimulation with asphyxia. The potentiated response, which revealed a true increase in absolute EMG activity, reveals compensatory plasticity in mdx either in the central brainstem respiratory network and/or at the level of the phrenic motor nucleus. Potentiated motor outflow in response to chemostimulation may serve to facilitate increased ventilation by providing greater neural drive via phrenic motor neurons to diaphragm, facilitating the transduction of neuromuscular-to-mechanical events during chemostimulation challenge. Perhaps in this manner the potentiated output facilitates equivalent increases in ventilation (delta V\text{E}) during chemostimulation despite the mechanical disadvantage presenting in mdx mice.

However, it is important to recognize that the force-generating capacity of the mdx diaphragm is severely compromised. It is evident from analysis of force-frequency relationship in mdx diaphragm that increased frequency of stimulation results in little gain in force. As such, there may be little mechanical advantage to increased activation of the diaphragm, which suggests that activation of accessory muscles of breathing may be especially important in mdx (and DMD) to support increased tidal volume during respiratory stimulation. We acknowledge that aberrant motor unit potentials in dystrophic mdx muscle (Han et al., 2006) could have contaminated diaphragm EMG recordings such that comparisons between wild-type and mdx may not be entirely appropriate in respect of the issue of neuroplasticity, an issue further complicated by potential changes at the neuromuscular junction (Pratt et al., 2015). Recordings of phrenic motor discharge are required to definitively determine if central respiratory drive is elevated in mdx and this is an area worthy of future study. Assessment of motor drive in accessory pathways contributing to ventilation is also worthy of pursuit. It would also be very interesting to characterize motor unit potentials in respiratory EMGs of young mdx mice.

**Limitations**

Whole-body plethysmography provides an estimate of tidal volume, which is dependent on a number of assumptions (Mortola & Frappell, 1998; Stephenson & Gucciardi, 2002)). The calculation depends on knowledge of chamber temperature and humidity, and animal airway temperatures. Whereas the former were measured in our studies (~22°C; relative humidity >80%), animal body
temperature, a surrogate for alveolar temperature, was estimated (37.5°C) for both groups in our study for the purpose of the calculation of tidal volume. This additional limitation raises concern over the accuracy of our estimation of tidal volume, important to address since we report that tidal volume was significantly different between wild-type and mdx mice. Direct airflow measurement with a pneumotachometer or assessment of breathing using head-out plethysmography would provide accurate measures of tidal volumes, worthy of pursuit into the future, although it is recognized that these techniques are not without limitations associated with anaesthesia and restraint, respectively. Of note for the present study, errors in body temperature could conceivably have accounted for a substantive proportion of the difference reported between wild-type and mdx mice (Mortola & Frappell, 1998). We determined that basal CO₂ production was equivalent in both groups, but we assumed for the purpose of tidal volume calculation that body temperature was also equivalent, which we acknowledge is an assumption of major significance in the calculation of tidal volume. We report a significant decrease in CO₂ production during both sustained and graded hypoxia. Body temperature also likely decreased during the hypoxic challenges. Whereas, the hypometabolic response (decreased CO₂ production) was equivalent in wild-type and mdx mice, we assumed (because we did not measure) that the likely hypothermic response was also equivalent between wild-type and mdx mice. Our assumption does not account for potential differences in body temperature between wild-type and mdx mice at rest and/or in response to gas challenge. Of interest, a significant difference in resting body temperature between wild-type and mdx mice was reported in a previous study (Helliwell et al., 1996). However, such a difference if it existed in our study would on average have resulted in an under-estimation of the magnitude of the reduction in tidal volume in mdx mice compared with wild-type during normoxia. It is also probable that our use of a single estimated body temperature for all animals in the study introduced errors and thus variability within and not just between groups, with potential consequences for comparisons between groups. It remains possible that a component — perhaps substantial — of the difference in tidal volume reported in the present study relates to differences in body temperature between wild-type and mdx mice and hence errors related to the assumptions made herein. This issue should be addressed in future studies, notwithstanding the inherent limitations of the technique of whole-body plethysmography for the estimation of tidal volume even with incorporation of body temperature. If differences exist in body temperature between wild-type and mdx mice, and their respective hypothermic response to hypoxia, this would be a fruitful area worthy of investigation.

We acknowledge the apparent discrepancy in the strength of the conclusions drawn from plethysmography and intra-cardiac blood gas analysis, particularly in the light of the limitations described above. We have favoured the conclusion that mdx animals hypoventilate based on the
significant decrease in $V_E/VCO_2$ measurements derived by plethysmography. Yet, the blood gas data, which are ordinarily taken as the gold standard, suggest modest differences in $PO_2$ and $PCO_2$ but with additional supporting evidence suggesting a compensated respiratory acidosis. We suggest that blood gas data should be viewed cautiously in our study, acknowledging that sample size was a limiting factor, but again we emphasize that the magnitude of the hypoventilation reported in our study by use of whole-body plethysmography may have been over-estimated. If ventilatory capacity is preserved in $mdx$, it is an impressive feat considering the profound diaphragm weakness that is evident in the model at an early age. Arterial blood gas sampling in conscious mice during ventilatory and metabolic assessment by plethysmography represents the gold standard for comparisons and would be required to convincingly demonstrate hypoventilation in young $mdx$ mice.

Our EMG study suggests compensatory neuroplasticity in respiratory motor output in $mdx$ mice in response to chemostimulation. We acknowledge that recordings of respiratory motor nerves in reduced preparations, complemented by respiratory EMGs in intact spontaneously breathing preparations, extending to contemporaneous recordings of respiratory EMGs and breathing across the sleep-wake cycle would lend further credence to this novel observation.

Relevance to DMD

Our study in the $mdx$ mouse raises interesting issues of potential relevance to human DMD. Dystrophin deficiency results in respiratory insufficiency early in life, which could establish blood gas disturbances with relevance to neuromuscular performance. Hypoxia-induced respiratory muscle dysfunction may be an important and under-recognised feature of DMD. Dystrophin deficiency may adversely affect basal sensory control of breathing, but the preserved capacity for carotid body chemosensitive nerves to respond to hypoxia and therefore presumably other stimulants, suggests that pharmacotherapies that enhance carotid body activity may have some application in the treatment of DMD. It is apparent that dystrophin deficiency results in profound diaphragm dysfunction, which appears early in the mouse model. The severe mechanical disadvantage presents across a range of stimulations, but a preserved capacity to raise ventilation suggests support from accessory muscles of breathing, which may have relevance to DMD. Our study revealed a potentiated neural drive to breathe in $mdx$ during maximal chemoactivation, suggesting compensatory neuroplasticity enhancing respiratory motor output to the diaphragm and perhaps other respiratory muscles, which should serve to facilitate ventilation in response to challenge. If neuroplasticity is a feature of DMD, it may be possible to boost motor facilitation of breathing through safe interventions, and in this way preserve or limit deficiencies in respiratory capacity.
5. References


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Dubach-Powell J. (2008). Quantitative determination of muscle fibre diameter (minimal Feret’s diameter) and percentage of centralised nuclei, pp. 14. TREAT-NMD Neuromuscular Network.


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6. Additional information

**Competing Interests**

The authors have no financial, professional or personal conflicts relating to this publication.

**Author Contributions**

DPB: experimental design; acquisition of data; data and statistical analysis and interpretation of data; drafting of the original manuscript; AR: carotid body studies: experimental design; acquisition

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of data; analysis; drafting of the original manuscript; DE: muscle histology: experimental design; data acquisition; interpretation of data; SG: muscle histology: data acquisition; data analysis; EFL: experimental design; acquisition of data; FBMcD: carotid body studies: experimental design; analysis; RJW: carotid body studies: experimental design; critical revision of the manuscript for important intellectual content; KDOH: experimental design; statistical analysis and interpretation of data; drafting and critical revision of the manuscript for important intellectual content.

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Translational Perspective

Duchenne muscular dystrophy (DMD) is an X-linked fatal neuromuscular disease, which commonly culminates in respiratory failure. Whereas respiratory muscle dysfunction is recognized in DMD, a comprehensive assessment of respiratory control is lacking. The dystrophin-deficient mdx mouse has proved to be a useful pre-clinical model of DMD, principally because diaphragm muscle dysfunction recapitulates features of the human disease. We set out to interrogate sensory and motor control of breathing in young mdx mice. We hypothesized that there would be evidence of plasticity in the neural control of breathing. Our study reveals that mdx mice hypoventilate as early as 8 weeks of age (equivalent to young adult). We revealed evidence of sensory deficit in mdx mice which may partly contribute to resting hypoventilation, but sensory responses to oxygen deprivation (hypoxia) are normal. Ventilatory responses to respiratory-relevant chemical activation of breathing are normal in mdx mice, revealing ventilatory reserve, although ventilation is lower in mdx mice compared with wild-type mice at all levels of ventilation. Assessment of diaphragm electromyogram activity revealed enhanced motor drive to the respiratory pump muscle during maximum chemoactivation, revealing a compensatory phenomenon in neuromechanical control of ventilation in mdx mice. Profound diaphragm weakness in young mdx mice suggests that enhanced recruitment of accessory muscles of breathing may be especially important in facilitating enhanced lung ventilation during chemoactivation. Our novel findings have implications for human DMD. Safe interventional therapies that serve to boost breathing may preserve or limit deficiencies in respiratory capacity in DMD patients reducing morbidity and mortality.

Translational Perspective Word Count = 249
Table legends

Table 1. Baseline ventilation and metabolic measurements

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<th>mdx (n=12)</th>
<th>Student t tests</th>
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<td>VO₂ (ml/min)</td>
<td>1.6 ± 0.2</td>
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<td>VO₂ (ml/g/min)</td>
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<td>Body mass (g)</td>
<td>22.0 ± 1.7</td>
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Definition of abbreviations: VO₂, oxygen consumption; VCO₂, carbon dioxide production; VCO₂/VO₂, respiratory exchange ratio (RER); Vₑ/VO₂, ventilatory equivalent for O₂; Vₑ/VCO₂, ventilatory equivalent for CO₂; WT, wild-type. Data are shown as mean ± SD and were statistically compared using unpaired Student t tests.
### Table 2. Intra-cardiac blood gas analysis

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<td><strong>pH</strong></td>
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<td>7.37 ± 0.01</td>
<td><em>P</em> = 0.5815</td>
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<td><strong>PCO₂ (mmHg)</strong></td>
<td>35.2 ± 4.7</td>
<td>38.3 ± 2.0</td>
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<td><strong>PO₂ (mmHg)</strong></td>
<td>107.1 ± 13.7</td>
<td>96.7 ± 6.2</td>
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<td><strong>HCO₃⁻ (mmol/L)</strong></td>
<td>20.0 ± 2.4</td>
<td>22.3 ± 1.4</td>
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<td><strong>TCO₂ (mmol/L)</strong></td>
<td>21.0 ± 2.4</td>
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<td><strong>SaO₂ (%)</strong></td>
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<td>97.3 ± 0.8</td>
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<td><strong>Na⁺ (mmol/L)</strong></td>
<td>143.0 ± 1.2</td>
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<td><strong>K⁺ (mmol/L)</strong></td>
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<td><strong>Haematocrit (%)</strong></td>
<td>39.7 ± 1.4</td>
<td>36.0 ± 1.5</td>
<td><em>P</em> = 0.0005</td>
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<td><strong>Hb (g/dl)</strong></td>
<td>13.5 ± 0.5</td>
<td>13.0 ± 2.1</td>
<td><em>P</em> = 0.5080</td>
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**Definition of abbreviations:** PCO₂, partial pressure of CO₂; PO₂, partial pressure of O₂; HCO₃⁻, bicarbonate; TCO₂, total CO₂; SaO₂, arterial oxygen saturation; Na⁺, sodium; K⁺, potassium; Hb, haemoglobin; WT, wild-type. Data are shown as mean±SD and were statistically compared using unpaired Student t tests.
Table 3. Carotid sinus nerve afferent discharge in normoxia and hypoxia

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<th>Discharge frequency (impulses/min)</th>
<th>WT</th>
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<th>60 mmHg</th>
<th>40 mmHg</th>
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<th>mdx</th>
<th>23.8 ± 11.5</th>
<th>36.2 ± 14.5</th>
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<th>80.0 ± 39.6</th>
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<th>Gene P = 0.327</th>
<th>Gas x gene P = 0.953</th>
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<td>Two-way RMANOVA</td>
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<tr>
<td>WT</td>
<td>37.4 ± 20.7</td>
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<tr>
<td>mdx</td>
<td>23.8 ± 11.5</td>
<td>36.2 ± 14.5</td>
<td>54.9 ± 22.4</td>
<td>80.0 ± 39.6</td>
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<tr>
<td>Δ discharge frequency (impulses/min)</td>
<td>WT</td>
<td>-</td>
<td>15.8 ± 7.8</td>
<td>44.9 ± 26.2</td>
<td>84.8 ± 59.6</td>
<td>Gas P &lt; 0.001</td>
<td>Gene P = 0.357</td>
<td></td>
<td>Gas x gene P = 0.228</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>mdx</td>
<td>-</td>
<td>12.5 ± 8.1</td>
<td>31.3 ± 16.2</td>
<td>56.3 ± 36.2</td>
<td></td>
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</tbody>
</table>

Definition of abbreviations: WT, wild-type. Data are shown as mean±SD and were statistically compared by repeated measures two-way ANOVA (gas x gene). Responses to hypoxia are expressed as Δ impulses per min from baseline (normoxia) values.

Table 4. Diaphragm muscle twitch force and contractile kinetics

<table>
<thead>
<tr>
<th></th>
<th>WT (n=7)</th>
<th>mdx (n=7)</th>
<th>Student t tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT (ms)</td>
<td>15.4 ± 1.3</td>
<td>18.9 ± 3.0</td>
<td>P = 0.0155</td>
</tr>
<tr>
<td>½ RT (ms)</td>
<td>19.9 ± 3.0</td>
<td>19.1 ± 2.9</td>
<td>P = 0.5975</td>
</tr>
<tr>
<td>P_t (N/cm²)</td>
<td>2.7 ± 0.7</td>
<td>1.8 ± 0.8</td>
<td>P = 0.0292</td>
</tr>
</tbody>
</table>

Definition of abbreviations: CT, contraction time; ½ RT, half-relaxation time; P_t, twitch force; WT, wild-type. Data are shown as mean±SD and were statistically compared using unpaired Student t tests.

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Figure legends

Figure 1. Baseline ventilation in conscious mice

A: representative respiratory flow traces during normoxic ventilation in a wild-type mouse (black) and mdx mouse (grey); inspiration downwards. B, C and D: breathing frequency (B), tidal volume (C) and minute ventilation (D) for wild-type (WT) (n=13) and mdx (n=12) mice during normoxic ventilation. Values (B-D) are expressed as box and whisker plots (median, 25-75% centiles and minimum and maximum values) and data were statistically compared by unpaired Student t tests with Welch’s correction used where appropriate. ***P<0.001 compared with WT.
Figure 2. *Ex vivo* carotid sinus nerve discharge

A and B: representative recordings of integrated carotid sinus nerve (CSN) activity *ex vivo* in a wild-type mouse (A) and *mdx* mouse (B) during normoxia (100 mmHg) and mild (80 mmHg), moderate (60 mmHg) and severe (40 mmHg) hypoxia. CSN activity was normalized to activity in hyperoxia (500 mmHg), illustrated by the horizontal dashed line. C: group data of CSN activity for wild-type (WT, n=6) and *mdx* (n=6) during normoxia (100 mmHg) and graded hypoxia (80, 60, 40 mmHg). Values are expressed as box and whisker plots (median, 25-75% centiles and minimum and maximum values). Data were statistically compared by repeated measures two-way ANOVA (gas x gene).

Figure 3. Ventilatory and metabolic responsiveness to graded hypoxia

A-H: group data for breathing frequency (A), tidal volume (B), minute ventilation (C), oxygen consumption (D; VO$_2$), carbon dioxide production (E; VCO$_2$), respiratory exchange ratio (F; VCO$_2$/VO$_2$), ventilatory equivalent for oxygen (G; V$_E$/VO$_2$) and ventilatory equivalent for carbon dioxide (H; V$_E$/VCO$_2$) for wild-type (WT, n=13) and *mdx* (n=12) mice during normoxia (21% inspired O$_2$; balance N$_2$) and graded hypoxia (15, 12, 10 and 8% inspired O$_2$; balance N$_2$). Values expressed as box and whisker plots (median, 25-75% centiles and minimum and maximum values). Data were
statistically compared by repeated measures two-way ANOVA (gas x gene) with Bonferroni post hoc test. *$P<0.05$, **$P<0.01$ compared with corresponding WT value.
Figure 4. Ventilatory and metabolic responsiveness to sustained hypoxia

A-H: group data (mean±SD) for breathing frequency (A), tidal volume (B), minute ventilation (C), oxygen consumption (D; VO$_2$), carbon dioxide production (E; VCO$_2$), respiratory exchange ratio (F; VCO$_2$/VO$_2$), ventilatory equivalent for oxygen (G; VE/VO$_2$) and ventilatory equivalent for carbon dioxide (H; VE/VCO$_2$) for wild-type (WT, n=13) and mdx (n=12) mice during baseline and after 5-20 minutes of exposure to hypoxia (10% O$_2$ inspired oxygen; balance N$_2$). Data were statistically compared by repeated measures two-way ANOVA (gas x gene) with Bonferroni post-hoc test. *P<0.05, **P<0.01 compared with corresponding WT value.
Figure 5. Ventilatory responsiveness to hypercapnia

A: group data (mean±SD) for minute ventilation in wild-type (WT, n=10) and mdx (n=11) mice during baseline (air) and hypercapnia (5% CO₂, balance N₂). Data were statistically compared using repeated measures two-way ANOVA. B: group data (mean±SD) for ventilatory responsiveness to hypercapnia (ΔVₑ) in WT (n=10) and mdx (n=11) mice. Values are expressed as box and whisker plots (median, 25-75% centiles and minimum and maximum values). Data were statistically compared by unpaired Student t tests.
Figure 6. Ex vivo diaphragm muscle contractile function

A and B: original traces of ex vivo diaphragm muscle twitch contraction (A) and force-frequency relationship (B) for wild-type (WT) (black) and mdx (grey) preparations. C, group data (mean ± SD; n=7 for both groups) for diaphragm muscle force-frequency relationship ex vivo in WT (open) and mdx (grey) muscle preparations. Data were statistically compared by repeated measures two-way ANOVA (frequency x gene) followed by Bonferroni post-hoc test. **P < 0.01, ***P < 0.001 compared with corresponding WT value.

Figure 7. Diaphragm muscle structure

A: representative images of diaphragm muscle immunofluorescently labelled for laminin from wild-type (WT) (top left) and mdx (top right) mice. B: representative images of diaphragm muscle immunofluorescently labelled for laminin (green) and myonuclei (blue) from WT (bottom left) and mdx (bottom right) mice. C: group data for coefficient of variation of diaphragm muscle fibre size as measured by minimal Feret’s diameter for WT (n=8) and mdx (n=8) mice. D: group data for percentage of fibres with centralised myonuclei in diaphragm muscle of WT (n=6) and mdx (n=5) mice. Values are expressed as box and whisker plots (median, 25-75% centiles and minimum and maximum values). Data were statistically compared by unpaired Student t tests. E and F: frequency distribution of WT and mdx diaphragm muscle fibre size as measured by minimal Feret’s diameter.
(E) and cross-sectional area (F). ***P = 0.0005, ****P < 0.0001 compared with corresponding WT value.
Figure 8. Diaphragm EMG

A: representative traces of diaphragm (Dia) muscle raw and integrated (Int.) EMG activity for a wild-type (WT) mouse (black) and mdx mouse (grey) during baseline (60% inspired O₂), hypercapnia (5% and 10% CO₂), hypoxia (15% O₂) and asphyxia (15% O₂/5% CO₂). B and C: diaphragm muscle integrated EMG activity expressed as amplitude (B) and area under the curve (C) for WT (n=8) and mdx (n=7) mice during baseline (inset), hypercapnia (5% CO₂ and 10% CO₂), hypoxia (15% O₂) and asphyxia (15% O₂/5% CO₂). Baseline data are reported as absolute units. Note that there was no significant difference in baseline data comparing WT and mdx mice. Gas challenges are expressed as % change from baseline. All values are presented as box and whisker plots (median, 25-75% centiles and minimum and maximum values). Baseline data were statistically compared by unpaired Student t tests. Gas challenges were statistically compared by repeated measures two-way ANOVA with Bonferroni post-hoc test. *P < 0.05 compared with WT.