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Characterization of Neuromuscular Transmission in Mice with Progressive Motoneuronopathy

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Summary

Progressive motoneuronopathy (PMN) is an autosomal recessive mouse disease, which is characterized by the development of hind limbs paralysis rapidly progressing to the anterior parts of the body, muscular atrophy, respiratory depression, and death at 6-7 postnatal weeks. Here, we recorded the resting membrane potential (RMP), spontaneous miniature endplate potentials (MEPPs), and quantum content of endplate potentials (EPP) at the diaphragm muscle fibers in controls and PMN mice aged 18 to 43 days. In control animals, there was a progressive increase in RMP, MEPP frequency and EPP quantum content, as well as a decrease in mean MEPP amplitude. In PMN mice, the developmental increase in frequency and decrease in the amplitude of MEPPs was practically stopped at the postnatal day 18, whereas RMP increased but only until the age of 31 days and then progressively decreased. The distribution histogram of RMP in PMN mice older than 35 days revealed the existence of two subpopulations of muscle fibers: one showing a denervation-like decrease in RMP and the second, which was matching controls. In addition, EPP quantum content was significantly attenuated in older PMN animals. These results indicate that neurotransmission is severely affected in advanced, but not in early stage of disease, which is apparently due to a partial denervation of the muscles.

Key words

Diaphragm muscle • Motoneuron disease • Resting membrane potentials • Miniature endplate potentials • Endplate potentials

Introduction

Mouse progressive motor neuronopathy (PMN) is a lethal autosomal recessive disease that was discovered and described by Schmalbruch *et al.* (1991). The mutation, which appears spontaneously, was initially detected in 1988 in a stock of Pan:NMRI mice at the Animal Department of the Panum Institute in Copenhagen. In homozygous mice (*pmn/pmn*), the paralysis of hind limb fingers represents the first sign of disease and appears in 17 to 18-day-old animals. During the third and fourth postnatal week, the paralysis spreads to the whole hind limbs, progresses in caudo-cranial direction and starts to be visible in the front limbs. The PMN disease is accompanied by severe muscular wasting, loss of weight and respiratory depression. Most of animals die up to 7 weeks of age, probably due to respiratory failure (Schmalbruch *et al.* 1991). Heterozygotes are clinically normal and breed normally, and about 25 % of their offspring are *pmn*/*pmn* homozygotes.

The PMN disorder is manifested by the

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degeneration and loss of motor axons that is most prominent in sciatic and phrenic nerves (Schmalbruch *et al.* 1991, Sendtner *et al.* 1992, Sagot *et al.* 1996), as well as by loss in the number of motoneurons in cranial nuclei during the late stage of disease (Sendtner *et al.* 1992, Holtmann *et al.* 1999, Haenggeli and Kato 2002). Detailed histopathological studies of phrenic nerve revealed that distal axons degenerate earlier, whereas proximal axons and cell bodies are relatively well preserved, a phenomenon termed „dying-back“ process. Sensory fibers and muscle spindles are not affected. Electromyography from gastrocnemius muscle and diaphragm of PMN mice showed first abnormalities at the end of the second postnatal week, when the disease was not yet phenotypically manifested. This included a decrease in the amplitude of compound muscle action potential, slower conduction velocity, reduced number of functional motor units, and abnormal spontaneous muscle activity with fibrillation potentials (Kennel *et al.* 1996, Holtmann *et al.* 1999).

However, at the present, there is no information about synaptic transmission in the neuromuscular junction during progression of the disease. To elucidate this issue, we monitored resting membrane potential (RMP), spontaneous miniature endplate potentials (MEPPs) and quantum content of endplate potential (EPP) in the diaphragm of control and PMN mice using intracellular glass microelectrodes and stimulation techniques. Measurements were done from the onset of the symptoms (18 days of age) to the terminal stage of the disease (43 days of age). Our results show reduced spontaneous and evoked neurotransmitter release and the denervation-like changes in RMP in PMN mice in advanced but not early stage of disease.

Methods

Animals

Heterozygous carrier (*pmn/+*, strain 129) mice were generously provided by Dr. R. Vejsada (Geneva University, Geneva, Switzerland), and were kept and crossbred in our animal facilities. The litters (usually 6-8 animals) contained ~25 % homozygous mutant (*pmn/pmn*) mice (hereafter PMN mice). Wild type, *pmn/+* mice from the same litter and mice from parents that never produced diseased offspring served as controls. As early as day 12, all animals were tested for motor activity. The genotype *pmn/pmn* was determined by testing whether the mice, held head-down by tail, could spread out toes of their hind limbs. At the age of 18 days, the weakness of hind limb toes was clearly visible and the rapid time-course of paralysis was accompanied with a massive muscular atrophy and general retardation of the growth (see Results). These symptoms of the disease were absent in control mice as well as in *pmn/+* mice. All experiments were carried out in accordance with the European Communities Council Directives (86/609/EEC) and with the approval of the Institutional Animal Care and Use Committee. All efforts were made to minimize animal suffering and the number of animals used for these experiments.

Muscle preparation

At different stages of their respective disease, animals were decapitated and the diaphragm muscle with a piece of phrenic nerve was isolated and immersed in an oxygenated (95 % O_2+5 % CO_2) physiological solution (in mM): NaCl 136, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, NaH_2PO_4 1, $NaHCO_3$ 12.8, glucose 10, pH=7.2-7.3. The diaphragms were cleaned and divided into two parts, which were stretched slightly to about 120 % of their resting length and fixed to a Sylgard-covered bottom of a 5 ml chamber. The chamber was continuously perfused at a rate of 1 ml/min. In some experiments, in which the resting membrane potential and spontaneous MEPPs were measured, contractile activity of muscles from PMN mice was blocked by perfusing the tissue with a medium containing 1 μM tetrodotoxin (Sankyo, Tokyo, Japan). Quantum content of EPP was measured in low calcium and high magnesium solution containing (in mM): NaCl 136, KCl 5, $CaCl_2$ 0.2, $MgCl_2$ 2.2, NaH_2PO_4 1, $NaHCO_3$ 12.8, glucose 10, pH=7.2-7.3. Experiments were performed at room temperature of 22±2 °C.

Electrophysiological recordings

Standard electrophysiological techniques were used for intracellular recording and nerve stimulation. Glass microelectrodes filled with 3 M KCl (resistance of 10-15 MΩ) were used for recording potentials. The resting membrane potential was measured immediately after the electrode insertion and only potentials from surface muscle fibers were recorded. For miniature endplate potentials recording, the electrode was inserted under visual control in an area close to the termination of small intramuscular branching of phrenic nerve. The presence of MEPPs with fast rise time (<1 ms) indicated the proximity of the nerve ending, whereas muscle fibers with a slower rise of MEPPs were omitted. The frequency

Pof MEPPs was calculated from 1 min records. The quantum content of EPPs was assessed by the method of failures (Del Castillo and Katz 1954a, Boyd and Martin 1956). The nerve was stimulated with suction electrode in the presence of solution containing low calcium and high magnesium (see Muscle preparation) to reduce quantum content of EPPs. The 100 s train of pulses was delivered to the nerve at frequency 1 Hz. Quantum content *m* was calculated according to the equation (Del Castillo and Katz, 1954a):

$$m = \log_e n/n_f$$

where n is the number of pulses delivered and n_f is the number of pulses which failed to evoke EPP.

Data storage and analysis

Recorded signals were digitalized using Digidata 1200 interface (Axon Instruments, Union City, USA) at 10 kHz and stored on a computer. The amplitude, time course, and frequency of MEPPs were analyzed using pClamp 6 software (Axon Instruments). Amplitudes of MEPPs, which are linearly dependent on membrane potential (Hubbard *et al.* 1969), were corrected to a standard resting potential of –70 mV to eliminate the differences in RMP between individual muscle fibers. Measured amplitudes of MEPPs were multiplied by a correction factor *f* calculated according to the equation:

$$f = V_S - V_r / RMP - V_r$$

where V_S is standard membrane potential (–70 mV), V_r is reversal membrane potential which was taken to be 15 mV (Fatt and Katz 1951, Del Castillo and Katz 1954a) and *RMP* is resting membrane potential at which the MEPPs were recorded.

The values are given as means ± S.E.M. The differences between means were tested by Student's t-test, and p<0.05 was considered as significant.

Results

Body weight

The separation of PMN mice from the controls was possible at postnatal day 18, and was based on the motor activity test (see Methods). Figure 1 shows body weight determined in six age groups: 18-20, 21-23, 24-28, 29-33, 34-37 and 41-43 postnatal days. During that period, the body weight of control animals increased from ~8 g to ~19 g. In contrast to the controls, the body weight of PMN mice remained unchanged during the 3-week period (between 6-7 g). As shown in Figure 1, there were significant differences in body weight between control and PMN mice already at the age of 18-20 days.

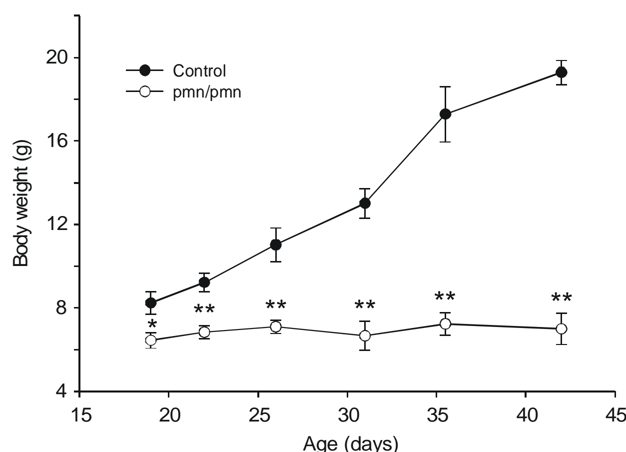

Fig. 1. Body weight of control and PMN mice during the 18-43 day postnatal period. The body weight was measured in six age groups: 18–20, 21-23, 24-28, 29-33, 34-37 and 41-43 postnatal days. In each group, 3-15 animals were measured and values are means±SEM. Asterisks indicate significant differences from controls [p<0.05 (*) and p<0.01 (**)].

RMP of diaphragm muscle fibers

The developmental changes in RMP of diaphragm muscle fibers were examined in 57 control and 53 PMN animals. In each muscle, the mean of individual measurements from 15-20 different muscle fibers was calculated and the grand mean was obtained from 5 to 13 muscles. As shown in Figure 2A, the RMP in control animals aged 18-20 days was –66.5±1.0 mV (n=13) and was hyperpolarized progressively during the subsequent two-week period to –73.5±1.1 mV (n=11), and then stabilized. In PMN mice, RMP was –64.3±1.3 mV (n=8) at day 18-20, was hyperpolarized more slowly and at day 27-31 was –68.9±2.0 mV (n=9, p<0.05 compared to controls of same age). In contrast to controls, there was no stabilization of RMP, but it was progressively depolarized to –65.1±1.7 mV (n=5) at age of 41-43 days, which was about 8 mV lower when compared with control muscles of the same age.

The distribution histogram of RMP in control mice older than 35 days was best fitted with a single Gaussian function with a peak at –72.7 mV (Fig. 2B), indicating the existence of a single muscle fiber population. In contrast, a double Gaussian function best fitted the distribution histogram of RMP in age-matched PMN mice (Fig. 2C), indicating the presence of two muscle fiber populations. The first peak was at –71.7 mV, similar to that recorded in the controls. The second peak

was –60.1 mV and indicated the presence of depolarized muscle fibers. This level of depolarization, by about 12 mV, was comparable with that observed in skeletal muscles after surgical denervation of normal animals (Card, 1977, Drachman *et al.* 1982, Zemková *et al.* 1987). These results are consistent with the hypothesis that a fraction (about 35 %) of muscle fibers from PMN mice was still innervated in the progressed stage of disease (higher RMP), whereas the residual fibers were functionally denervated (lower RMP).

Spontaneous neurotransmitter release

To investigate possible changes at the neuromuscular junction during the development of the disease, we measured the frequency, amplitude and the time-course of MEPPs in muscles from control and PMN mice of different ages. The frequency of MEPPs was measured in 78 control and 58 PMN animals, whereas the amplitude was tested in 18 control and 15 PMN animals. In control animals, the mean MEPP frequency was 0.32±0.03 s^{-1} (n=10) at 18-19 days of age and progressively increased to 0.82±0.05 s^{-1} (n=7) in 40- to 43-day-old animals. In PMN mice, the mean MEPP frequency at the age of 18-19 days was 0.32±0.07 s^{-1} (n=5), identical to that observed in controls. However, it practically remained unchanged, reaching 0.37±0.08 s^{-1} (n=4) at 40-43 days of age (Fig. 3A). The difference between MEPP frequency in control and PMN preparations was significant already at the age of 20-23 days. Distribution histogram of MEPP frequencies at the age of 20-23 days (Fig. 3C) showed a high percentage of muscle fibers with a very low frequency of MEPPs (below 0.1 s^{-1}), which were almost absent in control muscles (Fig. 3B).

No obvious difference was observed in the time-course of MEPP between control and PMN mice (Figs 4A and 4B). In contrast to frequency, the amplitude of MEPPs in control animals decreased progressively with age (from 2.3±0.2 s^{-1} at 18 days to 1.9±01 s^{-1} at 41-43 days of age). In PMN animals, the amplitude of MEPPs remained almost unchanged (2.4±0.2 mV at 18 days and 2.4±0.2 mV at 42 days, respectively). There were significant differences between the controls and PMN mice at the age of 35 and 43 days (Fig. 4C). The frequency of abnormally large and slow MEPP was very low in both PMN and control mice (data not shown).

Quantum content of EPP

The quantum content of EPP in controls and PMN

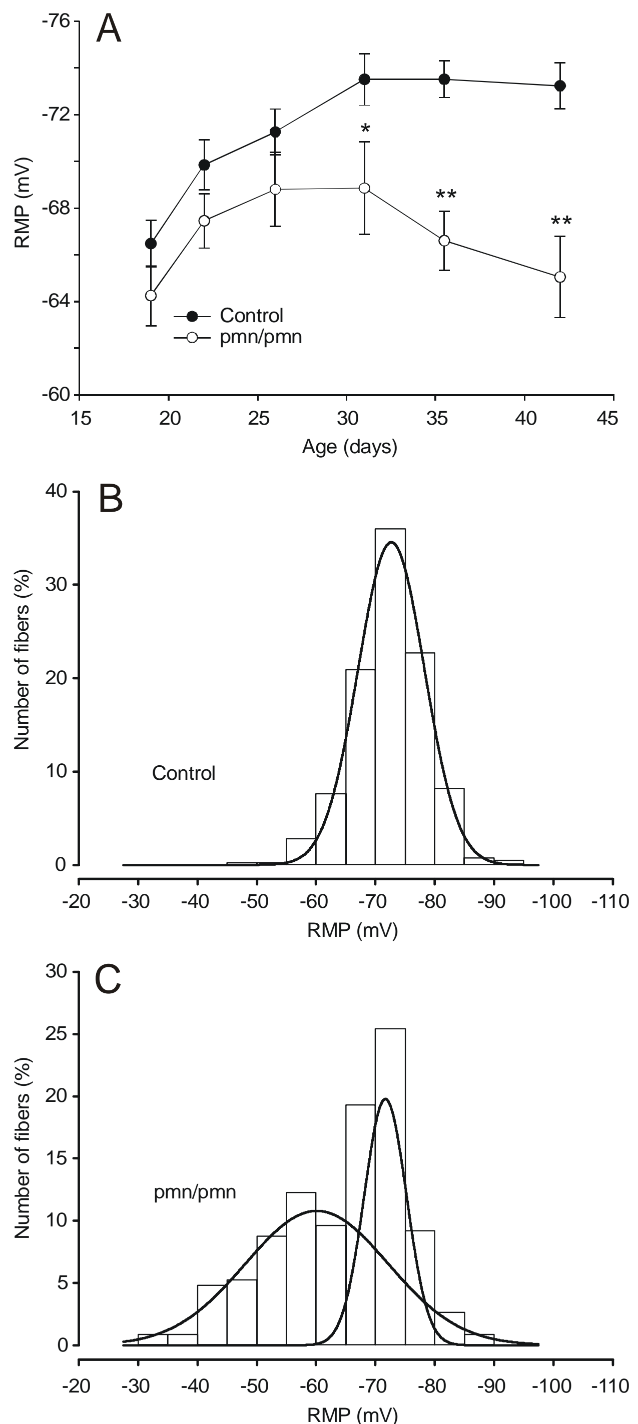

Fig. 2. Age-dependent resting membrane potential in diaphragm muscle fibers from controls and PMN mice. **A.** Time course of RMP changes. Data are means±SEM from 5-13 animals, with 15-20 muscle fibers measured from one diaphragm. After 31 postnatal days, the differences in RMP between control and PMN animals were significant as indicated by asterisks [$p<0.05$ (*) and $p<0.01$ (**)]. **B.** Distribution histogram of RMP from control 35-43 day-old mice (241 muscle fibers from 8 animals). The histogram was fitted with a single Gaussian function. **C.** Distribution histogram of RMP from PMN mice aged 35-43 days (100 muscle fibers from 5 animals) fitted with a double Gaussian function.

mice was estimated in two age groups: 19- to 24-day-old animals, i.e. in the early stage of disease manifested by mild weakness of hind limbs, and 29-32 days, i.e. in the advanced stage of disease manifested with complete hind limbs and pelvic girdle paralysis. Quantum content was measured in 9 controls and 6 PMN animals. As shown in Figure 5, the quantum content of EPP in control animals almost doubled (from 0.22±0.03 to 0.43±0.09). The same trend was also observed in preparation from affected mice. However, in PMN animals the quantum content of EPP was lower in both age groups (0.11±0.04 to 0.17±0.04).

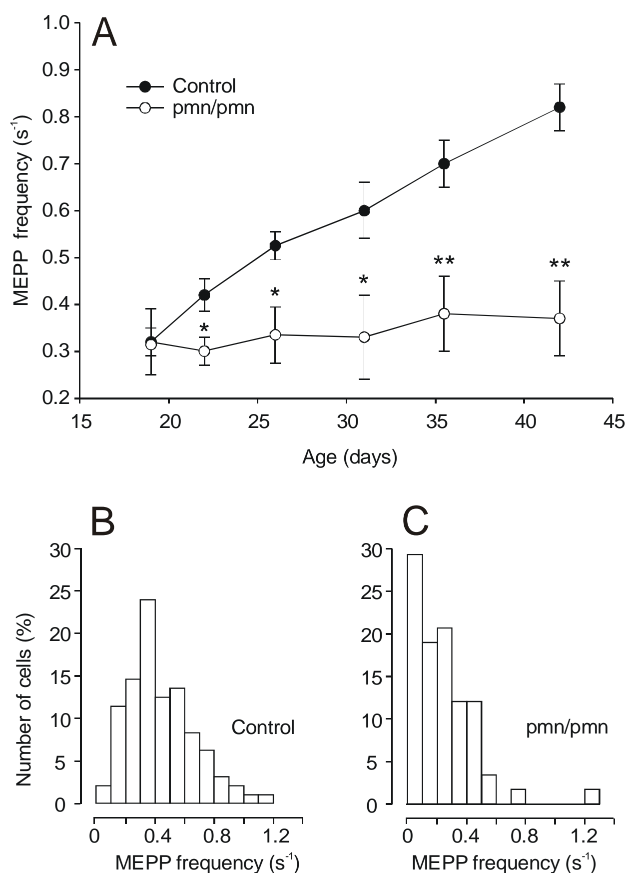

Fig. 3. Age-dependent frequency of miniature endplate potentials in diaphragm muscle fibers from controls and PMN mice. **A.** Frequency of MEPP recorded at neuromuscular junctions. Values are means±SEM from 4-20 animals, where 10-15 muscle fibers were recorded at each muscle. The difference between control and PMN animals was significant from the age of 20-23 days [p<0.05 (*) and p<0.01 (**)]. **B&C.** Distribution histograms of MEPP frequency at age of 20-23 days in control muscle (B panel; 96 muscle fibers from 6 animals) and muscles from PMN mice (C panel; 58 muscle fibers from 7 animals).

Discussion

In this study, we report that synaptic transmission in mice with PMN did not differ from controls until the age of 18 days, when the first signs of motor impairment were obvious. However, severe changes developed as disease progressed from 21 to 43 days of age. During that period, in healthy animals synaptic transmission developed to the adult stage, as it has been reported previously (Diamond and Miledi 1962, Kelly 1978, Miyata and Yoshioka 1980). RMP of muscle fibers increased (Fig. 2), as well as spontaneous (Fig. 3) and evoked (Fig. 5) release of neurotransmitter, reflecting the nerve terminal growth and increase in number of active zones (Slater 1982). The developmental increase in diameter and length of muscle fibers leads to a decrease in the input resistance (Katz and Thesleff 1957, Wareham *et al.* 1994). As a consequence of this, the amplitude of MEPPs also decreased in a few postnatal weeks (Fig. 4) (Kelly 1978, Wareham *et al.* 1994).

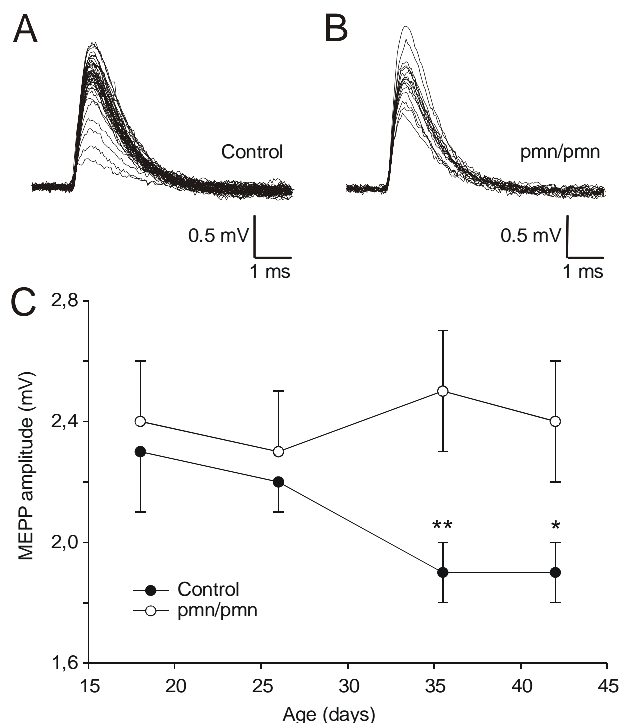

Fig. 4. Time course and amplitude of MEPPs in neuromuscular junction of diaphragm muscle from control and PMN animals. **A&B.** Superimposed MEPPs during 1 min recording from control (A) and PMN (B) neuromuscular junction of 43 day-old mice. **C.** Age-dependent changes in MEPP amplitude in control and PMN mice. Values are mean±SEM from 3-6 animals, where 3-9 muscle fibers were recorded at the endplate zone. Measurements of amplitudes were performed in four age groups: 18–20, 24-28, 34-37, and 41-43 postnatal days. Because the MEPP amplitude was influenced by the RMP of muscle fibers, it was always corrected for the membrane potential of –70 mV. The significant differences between control and PMN animals are indicated by asterisks [p<0.05 (*) and p<0.01 (**)].

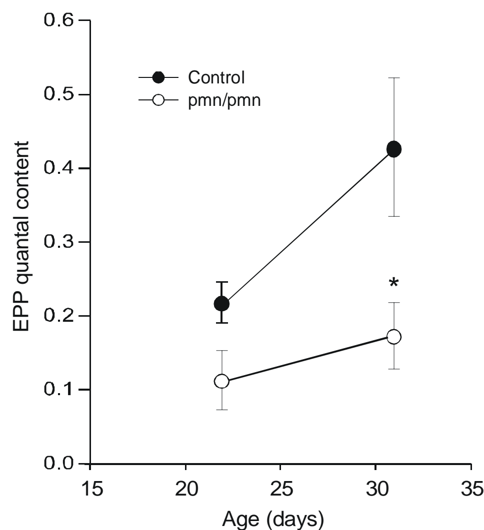

Fig. 5. Age-dependent quantum content of endplate potentials in diaphragm muscle fibers from controls and PMN mice. The phrenic nerve was stimulated with suction electrode in low calcium and high magnesium solution and the quantum content of EPP was assessed by the method of failures. Values are mean±SEM from 13 to 37 fibers from 3-6 animals. There was a significant difference between control and PMN mice [$p<0.05$ (*)].

In PMN mice, this process was severely affected. The developmental increase in the frequency of MEPPs was absent and evoked release of neurotransmitter was deteriorated within the third to sixth postnatal week. This finding is consistent with histopathological study of Schmalbruch *et al.* (1991), showing that many nerve terminals of PMN mice are disassembled and lack the synaptic vesicles. The developmental increase of RMP in PMN mice was also altered. It paralleled the rise observed in controls until the day 31, but than started to fall and at the end-stage of the disease the RMP was lower by about 8 mV as compared to controls. From the postnatal day 35, there were two populations of muscle cells: fibers with normal RMP and fibers with lower RMP. Depolarization by about 12 mV reflected a difference between mean values of these two populations. Similar difference in RMP was reported between innervated and denervated muscle fibers of rat diaphragm after cutting the nerve (Miledi and Slater 1970, Stanley and Drachman 1980, Zemková *et al.* 1987). Fibrillation potentials (Kennel *et al.* 1996) and expression of myogenic factors (Sedehizade *et al.* 1997) as markers of denervation have also been reported in this stage of PMN disease. These results strongly indicate that diaphragm of PMN mice is a mixed population of innervated and pathologically denervated muscle fibers. In contrast to controls, no developmental decrease in the amplitude of MEPPs was observed in PMN mice. As shown in Fig. 1, general retardation of the growth accompanied by muscle atrophy was apparent from the early stage of disease. We speculate that as a consequence of this, the diameter and input resistance of muscle fibers did not change in PMN animals and the amplitude of MEPPs remained unaltered. There were no changes in the time course of MEPPs indicating that neither acetylcholine receptor channel kinetics nor acetylcholinesterase activity at the endplate zone was changed in PMN mice.

Recently, a substantial advance was made in understanding the etiology of PMN disease. It has been found that PMN mice exhibit mutation in the gene for tubulin-specific chaperone (Bommel *et al.* 2002). Missense mutation in this gene leads to alterations in tubulin assembly and impairment of axonal transport, which was observed in PMN mice (Sagot *et al.* 1998). These observations suggest that impairment of axonal transport could deteriorate proper functioning of the neuromuscular synapses. However, blocking of tubulin-based axonal transport by colchicine evokes denervation-like changes in skeletal muscle without alteration in neuromuscular transmission (Albuquerque *et al.* 1972, Fernandez and Ramirez 1974), supporting the view that additional factors could also play a role in the etiology and progress of this disease.

In conclusion, our results indicate that neurotransmission is normal at the time of the first signs of motor impairment, suggesting it is highly unlikely that initiation of disease would be exclusively related to the nature of synaptic transmission. Other factors, including axonal transport, participate in etiology and development of disease, which is in accordance with the literature. Furthermore, we have shown that the neuromuscular transmission of PMN mice undergoes series of presynaptic and postsynaptic changes during the fourth to the sixth postnatal weeks that could contribute to the progress of disease. Based on our results, the following scenario of pathological changes at the neuromuscular endplate is suggested. Impaired axonal transport causes a decrease in spontaneous and evoked transmitter release. This leads to functional denervation of muscle, which results in membrane depolarization and muscle atrophy. At the end stage of PMN disease, motoneuron cell bodies, deprived from their target, also degenerate.

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Reprint requests

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