Progressive abnormalities in skeletal muscle and neuromuscular junctions of transgenic mice expressing the Huntington’s disease mutation

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Abstract

Huntington’s disease (HD) is a neurodegenerative disorder with complex symptoms dominated by progressive motor dysfunction. Skeletal muscle atrophy is common in HD patients. Because the HD mutation is expressed in skeletal muscle as well as brain, we wondered whether the muscle changes arise from primary pathology. We used R6/2 transgenic mice for our studies. Unlike denervation atrophy, skeletal muscle atrophy in R6/2 mice occurs uniformly. Paradoxically however, skeletal muscles show age-dependent denervation-like abnormalities, including supersensitivity to acetylcholine, decreased sensitivity to β-conotoxin, and anode-break action potentials. Morphological abnormalities of neuromuscular junctions are also present, particularly in older R6/2 mice. Severely affected R6/2 mice show a progressive increase in the number of motor endplates that fail to respond to nerve stimulation. Surprisingly, there was no constitutive sprouting of motor neurons in R6/2 muscles, even in severely atrophic muscles that showed other denervation-like characteristics. In fact, there was an age-dependent loss of regenerative capacity of motor neurons in R6/2 mice. Because muscle fibers appear to be released from the activity-dependent cues that regulate membrane properties and muscle size, and motor axons and nerve terminals become impaired in their capacity to release neurotransmitter and to respond to stimuli that normally evoke sprouting and adaptive reinnervation, we speculate that in these mice there is a progressive dissociation of trophic signalling between motor neurons and skeletal muscle. However, irrespective of the cause, the abnormalities at neuromuscular junctions we report here are likely to contribute to the pathological phenotype in R6/2 mice, particularly in late stages of the disease.

Introduction

Huntington’s disease (HD) is a fatal neurodegenerative disorder characterized by progressive decline in motor and cognitive function with insidious onset in the third to fifth decade. In adults, the first motor symptom of HD is usually chorea, but the motor deficits progress in advanced disease to rigidity, bradykinesia and dystonia. By the end stages of the disease, the HD patient is usually bedridden with very limited capacity for voluntary movement (for references, see Bates et al., 2002).

Although the genetic mutation causing HD has been identified (as an expanded CAG repeat that is translated into a polyglutamine repeat in the protein huntingtin), the mechanism underlying the pathology is unknown. The pathological changes in the brains of HD patients have been well described, and the striking neurodegeneration seen in the caudate and putamen has, for many years, focused attention on the mechanisms underlying pathology in these regions. However, with the cloning of the gene came the discovery that the expression of the HD gene and its protein product huntingtin is not restricted to the brain (The Huntington’s Disease Collaborative Research Group, 1993). Indeed, huntingtin expression is not even neuron-specific, but is found in many tissues including heart and skeletal muscle. The role of huntingtin in these tissues is unknown, although many patients exhibit signs of peripheral motor pathology, including abnormal eye movements, difficulty swallowing, gait abnormality, dystarthritis and skeletal muscle wasting (for references, see Bates et al., 2002). Further, skeletal muscle atrophy (which undoubtedly contributes to weight loss) is observed in many HD patients despite an adequate diet and feeding (Sanberg et al., 1981). Because the general health of HD patients declines as their disorder progresses, it has generally been assumed that changes in body mass are secondary to other symptoms. However, significant body weight changes are measurable in early (Djousse et al., 2002) as well as late stage HD patients. This suggests that weight loss due to atrophy is a significant pathological component of HD. Nevertheless, the cause of the atrophy remains unknown and, because motor function is under the control of the brain, the possibility that a peripheral pathology contributes directly to these symptoms has not been widely considered.

The dominant nature of the HD mutation has allowed the development of a number of mouse models (Hickey & Chesselet, 2003). The best characterized is the R6/2 line which is transgenic for the human HD mutation (Mangiarini et al., 1996). R6/2 mice exhibit a progressive neurological phenotype that includes abnormal...
involuntary movements, tremor and progressive deterioration of motor and cognitive function (Mangiariini et al., 1996; Carter et al., 1999; Lione et al., 1999). As well, they exhibit a number of peripheral symptoms which replicate those seen in HD, in particular, wasting of skeletal muscle (Sathasivam et al., 1999).

The cause of skeletal muscle atrophy in R6/2 mice and HD patients is unknown. However, skeletal muscle atrophy is an indicator of nerve and/or muscle pathology in a wide variety of diseases, and very subtle atrophies can be strongly indicative of pathology. In this study therefore we sought to establish whether the muscle atrophy observed in the R6/2 mouse is part of the primary pathology associated with the HD mutation or whether it is secondary to other previously unrecognized features of the disorder, for example, impairment of neuromuscular transmission. Our data suggest that both motor neurons and muscle fibers are independently affected by the HD mutation and that neuromuscular changes may contribute to the pathological phenotype in HD, particularly in late stages of the disease.

Materials and methods

Animals

All studies were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986. R6/2 mice (Mangiariini et al., 1996) and their wild-type (WT) littermates were taken from a colony established in the Department of Pharmacology, University of Cambridge, and maintained by back-crossing transgenic males onto female CBA × C57Bl/6 F1 mice. Some experiments were carried out using mice that were transferred to Edinburgh. In all cases, dry chow was supplemented by twice daily moist chow (mash) from weaning onwards, and lowered waterspouts were also provided. We have shown previously that this regime improved health and survival of R6/2 mice (for further details of husbandry see Carter et al., 2000). Genotyping was carried out using a polymerase chain reaction based on a modification of the method described by Mangiarini et al. (1996). Tissues from 50 WT and 42 R6/2 adult mice of both sexes were used in the study.

Surgery

R6/2 (N = 18) and WT (N = 16) mice were used for experiments to study the neuromuscular response to denervation or reinnervation. Reinnervation experiments were done after crushing the entire sciatic nerve. Partial denervation experiments were done by cutting the tibial nerve, and examining the sprouting response of endings of the intact (sural) nerve in the partially denervated fourth deep lumbrical muscles. The mice were anaesthetized by inhalation of halothane. The sciatic or tibial nerve was exposed and a 1–2-mm section was either cut (but not removed), or crushed using fine forceps. Mice were then kept for 1–40 days before being killed by cervical dislocation.

A number of different muscles from the hind limb and the chest were used in this study. The hind limb muscles were: flexor digitorum brevis (FDB), the peroneal, soleus and extensor digitorum longus (EDL) muscles from the anterior calf, and the deep lumbrical muscles of the hind foot. Different muscles were used as appropriate. For example, FDB is a superficial muscle from the foot which is well suited to location and electrophysiological recording of synaptic responses. (This is on account of the short length of the muscle fibers; <500 μm in length. As a consequence, they are ‘isopotential’ with respect to membrane potential changes induced locally at any point along their length. Thus, it does not matter where the intracellular micropipette tip is located with respect to longitudinal distance from the motor endplate, or where the synaptic currents originate; synaptic potentials of virtually identical magnitude and time course are recorded at all points along the length of the fiber.) The deep lumbrical muscles of the hind foot were used because they are particularly amenable to fluorescence immunocytochemistry (because they are very thin, and therefore there are minimal problems with penetration and diffusion of fixative and antibodies for immunostaining). Nerve terminals and endplates are readily observable in whole-mount preparations, either in regular fluorescence or confocal microscopes. Finally, the fourth deep lumbrical muscle has a dual nerve supply in rodents (Nakanishi & Norris, 1970; Betz et al., 1979; Taxt, 1983; Costanzo et al., 2000), one via an anastomosis of a branch of the sural nerve with the lateral plantar nerve, the other supplied by the same nerve (tibial nerve) as the FDB muscle. Section of the tibial nerve in anaesthetized animals thus renders FDB and the medial lumbrical muscles completely denervated, but the fourth deep lumbrical muscle is subject to a controlled partial denervation, making the muscle ideal for studies of motor nerve sprouting. We also made whole mounts of the triangularis sterni muscle, an exceptionally thin respiratory muscle, well suited to immunocytochemistry.

Histology

Peroneal muscles of the hind limb were dissected and snap-frozen in liquid isopentane cooled over liquid nitrogen. Serial transverse sections (20 μm) were cut from the belly of each muscle using a cryostat. For fiber typing, sections were stained histochemically for enzyme activity of NADH diaphorase or succinate dehydrogenase (SDH), both standard markers for the oxidative enzymes that characterize type I fibers (Filipe & Lake, 1983). Parallel sections were prepared for standard histological analysis by staining with Harris’s haematoxylin and eosin (stains cytoplasm and nuclei), Cresyl Violet (stains Nissl substance), Weigert’s iron haematoxylin (stains nuclei) and van Geison’s stain (stains connective tissue). Selected sections were also stained with Oil Red O (stains lipid) and the periodic acid–Schiff reaction (stains glycogen). For detailed methods, see Culling et al. (1985). Unless otherwise stated, all reagents for histological staining came from Sigma, UK.

Fiber diameters were measured in peroneal muscle using three sections from each of three WT and four R6/2 mice. Within each section, fibers were classified into type I or type II fibers according to their NADH diaphorase reaction, and the diameters of at least 75 type I and 75 type II fibers were measured and recorded using the methodology described by Patel et al. (1969). Proportions of fiber type were assessed in three sections from each muscle. All the fibers within three 500 × 500-μm square fields were classified according to their NADH diaphorase reaction, and the number of each type was counted using an overlaid grid. To visualize intranuclear inclusions, cryosections of fresh-frozen tissues were cut onto gelatinized slides, fixed for 30 min in 4% paraformaldehyde and processed for immunocytochemistry. Sections were incubated with a rabbit antiubiquitin antibody (DAKO) at 1:2000 dilution. A horseradish peroxidase-conjugated second antibody (1:1000 dilution, DAKO) was used and immunoreactive components were visualized using diaminobenzidine (Sigma, UK).

Neuromuscular junction staining

FDB, lumbrical and triangularis sterni muscles were prepared for immunocytochemistry by fixing in 0.1 m PBS containing 4% paraformaldehyde for 30–40 min. Acetylcholine (ACh) receptors were labelled by incubation for up to 30 min in 5 μg/mL TRITC-conjugated α-bungarotoxin (α-BTX: Molecular Probes, USA). Muscles were
washed in PBS and blocked in 4% BSA and 0.5% Triton-X in 0.1 M PBS for 30 min before incubation overnight in primary antibodies directed against either the 165-kDa neurofilament proteins (mouse monoclonal 2H3), the synaptic vesicle protein SV2 (mouse monoclonal; both 1:200 dilution; from the Developmental Studies Hybridoma Bank, Iowa, USA) or S100 antibody (rabbit polyclonal; Diagnostics Scotland, UK) or ubiquitin (rabbit polyclonal, 1:1000, DAKO). After washing for 30 min in blocking solution (see above), muscles were incubated for 4 h in 1:200 dilution of appropriate (antimouse IgG or antirabbit) secondary antibodies conjugated to either FITC or TRITC (Diagnostics Scotland or DAKO), as indicated in the relevant figures. Muscles were then whole-mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA). Some preparations were stained for cholinesterase by a modified Karnovsky–Roots method, described in Harris & Ribchester (1979). Fiber diameter and endplate area analysis were made using the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). For endplate area analysis, the region covered by the endplate was outlined and measured. This procedure overestimates the area of motor nerve terminal contact, but it is sufficiently robust to test the correlation between endplate size and muscle fiber size (Harris & Ribchester, 1979). Fiber diameter was measured within 100 μm of the endplate.

Some preparations were ‘vital’ stained with the dye FM1-43, a marker of endocytosis (Molecular Probes), a marker of endocytosis in functioning nerve terminals (Betz et al., 1992; Ribchester et al., 1994; Barry & Ribchester, 1995). Preparations were incubated in oxygenated depolarizing saline (K+ increased to 50 mM, Na+ decreased by 45 mM; concentrations of the other ions in the bathing medium were as given under Electrophysiology, below) containing 4 μM FM1-43, for 5–15 min, washed and then imaged in situ. Immunofluorescent and vitally stained preparations were imaged using either a standard fluorescence microscope (Olympus) or a laser scanning confocal microscope (Biorad Radiance 2000). Confocal z-series were merged using Lasersharp (Biorad) software.

**Electron microscopy**

FDB muscles were fixed in ice-cold 0.1 M phosphate buffer containing 4% paraformaldehyde and 2.5% glutaraldehyde for 4 h. Preparations were then washed in 0.1 M phosphate buffer, postfixed in 1% osmium tetroxide solution for 45 min and dehydrated through an ascending series of ethanol solutions before embedding in Durcupan resin (Sigma, UK) and sectioning at 75–90 nm. Sections were collected on Formvar-coated grids (Agar Scientific, UK), stained with uranyl acetate and lead citrate and viewed in a Philips CM12 TEM. EM negatives taken between 2000x and 60000x were scanned at 600 dpi using a Linsoscan 1200 (Heidelberg Instruments, Heidelberg, Germany) equipped with a transparency adaptor, before importing into Adobe Photoshop for analysis and presentation.

**Electrophysiology**

Intracellular recordings were made from isolated FDB nerve–muscle preparations. In initial experiments, FDB muscles were isolated in Cambridge early in the morning, bathed in oxygenated HEPES-buffered physiological saline and couriered the same day to Edinburgh, where they were then transferred to bicarbonate-buffered saline (below) for electrophysiological recording the same afternoon, as described previously (Mack et al., 2001). The isolated muscles were pinned out in a Sylgard (VWR International, Poole, UK)-lined bath and perfused with normal mammalian physiological saline (in mM: NaCl, 120; KCl, 5; CaCl2, 2; MgCl2, 1; NaH2PO4, 0.4; NaHCO3, 23.8; t-glucose, 5.6) bubbled to equilibrium with a 5% CO2–95% O2 mixture. In later experiments, batches of R6/2 mice were transferred from Cambridge to Edinburgh and killed, and muscles were directly dissected into the bicarbonate-buffered muscle. Muscle contractions were reduced or eliminated by bathing them in 2.5 μM μ-conotoxin (μ-CTX) GIIIB (Scientific Marketing Associates, UK) for 30–45 min. In some experiments, μ-CTX was retained in the bathing medium throughout subsequent recordings. Normally, up to 30 muscle fibers per muscle were sampled using microelectrodes filled with 4 M potassium acetate (impedance ≈ 40 MΩ), according to standard techniques. Spontaneous and evoked endplate potentials were recorded using either WPI M707 (WP Instruments Inc.) or Axoclamp 2B amplifiers (Axon Instruments) and stored and analysed on a PC using either WinWCP v3.0.8 software (developed and distributed by Dr John Dempster, Strathclyde University), Spike-2 (Cambridge Electronic Designs, Cambridge, UK), or MiniAnalysis (Synaptosoft, Atlanta, USA). In some experiments, input resistance was measured by applying current to the recording microelectrode via a Wheatstone-bridge circuit built into the recording amplifier. Constant current was applied to set the resting membrane potential at ~80 mV. The additional steady-state membrane hyperpolarization evoked by superimposed weak hyperpolarizing current pulses (1–5 nA, 30–100 ms duration) was then measured. In some fibers, stronger current pulses were injected to test for ‘anode-break’ excitation of the muscle fibers on termination of the current pulse (Marshall & Ward, 1974). In other experiments, iontophoretic responses to ACh were tested using micropipettes filled with 1 M ACh (>100 MΩ input impedance). A holding current of 1–3 nA was applied to the pipettes to prevent leakage of ACh. Carefully reducing the holding current produced slow depolarization of the muscle fiber impaled with the recording microelectrode when the tip of the iontophoretic pipette was in the vicinity of its endplate. After restoring the holding current, the electrode was displaced ~100 μm to place its tip over the extrajunctional membrane and brief outward current pulses (1–10 nA, 1 ms) were applied to the iontophoretic pipette. In other experiments, extracellular recordings of muscle fiber action potentials were made using blunt (1–2 MΩ) pipettes filled with 1 M NaCl. The pipette tip was inserted into the belly of the FDB muscles and electromyographic (EMG) responses were evoked by suprathreshold stimulation of the muscle nerve (nominally 10–30 V, 0.1–0.5 ms duration). In some experiments, muscle tension recordings were made by pinning the proximal tendon of FDB muscles to the Sylgard-lined base of the recording chamber. The distal tendons were gathered and tied with a short silk suture then attached to a sensitive force transducer (Akers, Oslo, Norway) connected to a custom Wheatstone-bridge circuit and preamplifier. The output was filtered and further amplified using Neurolog equipment (Digitimer, UK). Resting muscle length was adjusted to maximize the twitch tension and/or tetanic (50 Hz) tension responses evoked by nerve stimulation. ACh chloride (10 μM) and μ-CTX (2.0 μM) were added directly to the bathing medium during recording of either evoked EMG or muscle tension responses.

**Statistical analysis**

Data are presented as mean ± SEM in each group, when n = number of muscle fibers and N = number of muscles or animals used, or as median, interquartile range and 5–95% outliers. Where appropriate, data were analysed either by unpaired Student’s t-test, Mann–Whitney test or one-way ANOVA. Dunnett’s post hoc test was used to determine levels of significance following ANOVA.
Results

Experiments were performed over a period of 44 months, from 2000 to 2004. Initially, muscles for physiological recording were dissected in Cambridge and couriered to Edinburgh in physiological saline for analysis and further processing the same day. In later experiments, surgery, physiological measurements, immunocytochemistry, histochemistry and electron microscopy were made on muscles isolated from R6/2 mice and their WT littermates following their delivery to Edinburgh. Over the course of the study, it became evident that the delay to onset of discernible weight loss and muscle atrophy increased, from \( \approx 9 \) weeks of age at the start of the study to \( > 11 \) weeks of age by the end. Mice did not normally live beyond 16 weeks at the start of the study, but routinely survived to 18 weeks or older by the end. This improved longevity occurred in spite of a spontaneous and progressive increase in the CAG repeat length in the colony over the period of the study (data not shown) and is attributed primarily to improvements in husbandry (Carter et al., 2000). None the less, the data are presented here in relation to the chronological age of the mice, with 'severely affected' mice in all cases being older than 15 weeks.

Clinical presentation of R6/2 mice

R6/2 mice show no overt behavioural phenotype until they are \( \approx 8 \) weeks of age (Carter et al., 1999). Thereafter, growth of R6/2 mice begins to slow and by 12 weeks they begin to lose weight (see below). At the same time they show an increasing number of abnormal motor signs, such as excessive hind limb grooming and tremor. Like many other mutant mice with neurological dysfunction, R6/2 mice also exhibit abnormal hind-limb reflex responses to tail lifting: hip flexion and toe claspig rather than extension. By 12 weeks, R6/2 mice are noticeably weaker and less active than their WT littermates and they perform poorly in behavioural tasks requiring strength. For example, they have difficulty swimming, performing on the rotorod or lifting their own body weight out of water onto an elevated platform, tasks that pose no difficulty to age-matched WT mice (Carter et al., 1999; also A.J. Morton, unpublished observations). However, in their home cage they appear generally healthy, and are not overtly different from their WT littermates.

In the present study, body weights of R6/2 mice at 12 weeks were \( 21.9 \pm 0.4 \) g (\( N = 17 \)) compared to \( 23.6 \pm 0.6 \) g (\( N = 16 \) WT mice). By 16 weeks their body weight dropped significantly (\( 17.5 \pm 0.5 \) g compared to \( 24.5 \pm 1.2 \) g for R6/2 and WT mice, respectively). Thus, the R6/2 mice lost \( \approx 20\% \) of their body weight while WT mice continue to grow. By 15 weeks, the mice show a marked lordosis (hunchback spine; Fig. 1a and b), poor mobility and pronounced muscle weakness. However, flexion reflex responses to tail lifting persisted. Overt motor symptoms such as spontaneous hindlimb grooming are present by 12 weeks. In R6/2 mice, 1.2 \( \pm 0.6 \) instances of hindlimb grooming were recorded (in a 10-min observation period in the open field; \( N = 17 \)) compared with 0.3 \( \pm 0.3 \) instances recorded in WT mice (\( N = 16 \)). By 15 weeks, 7.7 \( \pm 2.7 \) instances of hindlimb grooming were recorded in R6/2 mice (in 10 min) compared with 0.2 \( \pm 0.2 \) instances in WT mice. The motor symptoms progress until the premature death of the mice, usually between 16 and 20 weeks of age.

Skeletal muscle atrophy

By 12 weeks of age, R6/2 mice show visible atrophy of hind limb skeletal muscles compared with WT littermates and, by 15 weeks, this muscle atrophy is very severe (Fig. 1c and d). To examine the pathology of the skeletal muscle in more detail, cryosections of gluteus maximus and peroni from 15-week-old mice were processed and examined using a number of histological stains for myopathies (Anderson, 1985). Apart from the reduced diameter of fibers, microscopic examination of sections from R6/2 mouse showed normal morphology with haematoxylin and eosin, Cresyl Violet, Van Geison's, Oil Red O and periodic acid–Schiff stains. The frequency and prominence of nuclei was normal, and there were no centrally located nuclei. Other pathological signs that were sought, but not found, included central cores, nemaline rods, granular cytoplasm (in haematoxylin and eosin staining), basophilic rims, target fibers, ring fibers, cytoplasmic vacuoles, basophilic blebs, concentric lamellae, fibrosis and thickened endomysium (data not shown). Although atrophic, the muscle fibers looked healthy and there was no evidence of cell death, such as inflammatory cell infiltrates, fibro-fatty change, or regenerating fibers. Oil Red O revealed no evidence of lipid accumulation. Apart from the diffuse atrophy and fiber type changes (see below), there was no evidence of any of the other changes typically associated with muscle pathology.

The extent of muscle atrophy was measured in both transverse sections (Fig. 1e and f) and teased muscle fiber preparations of peroneal, SOL and EDL muscles (Fig. 2). Some transverse sections were stained histochemically for NADH diaphorase to distinguish type I from type II muscle fibers (Fig. 1c and d). By 8 weeks, muscle fiber diameter was slightly, though significantly, smaller in R6/2 mice than WT mice (Fig. 1i and j). However, muscle fiber diameter collapsed between 8 and 12 weeks. By 16 weeks, there was a severe, generalized atrophy of all muscle fibers in R6/2 mice that we examined and diameters of muscle fibers from R6/2 mice were half the size of age-matched WT muscles. For instance, in teased muscle fiber preparations of EDL muscles, fiber diameter of muscles from R6/2 mice was \( 28.32 \pm 0.78 \) \( \mu \)m (mean \( \pm \) SEM; \( n = 84 \) fibers, \( N = 2 \) muscles), compared with \( 45.33 \pm 1.06 \) \( \mu \)m (\( n = 80 \) fibers, \( N = 2 \) muscles) from WT muscle. Notably, both type I and type II muscle fibers were atrophic. In transverse sections of the peroneal muscles, the diameter of type I and II muscle fibers in WT muscles were \( 25.4 \pm 0.3 \) and \( 41.3 \pm 0.3 \) \( \mu \)m, respectively, compared with \( 15.9 \pm 0.2 \) and \( 17.4 \pm 0.2 \) \( \mu \)m in R6/2 (\( N = 675 \) muscle fibers in each case). Although atrophy occurred in both fiber types (see also Sathasivam et al., 1999), the proportion of type I and II fibers was altered. In WT peroneal muscles, \( 51 \pm 2\% \) of fibers were type I compared with \( 81.8 \pm 2.1\% \) in the R6/2 mouse. However, there were no signs of degeneration, nor was there any marked change in grouping of muscle fibers within the muscle. Thus it appears that type II fibers were converted to type I during the process of muscle atrophy rather than the change occurring by another process (e.g. fiber splitting).

Fiber diameter and motor endplate area

Muscle atrophy was measured in teased fiber preparations of R6/2 mouse EDL, soleus and FDB muscles, after staining histochemically for cholinesterase at motor endplates (Harris & Ribchester, 1979). This method typically stains the rims of junctions, delineating the extent of secondary folds. There were no discernible differences in the pattern or density of cholinesterase staining in R6/2 muscles (Figs 2a and b, and 3). Every fiber showed a single motor endplate, and the ‘pretzel’-like structure of the endplates were similar to those seen in WT muscle. Further, there was no overt fragmentation or expansion of endplate areas as observed in other neurological mutants with muscle atrophy (Harris & Ribchester, 1979; Blanco et al., 2001; Lin et al., 2001). Muscle fiber diameters declined uniformly as the animals deteriorated.
By 16 weeks, R6/2 muscle fiber diameters were 20–40% less than WT muscle diameters. This atrophy was evident in all three muscles but greatest in EDL (Fig. 2c). Interestingly, endplate areas also shrank in R6/2 muscles as muscle atrophy progressed (Fig. 2d), but the ratio of endplate area to fiber diameter was unaltered, as evident from plots of endplate area against muscle fiber diameter (Fig. 2e and f).

Ubiquitinated inclusions in myonuclei

One of the hallmarks of pathology in HD brain and brains of mouse models of HD is the presence of abnormal aggregates of ubiquitinated protein (Davies et al., 1997). Inclusions have been observed in quadriceps muscle (Sathasivam et al., 1999). To check for the presence of inclusions in the muscles we immunostained for ubiquitin in both whole mounts and transverse muscle sections. WT muscles showed no evidence of ubiquitin accumulation. Some myonuclei in R6/2 EDL and soleus muscles stained immunopositive for intranuclear inclusions. The morphological appearance of these inclusions was similar to those seen in the brains of R6/2 mice (Morton et al., 2000). They were infrequent up to 12 weeks of age but by 16 weeks they were present in ≈20% of EDL muscle fibers (Fig. 1g and h). A similar percentage (≈20%) of myonuclei was immunopositive for ubiquitin in whole-mount immunofluorescent specimens of the triangularis sterni muscle. This included nuclei in the motor endplate region of the muscle fibers (Fig. 3b).

Morphology of neuromuscular junctions

ACh receptors at motor endplates of R6/2 muscles were stained with TRITC-α-BTX. Co-immunostaining for NF/SV2 showed that, at all ages and in all muscles examined, the endplates in R6/2 muscles were pretzel-shaped and mononeuronally innervated by single axon collaterals in alignment with postsynaptic junctional folds, as in WT muscles (Fig. 3c–h). This appearance suggests that the adult neuromuscular innervation pattern develops normally, and this pattern is sustained throughout the life of R6/2 transgenic mice. Myelinating and perisynaptic terminal Schwann cells were also of normal appearance and there was no evidence of any constitutive Schwann cell or nerve terminal sprouting (see section on sprouting in response to nerve injury, below).

Several distinctive abnormalities became apparent in R6/2 mice from the age of ≈12 weeks. Most were not quantified because their incidence was generally very low (estimated at fewer than 5% of endplates in all muscles). The abnormalities included: long preterminal axon branches (Fig. 4a); thin, wispy or ‘untwisted’ neurofilaments (Fig. 4b); poor penetration of neurofilaments into nerve terminals (Fig. 4c); and accumulations of neurofilaments in preterminal axonal swellings (Fig. 4d). Similar abnormalities have been reported in mouse models of dying-back neuropathy (Miura et al., 1993) and motor neuron disease (Frey et al., 2000; Fischer et al., 2004). Motor nerve terminal abnormalities included partially occupied motor endplates, with exposed regions of ACh receptor staining (Fig. 4e). Although this was observed rarely (in three out of >600 neuromuscular junctions in three lumbrical muscles examined: 0.5%), this feature is notable because it has also been reported in mouse models of motor neuron disease (Cifuentes-Diaz et al., 2002; Ferri et al., 2003).

A remarkable feature of immunostained triangularis sterni muscles observed in three out of six severely atrophic R6/2 mice was a significant reduction in or absence of endplate staining of ACh receptors (Fig. 4f). This abnormal feature was not observed in lumbrical muscles of R6/2 mice at any stage although it is consistent with physiological failure of synaptic transmission observed in recordings from FDB muscles (see below). Absence of receptor staining could not be explained by failure of the TRITC-α-BTX toxin to penetrate the muscle because endplates in the same confocal plane were clearly stained (Fig. 4f).

Some lumbrical muscles were vitally stained with FM1-43, a dye which highlights regions of nerve terminals that contain recycling synaptic vesicles (Ribchester et al., 1994; Gillingwater et al., 2002). Almost all neuromuscular junctions in R6/2 mice took up FM1-43 in response to depolarizing stimuli, indicating they were able to recycle synaptic vesicles. As in the immunostained material, we saw occasional instances of partial occupancy of endplates by FM1-43-loaded terminals (data not shown).

Electron microscopy

We examined endplates in FDB muscles from two 12-week R6/2 mice, seeking evidence for early changes in nerve terminal ultrastructure. Of 44 endplates examined in detail, 33 appeared normal and 11 showed abnormalities, including membrane inclusions or vacuoles, pre- or postsynaptic detachment, and insinuation of terminal Schwann cell processes into synaptic cleft (arrows, Fig. 5a). Two endplates were found where junctional folds appeared abnormally wide (Fig. 5a and b). Synaptic vesicles appeared normal but there were vacuolated inclusions in the presynaptic nerve terminals and the density of mitochondria appeared abnormally high (Fig. 5a and b).

Myelinated axons in intramuscular nerves appeared normal (Fig. 3b, inset), confirming the normal appearance of axons in whole-mounts immunostained and studied using confocal microscopy.

Nerve regeneration and sprouting

Motor nerve endings sprout when muscles atrophy as a result of muscle paralysis or disuse (Betz et al., 1980; Brown et al., 1981; Pun et al., 2002). We were therefore surprised by the normal appearance of most R6/2 neuromuscular junctions in spite of the significant, uniform and progressive atrophy of the muscle. We hypothesized that atrophic R6/2 muscles might be incapable of generating nerve regeneration or sprouting signals, or that motoneurons in these muscles were incapable of responding to such stimuli. To test this, we first examined the ability of injured axons in R6/2 mice to regenerate.
Abnormal neuromuscular junction function in HD mice

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Fig. 2. Cholinesterase staining in atrophic R6/2 muscle appears normal. Examples of teased EDL muscle preparations from (a) 16-week-old WT and (b) R6/2 littermate stained histochemically for cholinesterase. There were no discernible differences in the staining pattern, although muscle fiber diameter and endplate area were both reduced in the R6/2 muscle. (c) Muscle fiber diameter and (d) end-plate area in FDB, EDL and soleus muscles were all significantly reduced (P < 0.05; t-tests) comparing R6/2 with WT in each case. The correlation between endplate area and muscle fiber diameter was sustained during progression of muscle atrophy. (e and f) Examples of WT (○) and R6/2 (●) from littermates aged (e) 13 weeks and (f) 16 weeks. Calibration bar in (b) 30 µm (for a and b).

Fig. 3. Most immunostained neuromuscular junctions appeared normal. Examples of confocal projections from whole-mount preparations of (a,c,e,g) WT and (b, d, f and h) R6/2 mice aged 12–15 weeks. (a–d) Triangularis sterni and (e–h) lumbrical muscles were costained with TRITC-a-BTX to label ACh receptors (red). In panels a and b, nuclei were stained using ToPro (blue) and immunostained for ubiquitin (green). (a) Ubiquitin staining was completely absent from WT muscles. (b) About 20% of myonuclei stained positive for ubiquinated inclusions (small arrows), similar to that seen in transverse sections of R6/2 mouse muscle (Fig. 1) or brain. Inset in (b) is a higher magnification of the nucleus indicated by the arrowhead in the main panel showing an inclusion in the same confocal plane. Axons and synaptic terminals in R6/2 muscles also showed diffuse immunostaining for ubiquitin. (c and d) Immunostaining for S100 (green) shows no discernible abnormality in the organization of either myelinating or terminal Schwann cells. (e–h) Neurofilament–SV2 staining (green) shows near-normal appearance of axons, motor nerve terminals and motor endplates in limbinal (12 weeks old; e and f) and overtly (16 week; g and h) atrophic muscles of R6/2 mice. Calibration bar in (h), 30 µm (a, b, c, f, g and h), 50 µm (c and d).
by crushing the tibial nerve in 6-week-old animals, and studied the innervation pattern of muscles physiologically using FM1-43 staining and microelectrode recording. In six R6/2 mice studied 5–6 weeks after the nerve crush was done, flexion reflexes had returned and, in isolated preparations, FM1-43 stained regenerated motor nerve terminals (Fig. 5c and d). Intracellular recordings from two of the muscles showed spontaneous miniature endplate potentials (mEPPs), evoked EPPs and/or action potentials in 15/20 and 19/20 fibers, respectively. Thus, motor neurons in young R6/2 mice are capable of axon regeneration and neuromuscular synapse repair.

Reinnervation studies were not possible in the oldest, most severely affected R6/2 mice because they did not live long enough for axon regeneration to occur after complete nerve section. However, we

Fig. 4. By 16 weeks, some R6/2 motor endplates showed distinctive diverse abnormalities. Endplates were immunostained for (a–c) neurofilaments or (d–g) neurofilaments and SV2. All preparations costained with TRITC-α-BTX. Examples of abnormalities observed include (a) a neuromuscular junction in a triangularis sterni muscle whose motor nerve terminal is supplied by a long preterminal axon branch (arrow); (b) neurofilaments that are ‘untwisted’ in this triangularis sterni terminal (arrow), a feature of ≈5% of terminals; (c) thinning of neurofilaments with poor penetration into a lumbrical nerve terminal; (d) a lumbrical terminal supplied by an axon showing swollen accumulations of neurofilaments (arrows), with an ultra-fine preterminal branch supplying the motor endplate; (e) a partially occupied endplate (arrow) (the inset shows a partially occupied lumbrical neuromuscular junction contacted by only two synaptic boutons, leaving the remainder of the endplate vacant; arrow); (f) a triangularis sterni neuromuscular junction with intact nerve terminal but complete absence of junctional ACh receptors (inset); (g) a neuromuscular junction with absent ACh receptors in a small fraction of the endplate (inset, arrow). Calibration bar in g, 20 μm (a and c–f), 10 μm (b and g).
examined whether motor axons in R6/2 muscles older than 12 weeks were able to generate compensatory nerve sprouts in response to partial denervation. Sprouting of motor axons is normally preceded by sprouting of terminal Schwann cells (Reynolds & Woolf, 1992; Son et al., 1996) and also, in mice, by down-regulation of the intensity of S100 immunostaining (W.J. Thompson, personal communication). The fourth deep lumbrical (4DL) muscles in rodents receive motor innervation by axons running in both the tibial–lateral plantar and sural nerves (Nakanishi & Norris, 1970; Betz et al., 1979; Taxt, 1983). Section of the tibial nerve in anaesthetized animals thus renders FDB and the medial lumbrical muscles completely denervated but the 4DL muscle is subject to a controlled partial denervation, making the muscle ideal for studies of motor nerve sprouting of the sural nerve.

Tibial nerves were cut under anaesthesia in nine R6/2 mice (four at 9–14 weeks and five at 15–18 weeks) and eight age-matched WT mice. 4DL muscles were immunostained 7 days later and examined for sprouting of sural nerve motor axons.

The results showed that axons in partially denervated R6/2 muscles from 9–14-week-old mice retained their competence to sprout as their muscle became atrophic. Figure 6a–d shows images taken from 4DL muscles 7 days after partially denervation, stained for S100 protein. Taking account of the settings of laser power and the gain and offset on the photomultiplier tubes in the confocal microscope, the attenuation of the S100 immunostaining signal was similar in WT and R6/2 mice. All muscles showed strong indications of Schwann cell and axonal sprouting (Fig. 6e–h). To
quantify the sprouting of axons, we scored the number of sprouts growing either into or out of α-BTX-stained ACh receptor pretzels. Collateral sprouting from nodes of Ranvier of intact intramuscular nerve axons was observed in all 9–14 week muscles and in four out of five muscles from severely affected mice older than 15 weeks (Fig. 6e–h). We pseudorandomly selected confocal microscope fields straddled by an intramuscular nerve branch. In the four muscles from the 9–14-week-old group of R6/2 mice, 17 endplates out of 71 sampled (24%) received collateral axonal sprouts from intact sural nerve motor axons, compared with 12 out of 53 (23%) of endplates in WT littermates. In the muscles from R6/2 mice older than 15 weeks, we observed that 13 endplates out of 50 analysed (26%) received collateral axonal sprouts. However, the sprouts were discernibly finer than in WT muscles, suggesting a qualitative impairment. Interestingly, in the WT muscles we also saw several instances of terminal sprouting (outgrowths from nerve terminals extending to nearby denervated endplates; arrows in Fig. 6f). However, we did not observe any of this form of sprouting in any of four partially denervated muscles from two severely affected R6/2 mice older than 16 weeks.

Together, the morphological data suggest that the innervation pattern and morphological appearance of neuromuscular junctions in a variety of R6/2 muscles is quite normal between 8 and 12 weeks of age, despite the decline in muscle mass during this period. However, significant and increasing instances of abnormal nerve terminals were detected in coincidence with increasing severity of the disease and there was a weakened sprouting response following partial denervation in muscles from mice older than 12 weeks. Most notably, whereas a compensatory sprouting reaction in motor nerve terminals might have been expected in response to the pronounced atrophy seen in mice older than 12 weeks, none was observed.

**Synaptic transmission at R6/2 mouse neuromuscular junctions**

Previous physiological studies of motor function in R6/2 mice have focused on central nervous system defects in synaptic transmission, as measured behaviourally and electrophysiologically. Because R6/2 mice show deteriorating cognitive and behavioural function yet remain mobile despite their severe muscle wasting and weight loss, it is unclear whether muscle atrophy is a consequence of central or peripheral neural defects or whether it is caused by intrinsic changes in skeletal muscle properties. In preliminary tests we measured forelimb grip strength. These showed that both WT and severely affected (15–17-week-old) R6/2 mice were motivated to exert consistent voluntary force, but R6/2 mice showed some fatigue on repeated testing (data not shown). However, such tests do not distinguish neuropathic changes from myopathic ones. We therefore studied passive membrane properties of R6/2 muscle fibers (resting membrane potential, input resistance and time constant) as well as synaptic properties (mEPPs and EPSPs). We also examined the sensitivity of muscle fibers to bath-applied and iontophoretically applied Ach, and the sensitivity of action potentials to the selective muscle NaV1.4 sodium channel blocker μ-CTX.

**Resting membrane potentials**

The mean resting membrane potential of FDB muscle fibers from WT mice was $-72.2 \pm 3.2$ mV (SEM, $N = 6$ muscles). This was significantly different from muscle fibers of presymptomatic R6/2 mice aged $<14$ weeks ($-62.8 \pm 1.52; P < 0.001; N = 10$ muscles). There was a further deterioration of resting membrane potential in R6/2 muscle fibers as the animals became overtly symptomatic and the muscle fibers more atrophic (Fig. 7a). By 15–18 weeks, muscle fiber resting potentials were $-55.2 \pm 4.3$ mV ($N = 10$ muscles). This decline was not an artefact of impalement of the atrophic muscle fibers because we made the recordings using high impedance microelectrodes filled with potassium acetate ($> 40$ MΩ). Low resting membrane potentials were also evident in R6/2 muscles when using ultra-sharp, very high impedance ($\approx 100$ MΩ) microelectrodes, which also registered normal resting potentials in WT muscles.

**Input resistance and time constant**

The small diameter of atrophic muscle fibers would be expected to confer a high input resistance and prolonged membrane time constant, as measured by the ratio and time course of the steady-state change in membrane potential in response to injection of rectangular constant-current pulses. Thus, input resistance is proportional to $d^{-3/2}$, where $d$ is the muscle fiber diameter (Katz & Thesleff, 1957). FDB muscle fibers in 15–18-week R6/2 mice are $\approx 40\%$ narrower than in WT mice (see above). As a consequence, input resistance should be about twice as great. Input resistance and membrane time constant were estimated using a single microelectrode and Wheatstone bridge method (Fig. 7b–e). Though imperfect, the single-electrode and bridge method minimizes damage to muscle fibers and allows effective comparisons between experimental and control groups of muscles (Barry & Ribchester, 1995). There was no significant difference between input resistances of presymptomatic, nonatrophic R6/2 muscles and those of WT (data not shown). However, by 15–18 weeks, as expected from the degree of muscle atrophy, the input resistance of R6/2 fibers was $\approx 4.38 \pm 0.36$ MΩ (SEM; $n = 21$ fibers, $N = 3$ muscles and three mice) compared with WT (2.34 $\pm 0.27$ MΩ; $n = 27$ fibers, $N = 3$ muscles and three mice; $P < 0.001$, t-test).

The membrane time constant in atrophic R6/2 muscle fibers was extended to more than twice that of WT fibers (Fig. 7b–e). FDB muscle fibers are equivalent to short cables with open-circuited ends, and are isopotential along their length in response to focal constant current injection (e.g. by synaptic currents, or via intracellular micropipettes; Bekoff & Betz, 1977). Biophysiologically, they are similar in their overall passive membrane behaviour to spherical cells. The membrane time constant was therefore estimated from the time taken to reach 63% of the steady-state membrane potential displacement during the same constant current injections (Jack et al. 1974). In presymptomatic R6/2 muscles the membrane time constant was not significantly different from WT (data not shown). However, in atrophic muscle fibers from 15–18-week R6/2 mice, with higher input resistances than normal, the membrane time constant was about...
Abnormal neuromuscular junction function in HD mice

Power = 4.7%
Gain = 4.9
Offset = -3.9

Power = 8.1%
Gain = 4.9
Offset = -3.9

Power = 6.5%
Gain = 4.1
Offset = 1.9

Power = 6.5%
Gain = 4.1
Offset = 1.9

Abnormal neuromuscular junction function in HD mice

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Spontaneous transmitter release

Measurements of spontaneous mEPPs were made in 137 muscle fibers from 12 muscles of R6/2 mice aged 8–18 weeks. In the younger mice, mEPP frequencies were in the normal range (mean ± SEM 0.23 ± 0.2/s at 18 °C, n = 40 fibers from two muscles; compared with 0.16 ± 0.04/s, 25 fibers from two age-matched WT muscles). mEPP amplitude, rise time and decay time constants were also within the normal range.

Analysis of mEPP frequency, amplitude and time course was performed on a subset of fibers in older, atrophic R6/2 muscles. Examples of averaged mEPSPs are shown in Fig. 8a and b. Between-event interval histograms of mEPSPs in 18-week-old mice showed an exponential time course, indistinguishable from the Poisson
distribution that characterizes normal mEPP interval histograms (Fig. 8c and d). However, though highly variable between fibers, mEPP frequencies were reduced overall by about half; median frequency was 0.27/s (interquartile range 0.17–0.5/s) compared with 0.60/s overall in WT (interquartile range 0.47–0.72/s; \( P < 0.001 \), Mann–Whitney). Overall, these data suggest that the stochastic determinants of synaptic vesicle exocytosis are not altered in R6/2 mouse neuromuscular junctions. However, further analysis of the characteristics of spontaneous transmitter release frequency may be warranted, especially in the light of the finding that at least one synapse-specific protein thought to modulate exocytosis (complexin II) is down-regulated in the R6/2 mouse brain (Morton & Edwardson, 2001).
As expected from the measurements of input resistance, mEPP amplitudes were markedly increased as the phenotype of the R6/2 mice deteriorated. In R6/2 mice older than 15 weeks, mEPPs were typically 2–4 times greater in amplitude than in WT mice (Fig. 8c). Overall, the median mEPP amplitude in R6/2 fibers was 1.96 mV (interquartile range 1.58–2.16 mV, n = 21 fibers from six muscles) compared with 0.54 mV (interquartile range 0.49–0.74 mV n = 11 fibers) from two age-matched WT muscles (P < 0.001, Mann–Whitney). The coefficient of variation of mEPP amplitude in some R6/2 muscle fibers was numerically higher (mean ± SEM 0.47 ± 0.27) but not statistically significantly different from WT (0.34 ± 0.06; P > 0.1, t-test). The instances where the variability seemed higher may thus have been merely a consequence of the better signal-to-noise ratio for detection of mEPPs in R6/2 muscle fibers on account of their high input resistance. However, we cannot rule out at this stage a real difference in the distribution of quantal sizes.

Also as expected from the increase in membrane time constant, time-to-half-decay of mEPPs were longer in R6/2 than in WT fibers (Fig. 8f). The decay time constants of exponential curve fits to the repolarization phase of the normalized average mEPP were consistently 2–3 times longer in R6/2 than WT.

**Evoked EPP characteristics**

Muscles were pretreated with μ-CTX GIIIB (2.5 μM), with the intention of blocking muscle action potentials and thereby exposing the full amplitude and time course of nerve-evoked EPPs. The μ-CTX was very effective in WT muscles, but we experienced considerable difficulties with older R6/2 muscles because they were resistant to the blocking effects of the toxin (see below). Movement artefacts were evident in many of the recordings, and action potentials rather than EPPs were frequently registered. However, continuous incubation in μ-CTX eventually produced sufficient block to enable recording of EPPs (Fig. 9). In most fibers, robust EPPs with a low coefficient of variation were recorded, indicating a high quantal content (Fig. 9b). We found no instances of random ‘failures’ of synaptic transmission in any of the trains of EPPs recorded from single muscle fibers, at any stage in the deterioration of R6/2 mice. For instance, EPP trains were sustained during repetitive high-frequency (> 30Hz) stimulation (Fig. 9c) as in WT muscles and there was no evidence for any systematic change in mean quantal content. Highly variable responses (including random occurrences of ‘failures’ in response to nerve stimulation) would have indicated a low quantal content and weak synaptic transmission. This was never observed in any of the recordings from >500 muscle fibers recorded during the course of this study. Analysis of EPP amplitudes using the coefficient of variation method indicated that the overall mean quantal content of EPPs in atrophic muscle fibers was not statistically significantly different from WT (data not shown). Voltage-clamp analysis of synaptic currents would perhaps clarify whether there are any subtle differences in quantal content of EPPs in R6/2 muscle fibers but, based on the voltage recordings, any such differences are unlikely to be physiologically significant.

Despite the absence of significant progressive deterioration in EPP quantal content, there was an increase in the number of muscle fibers that did not respond at all to nerve stimulation in severely affected R6/2 mice (older than 14 weeks). Only two out of 206 fibers in 16 WT muscles did not show evoked EPPs or action potentials when the muscle nerve was stimulated supramaximally. However, in 269 recordings from 18 muscles in R6/2 mice aged 15–18 weeks, 52 of the fibers (i.e. ≈20% overall) were consistently unresponsive to repeated stimulation and failed to show evoked EPP responses. This was not a consistent feature between mice or muscles because in eight out of 18 of the muscles all fibers impaled gave evoked responses. Nevertheless, complete failure of evoked synaptic transmission was evident in muscles from half of the severely affected R6/2 mice. It remains to be established whether this was due to abnormal nerve conduction or complete pre- or postsynaptic failure of neurotransmitter release or action.

In spite of the high input resistance of atrophic R6/2 muscle fibers and the concomitant increase in mEPP amplitudes, the amplitudes of evoked EPPs were not significantly different from those in WT muscles (Fig. 10a). However, resting potentials of the R6/2 muscles were reduced (see above, Fig. 8), and differences in EPP amplitude could have been obscured by the nonlinear summation of synaptic potentials as the membrane potential approached the transmitter null (i.e. reversal) potential (McLachlan & Martin, 1981). We therefore measured the membrane potential at the peak of the EPP response. A nonlinear polynomial curve fitted to the data showed a trend towards increased synaptic efficacy in the ageing R6/2 mice that did not reach statistical significance (EPPs depolarized muscle fibers to –40 ± 5 mV (N = 10 muscles) in older R6/2 mice compared with –48 ± 4 mV in WT mice (N = 4 muscles; P > 0.05; t-test). There were similar trends in EPP latency and time-to-peak (Fig. 10c and d), although only the time-to-half-decay of EPPs in atrophic R6/2 muscles was statistically significantly different: about three times longer in R6/2 mice older than 15 weeks compared with WT.

**Effect of anticholinesterase**

The prolongation of EPPs as R6/2 mice age is not a consequence of any functional reduction in cholinesterase activity. Histochemically, cholinesterase distribution at endplates was unaffected (see Fig. 2). Further, the addition of the cholinesterase inhibitor neostigmine...
to the bathing medium prolonged the decay time of EPPs in R6/2 mice about seven-fold, showing that the cholinesterase in unblocked muscles was functional. The mean ± SD half-decay time was 22.95 ± 7.06 ms (n = 4 fibers) in one 16-week R6/2 muscle after neostigmine compared with 3.34 ± 1.54 ms (n = 7) in the same muscle before neostigmine (see Fig. 8d).

Resistance of muscle action potentials to μ-CTX

Voltage-gated sodium channels in mammalian skeletal muscle (NaV1.4 channels) are normally blocked by μ-CTX GIIIB at concentrations of 1–3 μM (Cummins et al., 2002; Li et al., 2003). At this concentration, the toxin is ineffective on axons and motor nerve terminals, which therefore release physiologically normal amounts of neurotransmitter in response to nerve stimulation (Wood & Slater, 1997; Costanzo et al., 1999; but see Braga et al., 1992).

Incubation of WT muscles in μ-CTX routinely and visibly blocked nerve-evoked muscle contractions within 5–30 min and this block normally persisted for 1–4 h after returning muscles to normal physiological saline. As described above, robust endplate potentials were recorded from virtually all WT muscle fibers as R6/2 mice deteriorated compared with WT (data are mean ± SEM, N = 7 muscles). There was a no statistically significant change in (c) EPP latency and (d) time-to-peak, but (e) time-to-half-decay increased significantly from ≈13 weeks of age (P < 0.01, Mann–Whitney). Lines in a–e are best-fit second-order polynomials. (f) Cumulative distributions of relative frequencies of EPP half-decay times from all muscle fibers recorded in muscles from R6/2 mice (solid line) aged 15–18 weeks were significantly different from WT (dotted line; P < 0.01, Kolmogorov–Smirnov test).

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response (Fig. 11b), still producing visible contractions that appeared quite strong under the dissecting microscope.

Next, we recorded extracellular action potentials using 1 M NaCl-filled blunt-tipped (∼2–20 μm) glass micropipettes inserted into the belly of the FDB muscles. In three R6/2 preparations recorded in this fashion, μ-CTX depressed nerve-evoked muscle fiber inward currents by <5%, 28% (Fig. 12a) and 48%, whereas extracellular responses were reduced by 54% and 76% of initial current amplitude in two WT muscles (Fig. 11c and d). No contractions were visible in the case of these two WT muscles. The residual responses were therefore caused by endplate currents. As expected, adding 10 μM α-BTX to the bathing medium completely blocked junctional ACh receptors and abolished all evoked responses and contractions in both R6/2 and WT muscles (data not shown).

Lastly, we measured the amount of μ-CTX resistance in intracellular recordings. In 15–18-week-old R6/2 muscles, some fibers showed...
full-blown action potential responses, apparently unaffected by \(\mu\)-CTX treatment. However, others showed nonovershooting action potentials, with positive-going inflexions on the upstroke of the EPP (Fig. 11e). In some fibers, large mEPPs triggered action potentials that were also resistant to \(\mu\)-CTX (Fig. 11f).

To quantify the extent of \(\mu\)-CTX resistance, we measured the maximum rate of rise of the first intracellularly evoked response recorded in each muscle fiber. FDB muscle fiber action potentials typically have rates of rise > 60 mV/ms at room temperature, before treatment with \(\mu\)-CTX. In WT muscles, 90% of EPPs evoked by nerve stimulation after treatment with conotoxin had maximum rates of rise (dV/dt) < 30 mV/ms (53/58 muscle fibers from eight muscles).

Overall, \(\mu\)CTX reduced the rate of rise of intracellularly recorded muscle fiber responses in older R6/2 mice: \(\approx\) 42% of muscle fibers in 15–18-week-old R6/2 mice (38/90 fibers from eight muscles) showed rates of rise in their nerve-evoked responses that remained > 30 mV/ms; that is, below the lower bound for most EPPs in unblocked WT muscles. In the remainder dV/dt was < 30 mV/ms. Thus, almost half of the muscle fibers in severely affected R6/2 mouse FDB muscles showed evidence of significant \(\mu\)-CTX resistance.

Anode-break action potentials

Resistance to tetrodotoxin and \(\mu\)-CTX are properties of rSkM2 (NaV1.5) sodium channels that appear in WT muscle fiber membranes after denervation (White et al., 1991; Chen et al., 1992). Because atrophic muscle fibers from R6/2 mouse FDB muscles resembled denervated or paralysed muscles with respect to the \(\mu\)-CTX resistance, we tested them for anode-break excitation (Marshall & Ward, 1974). In a sample of 10 fibers recorded from one 16-week R6/2 FDB muscle, all 10 fibers showed clear anode-break excitation (Fig. 12). By comparison, > 90% of WT muscle fibers showed no anode-break excitation.

ACh responses

In the light of the other denervation-like physiological and pharmacological characteristics of R6/2 muscles, we examined them for ‘supersensitivity’ to ACh. Figure 12c shows alternating cycles of responses to single twitch and 30 Hz tetanic stimulation in an R6/2 muscle before, during and after adding ACh (10 \(\mu\)M) to the bathing medium. This caused a muscle contracture equivalent in magnitude to the resting twitch response, that is, about one-third of the tetanic tension response. Similar contractures were evoked in two other 18-week R6/2 muscles. In two WT muscles, as expected, the ACh contracture was < 10% of the nerve-evoked tetanic tension. Thus, R6/2 muscles were more than three times as sensitive as WT muscles to bath-applied ACh.

Finally we tested the response of individual fibers to iontophoretic application of ACh to extrajunctional muscle fiber membranes (Fig. 12d). Extrajunctional sensitivity to ACh was not seen in WT muscle fibers (data not shown). However, graded depolarizations were produced in three out of 10 fibers in one 18-week-old R6/2 muscle. These fibers were innervated, because we also observed spontaneous mEPPs in the recordings. This incidence of supersensitive fibers is consistent with the sensitivity to bath-applied ACh and the

Fig. 12. Atrophic R6/2 muscles showed anode-break excitation and ACh supersensitivity. (a and b) These characteristics were absent from WT muscles (a) and presymptomatic R6/2 mice (not shown), but strong hyperpolarizing current injections induced action potentials on break of the anodal current in virtually all severely affected R6/2 mouse FDB muscle fibers (b). (c and d) Muscles from mice at late stages of the disease (15-week-old) were supersensitive to ACh, as shown by (c) contractures in response to bath-applied ACh or (d) graded, iontophoretic application of ACh to muscle during intracellular recording. In (c), FDB muscle twitches and tetani were induced by nerve stimulation, then the bathing medium was substituted with medium containing 10 \(\mu\)M ACh. This produced a muscle contracture that reached \(\approx\) 80% of the twitch contraction response (\(\approx\) 30% of the tetanus evoked by 50 Hz stimulation). Traces in (d) show membrane depolarization in response to graded 2-ms iontophoretic pulses of ACh applied from a micropipette manipulated over the extrajunctional membrane of the impaled fiber.

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other denervation-like properties measured above, such as \( \mu \)-CTX resistance.

**Discussion**

The R6/2 transgenic mouse model of HD shows motor abnormalities that appear at \( \approx \) 8 weeks of age and progress insidiously until they are manifest overtly by \( \approx \) 12 weeks of age (for summary, see Fig. 13). From this time onwards, the mice deteriorate steadily until their untimely death at \( \approx \) 16–20 weeks of age. The present study shows that the R6/2 mouse has a complex phenotype with respect to neuromuscular structure and function. Both neural and muscular abnormalities were detected. These changes were paradoxical. For example, there is no evidence for paralysis or functional denervation until the latest stages of the disease, yet R6/2 muscle fibers undergo profound atrophy and many show membrane characteristics of denervated or paralysed muscle fibers. Conversely, intact nerve terminals and terminal Schwann cells show no evidence of constitutive sprouting, in spite of the atrophy and other denervation-like state of R6/2 muscle fibers. In addition, synaptic transmission fails completely at increasing numbers of neuromuscular junctions as the disease progresses. Together, these data suggest that neuromuscular abnormalities could contribute deleteriously to motor function in R6/2 mice and may also help explain their sudden deaths.

**Denervation-like characteristics of R6/2 muscles and their role in muscle atrophy**

Remarkably, in spite of their strong, functional motor innervation, R6/2 muscle fibers resemble denervated muscles in several important respects (atrophy, reduced resting membrane potential, resistance to an Na\( ^+ \) channel blocker, anode-break action potentials). However, other features of R6/2 muscle (most motor nerve terminals fully occupying Ach receptor prezets at motor endplates, robust EPP responses to nerve stimulation, absence of spontaneous fibrillation) were at odds with a denervated state. Thus, while the atrophy was pronounced, the absence of characteristic muscle pathology would appear to rule out the possibility that the atrophy was due to a denervation-like pathology. For example, there was no fiber-type grouping or compensatory hypertrophy of innervated fibers seen (Pachter & Eberstein, 1992). We also ruled out other neuropathological causes of the atrophy. Neurogenic disorders with skeletal muscle involvement show characteristic abnormalities such as angulated atrophic fibers, fiber-type grouping, group atrophy or target fibers. However, none of these abnormalities were found. There was also no pathology consistent with a myopathic origin of the atrophy because this would establish the extent of differences in pattern of neurogenic disorders with skeletal muscle involvement to show characteristic abnormalities such as angulated atrophic fibers, fiber-type grouping, group atrophy or target fibers. However, none of these abnormalities were found. There was also no pathology consistent with a myopathic origin of the atrophy because this would establish the extent of differences in pattern of morphological and physiological impairment in nerve terminal structure and function in other neurological mutants which undergo muscle atrophy, such as wobbler mice (Harris & Ward, 1974) smn mice (Cifuentes-Diaz et al., 2002) pnn mice (Ferri et al., 2003) and wasted mice (R.R. Ribchester, T.H. Gillingwater and C.M. Abbott, unpublished observations). There was also little resemblance to the widespread retraction of motor nerve terminals that occurs, with accompanying progressive weakening of synaptic transmission, in mutant or transgenic mice with slow Wallerian nerve degeneration (Ribchester et al., 1995; Gillingwater et al., 2002; Gillingwater et al., 2003). There were a number of morphological abnormalities in R6/2 muscles that might have contributed to abnormal physiology. For example, ultrastructural and fluorescence microscopy of motor endplates in mice older than 12 weeks showed several unusual features, including invagination of Schwann cell processes into the terminal, and abnormal neurofilament organization. Further, in some muscles, only slender neurofilaments penetrated the motor nerve terminals; in others, we saw wispy assemblages of untangled neurofilaments in preterminal axons. There was also evidence at some junctions for abnormal preterminal axon branching and the surprising finding of some endplates in older muscles that appeared devoid of Ach receptors. These changes would be consistent with aberrant function of the endplates. However, none of these adequately explain the electrophysiological deficits.

Although nerve function is abnormal, the electrophysiological data suggest that most of the muscle atrophy in R6/2 muscle up to 12–14 weeks of age is caused by an intrinsic deterioration in muscle properties, rather than by progressive structural or functional denervation. At late stages there was concurrent physiological evidence of increasing numbers of junctions showing progressive failure of neuromuscular transmission, which could reflect independent neural abnormalities. Thus, one possible explanation for the morbidity and
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**Fig. 13.** Summary diagram showing the relationship between timing of appearance of pathological features in the brains and skeletal muscle in R6/2 mice. Data used in compiling this figure come from (a) this study and from the studies of (b) Mangiarini *et al.* (1996), (c) Carter *et al.* (1999), (d) Lione *et al.* (1999), (e) Sathasivam *et al.* (1999), (f) Murphy *et al.* (2000), (g) Morton *et al.* (2000), (h) Meade *et al.*, 2002 and (i) Klapstein *et al.*, 2001). Abbreviations used are MWM, Morris water maze; NII, neuronal intranuclear inclusion; ENNI, extranuclear neuronal inclusion.

**Pathology at 6 weeks**
- **Brain**
  - mild atrophy
  - nuclear inclusions in striatum, hippocampus (CA1>>CA3,DG), thalamus, cortex, cerebellum
  - extranuclear inclusions rare
- **Skeletal muscle**
  - no atrophy
  - nuclear inclusions rare
- **Neuromuscular junctions**
  - No abnormalities seen

**Pathology at 12 weeks**
- **Brain**
  - atrophy significant
  - abundant nuclear and extranuclear inclusions in >90% of neurons in all regions
- **Skeletal muscle**
  - mild atrophy (<10%)
  - occasional nuclear inclusions (<5%)
- **Neuromuscular junctions**
  - few neurofilament abnormalities (<10%)
  - ACh receptors normal
  - nerve sprouting/regeneration normal

**Pathology at 15 weeks**
- **Brain**
  - atrophy significant
  - abundant nuclear and extranuclear inclusions in >90% of neurons in all regions
- **Skeletal muscle**
  - pronounced atrophy (~50%)
  - nuclear inclusion common (~20%)
- **Neuromuscular junctions**
  - neurofilament abnormalities (~20%)
  - ACh receptors absent (~20%)
  - sprouting absent after partial denervation

**Electrophysiological changes**
- **Striatum**
  - 5-7 weeks: spontaneous EPSCs
- **Hippocampus**
  - 8-12 weeks: μ-conotoxin resistance
  - 12-16 weeks: EPP failure
- **Neuromuscular junction**
  - 8-12 weeks: μ-conotoxin resistance
  - 12-16 weeks: EPP failure

**Other aspects of R6/2 phenotype**
- **Weight loss**
  - weight loss pronounced by 15 weeks
- **Diabetes**
  - first seen at 9 weeks, ~75% by 12 weeks
  - common by 15 weeks (>50% of mice)
- **Overt motor symptoms**
  - (hindlimb grooming, tremor)
  - rotorod (mild at 6 weeks, pronounced by 12 weeks)
- **Motor deficits**
  - MWM (mild at 4 weeks, pronounced by 12 weeks)
- **Cognitive deficits**
  - MWM (mild at 4 weeks, pronounced by 12 weeks)

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mortality of R6/2 mice over the age of 15 weeks is a collapse of skeletal muscle function, caused by both myogenic atrophy and neurogenic failure of neuromuscular transmission.

HD is considered to be a disease solely of the brain, despite the peripheral expression of huntingtin. However, our findings here suggest that abnormalities of either motor neuron or skeletal muscle function could contribute directly to motor symptoms in this disease. In addition to the uniform atrophy observed in R6/2 mouse muscles, we found some significant abnormalities in sprouting responses. In grade 2–4 HD brain, dendritic changes consistent with both regeneration and degeneration (Graveland et al., 1985; Ferrante et al., 1991; Soret et al., 1993) have been reported. However, in two transgenic models where dendritic changes have been studied (R6/2 and HD full-length cDNA mice), degenerative changes but not sprouting responses have been reported (Guidetti et al., 2001; Klapstein et al., 2001). It would be interesting to see whether the neurons in R6/2 brains show a typical sprouting response to direct injury or whether they, like the peripheral neurons, show abnormal sprouting responses.

The most parsimonious explanation for our findings is that the polyQ-huntingtin transgene affects motoneurons, their presynaptic motor nerve terminals and skeletal muscle fibers independently. In fact, the peculiar combination of pathology we see in the neuromuscular junction suggests that there is a functional disconnection of the homeostatic feedback mechanisms that normally regulate neuromuscular function. Thus, skeletal muscle fibers do not appear to respond normally to the trophic input from their motor nerve terminals, and/or the motor neurons do not respond to neurotrophic feedback from the muscle fibers. It is tempting to speculate that a similar dysregulation of feedback mechanisms may also be present in the CNS. This may constitute (or produce) breakdown in the mutual trophic interactions that normally maintain nerve and muscle. It has been shown previously that skeletal muscle is a target of polyglutamine-related perturbations in gene expression (Luthi-Carter et al., 2002). Further analysis of patterns of gene expression in R6/2 muscle could help resolve this issue, or at least show to what extent the Huntington’s mutation causes dysregulation of muscle gene expression. For example, it would be interesting to determine whether the changes in μ-CTX sensitivity were due to changes of sodium channel subunit expression or subunit composition. Targeted expression of the R6/2 transgene selectively in muscle or motoneurons, or utilization of chimeric mice or mice with xenografts of muscle, might also help to establish whether muscle atrophy is a direct consequence of the mutation. In the light of published abnormalities in synaptic vesicle proteins in the brains of R6/2 mice, notably complexin II (Morton & Edwardson, 2001), it is interesting to speculate that the causes of the diverse array of neuromuscular abnormalities may be a consequence of abnormal trafficking, integration or function of other synapse-specific proteins. Such abnormalities might, for example, underpin the reduced mEPP frequency and abnormally high coefficient of variation in the amplitude distribution of mEPPs we observed in some R6/2 muscle fibers.

For the time being, the clinical relevance of our studies remains speculative. While some studies on human skeletal muscle function have been done, most of those have focused on descending control of motor function. Interestingly, EMG studies in HD patients have reported abnormalities in motor unit activity (Harper et al., 1991) and long latency responses (Noth et al., 1985; Leblhuber et al., 1991; Berardelli et al., 1999; Siedenberg et al., 1999). Although these deficits have usually been attributed to defects in cortical processing (Noth et al., 1985; Abbruzzese & Berardelli, 2003), not all of them can be explained by alterations in the primary sensory pathways (Siedenberg et al., 1999). However, systematic studies of skeletal muscle pathology or function in HD patients have not been carried out. In the light of the present study, this would clearly be worth doing in the future.

Conclusions

When taken together our data suggest that, despite the inexorable and uniform progression of muscle atrophy in R6/2 mouse from ≈8 weeks of age, most neuromuscular junctions remained competent in their function. However, the function of increasing numbers of these junctions deteriorated with age, evidently through a combination of pre- and postsynaptic impairment. The progression of abnormalities was not linear; indeed, most abnormalities were not seen consistently until 15 weeks of age, that is, during the last 1–2 weeks of life. This suggests that one possible explanation for the morbidity and mortality of R6/2 mice over the age of 15 weeks is failure of skeletal muscle transmission subsequent to a collapse both in muscle fiber strength and in neuromuscular transmission. Irrespective of the cause, it is clear that there is significant and progressive pathology of muscles and neuromuscular junctions in the postsymptomatic R6/2 mouse which seems likely to contribute to several aspects of motor dysfunction. If the neuromuscular junction deficits we see in the mice are present in humans then it seems likely that abnormalities in peripheral neuromuscular function would contribute significantly to the progression of HD, particularly in the later stages.

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Abbreviations

CTX, μ-conotoxin; 4DL, fourth deep lumbral (muscles); α-BTX, α-bungarotoxin; ACh, acetylcholine; EDL, extensor digitorum longus; EMG, electromyograph(ic); EPP, endplate potential; FDB, flexor digitorum brevis; mEPP, miniature endplate potential; SDH, succinate dehydrogenase; WT, wild type.

References


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