Persistence of Neuromuscular Junctions after Axotomy in Mice with Slow Wallerian Degeneration (C57BL/WldS)

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Abstract
The present study was undertaken to examine the fate of neuromuscular junctions in C57BL/WldS mice (formerly known as OLA mice) after nerve injury. When a peripheral nerve is injured, the distal axons normally degenerate within 1–3 days. For motor axons, an early event is deterioration of motor nerve terminals at neuromuscular junctions. Previously, the vulnerability of motor terminals has been attributed either to a 'signal' originating at the site of nerve injury and transported rapidly to the terminals or to their continual requirement for essential maintenance factors synthesized in the motor neuron cell body and supplied to the terminals by fast axonal transport. Mice of the WldS strain have normal axoplasmic transport but show an abnormally slow rate of axon and myelin degeneration. Structure and function are retained in the axons of distal nerve stumps for several days or even weeks after nerve injury in these mice. The results of the present study show that WldS neuromuscular junctions are also preserved and continue to release neurotransmitter and recycle synaptic vesicle membrane for at least 3 days and in some cases up to 2 weeks after nerve injury. Varying the site of the nerve lesion delayed degeneration by ~1–2 days per centimetre of distal nerve remaining. These findings suggest that the mechanisms of nerve terminal degeneration after injury are more complex than can be accounted for simply by the failure of motor neuron cell bodies to supply their terminals with essential maintenance factors. Rather, the data support the view that nerve section normally activates cellular components or processes already present, but latent, in motor nerve endings, and that in WldS mice either the trigger or the cellular response is abnormal.

Introduction
When a peripheral nerve is cut or crushed, the distal axons degenerate by a process first described in frogs, known as Wallerian degeneration (Waller, 1851; Langley, 1909). Normally, neuromuscular transmission fails early on in this process (Birks et al., 1960). In rodents, transmission fails within ~12–36 h, while axons and myelin sheaths deteriorate over a further 24–48 h (Miledi and Slater 1970; Winlow and Usherwood, 1975). Thus by 3 days after nerve injury, both neuromuscular transmission and nerve conduction in the distal stump have normally failed completely. Miledi and Slater (1970) proposed that the rapid rate of nerve terminal degeneration might be due to a process or 'signal' originating at the site of injury and that this signal is normally transported to the nerve terminal by fast axoplasmic transport. Their interpretation was based on the finding that the onset of degeneration of nerve terminals in the rat diaphragm depended on the length of the phrenic nerve stump. Neuromuscular transmission failed ~2–2 h later per additional centimetre of distal nerve that was left after nerve injury. Other contemporaneous studies favoured a different interpretation, however: namely that motor terminals normally degenerate rapidly after nerve section because the axotomized neuronal cell bodies are no longer capable of supplying their terminals with the essential cellular components required to maintain synaptic transmission and function (Albuquerque et al., 1972; Perisic and Cuenod, 1972). For example, Hudson et al. (1984) concluded that the effects of topical administration of batrachotoxin to the sciatic nerve of rats caused motor terminals to degenerate then recover at a rate that was consistent with interruption then resumption of fast axonal transport.

Wallerian degeneration of axons occurs much more slowly than normal in mice of the C57BL/WldS strain (WldS) (Lunn et al., 1989). Following nerve section, the structure of axons and myelin sheaths is maintained, and the axons continue to conduct action potentials for several weeks (Perry et al., 1990a; Brown et al., 1992; Tsao et al., 1994). The rate of fast axoplasmic transport in isolated segments of WldS peripheral nerves appears to be normal (Smith and Bisby, 1993). The defect in these mice has recently been traced to an autosomal dominant gene, located on chromosome 4 (Perry et al., 1990b; Lyon et al., 1993). This gene seems to affect an intrinsic property of the axons rather than any important properties of glia, supporting cells or myelomonocytic cells, but the molecular basis of the slow Wallerian degeneration in these mice remains obscure (Perry et al., 1990a;
1642 Neuromuscular junctions in WldS mice

Brown et al., 1991b; Perry and Brown, 1992; Glass et al., 1993; Buckmaster et al., 1994; Deckwerth and Johnson, 1994).

The aim of the present study was to use WldS mice to investigate further the possible nature of the trigger for the rapid degeneration of motor nerve terminals that normally follows nerve injury. If terminals depend on a 'maintenance factor' supplied by fast axonal transport from the cell body, then we would expect the rate of degeneration of motor nerve terminals to be the same in WldS and control mice. Equally, if nerve terminal degeneration were due to the active transport of a 'degeneration factor' propagated from the site of nerve injury, we should expect the same dependence on nerve stump length in WldS as in control mice.

We show that in young adult WldS mice, degeneration of motor nerve terminals is abnormally slow. Terminals disconnected from their cell bodies continue to transmit and recycle synaptic vesicles, in some cases up to 2 weeks after nerve section. Degeneration is also more protracted when a long nerve stump is left compared with a short stump: but by ~1–2 days per cm of distal nerve, rather than h per cm as normally found in wild-type animals (Miledi and Slater, 1970). We also found that in older WldS mice, however, the rate of degeneration of motor terminals approaches that in wild-type mice.

Some of the observations reported here have appeared in abstract form (Ribchester et al., 1994a, b).

Materials and methods

WldS mice

We used two separate breeding colonies of C57BL/WldS mice for the present experiments: one housed in Oxford (formerly designated C57BL/6/Ola) and one built up and maintained over several years in the Department of Medical Microbiology in Edinburgh (C57BL/6/EUMM). Both colonies were originally derived from stock supplied by Harlan-Olac (Bicester, UK). Mice from both colonies shared the 'wild-type' strains (normal degeneration) used as controls in the present experiments: one housed in Oxford (formerly designated CBA and C57BL/6J). We studied structure and function of neuromuscular junctions in the soleus (SOL), flexor digitorum brevis (FDB) and triangularis sterni (TS) muscles after injury to the sciatic nerve, tibial nerve or the left side, −0.5 cm lateral to the vertebral column.

For each fibre, the resting membrane potential, presence of miniature end-plate potentials (MEPPs) and latency of any end-plate potential (EPP) or action potential evoked by stimulation of the FDB muscle nerve were recorded. The muscle nerve was stimulated using a suction electrode, with pulses of 0.2 ms duration and up to 100 V in amplitude (normally <10 V). The occurrence of MEPPs was monitored for each fibre in one or more 10 s sweeps on a slow oscilloscope time base. MEPP frequency was categorized as >1 MEPP/s; 0.1–1 MEPP/s; and <0.1 MEPP/s (no MEPPs). Recordings were made at room temperature (17–22°C). Recordings from some fibres were digitized using a modified SONY PCM 701ES and stored on VHS videotape. MEPP amplitudes were measured off-line from these tapes, using a CED1401 interface and a personal computer running the WCP software package, written and supplied by J. Dempster (University of Strathclyde, Glasgow, UK).

Surgery

Mice were anaesthetized either by inhalation of halothane or ether, by i.p. injection of sodium pentobarbitone (0.5 mg/10 g) or by injection of Avertin (2.4 mg/10 g). The sciatic nerve was sectioned on one or both sides at one of four different levels: at the emergence of the nerve at the sciatic notch; at the mid-thigh level; distal to the popliteal fossa (denervating the SOL muscle only); or at the mid-calf level (tibial nerve), denervating the foot only. Electron microscopy of distal sciatic nerve stumps, prepared 5–6 days after nerve section, confirmed the presence of myelinated axons with few overt signs of degeneration, in contrast to the distal stumps of sectioned nerves in wild-type strains of mouse (see, for example, Tsao et al., 1994). In some mice, the second to sixth intercostal nerves were sectioned on the left side, −0.5 cm lateral to the vertebral column.

Isometric tension

Isolated SOL nerve–muscle preparations were bathed in a solution of the following composition in mM: Na+ 144.5, K+ 5, Ca2+ 2, Mg2+ 1, Cl− 131, HCO3− 23, H2PO4− 1.5, D-glucose 11. The Achilles tendon was pinned to the base of a Sylgard-lined chamber and the proximal tendon was attached with a short length of silk thread to a silicon strain gauge. The nerves were stimulated using suction electrodes with 0.1–1.0 ms pulses, up to 100 V.

Intracellular recording

Intracellular recordings were made between 1 and 15 days after nerve section. Isolated FDB nerve–muscle preparations were bathed and bathed in physiological saline as defined above for tension measurements. Normally, 20 muscle fibres from each muscle were impaled with glass microelectrodes filled with 4 M potassium acetate, of ~40 MΩ resistance. For each fibre, the resting membrane potential, presence of miniature end-plate potentials (MEPPs) and latency of any end-plate potential (EPP) or action potential evoked by stimulation of the FDB muscle nerve were recorded. The muscle nerve was stimulated using a suction electrode, with pulses of 0.2 ms duration and up to 100 V in amplitude (normally <10 V). The occurrence of MEPPs was monitored for each fibre in one or more 10 s sweeps on a slow oscilloscope time base. MEPP frequency was categorized as >1 MEPP/s; 0.1–1 MEPP/s; and <0.1 MEPP/s (no MEPPs). Recordings were made at room temperature (17–22°C). Recordings from some fibres were digitized using a modified SONY PCM 701ES and stored on VHS videotape. MEPP amplitudes were measured off-line from these tapes, using a CED1401 interface and a personal computer running the WCP software package, written and supplied by J. Dempster (University of Strathclyde, Glasgow, UK).

FM1-43 imaging

Triangularis sterni muscles and, in some cases, FDB muscles were stained with FM1-43 (Molecular Probes, Eugene, USA) by bathing isolated preparations in a 2 μM solution of the dye in mammalian saline (composition as above) and stimulating the distal nerve stump at 30 Hz for 5 s every 15 s, for 10 min (Ribchester et al., 1994c). After washing, nerve terminals were viewed in a fluorescence microscope using a 100 W xenon lamp attenuated with 1–10% transmittance neutral density filters, via a 435 nm excitation filter, 455 nm dichroic mirror and 515 nm interference barrier filter. Terminals were destained either by continuous nerve stimulation or by bathing preparations in saline with [K+] raised to 50 mM ([Na+] reduced by 45 mM). Images were captured through a Falcon LTC1160 SIT camera (Custom Cameras, Wells, UK) and digitized on an Apple Macintosh Quadra 840AV microcomputer fitted with a Perceptrics PixelPipeline frame store. Images were averaged (32–64 frames) and processed using either NIH Image (public domain software) or IonVision (Improvision, Coventry, UK). Successive images were aligned and linear contrast stretched between equal limits. Regions of interest were outlined and the mean pixel intensity calculated.
Photographic quality images were prepared using Adobe Photoshop and printed on a Kodak ColorEase printer.

Zinc iodide–osmium tetroxide staining
Some muscles were stained with zinc iodide and osmium tetroxide (ZIO), teased into bundles of a few fibres, and mounted in glycerol as described previously (Harris and Ribchester, 1979). Images were captured and processed using a Sony CCD camera and the same digitization and processing software as for FM1-43 stained preparations.

Electron microscopy
FDB muscles were fixed overnight in 2% glutaraldehyde/0.2% picric acid in 0.1 M phosphate buffer, pH 7.4. After rinsing in buffer, the muscles were post-fixed in 2% osmium tetroxide in buffer for 2 h. Rinsed in buffer, stained for 1 h in 2% uranyl acetate, dehydrated and embedded in acrylic LR white resin (London Resins, UK). Ultrathin sections were viewed with a JEOL 100CX electron microscope. Enlarged electron photomicrographs were scanned at 150–300 d.p.i., enhanced and reprinted using Adobe Photoshop on the same computer system as described above.

Results

Wild-type animals
In common with previous findings (e.g. Miledi and Slater, 1970; Winlow and Usherwood, 1975), nerve sectioning in the two wild-type strains we studied (CBA and C57BL/6J) caused rapid degeneration of motor terminals and axons. By 48 h after nerve section, the distal nerve stump had adopted a uniform, opaque appearance that contrasted with the regular banding pattern of intact nerves. Stimulation of the distal nerve stump caused no contractile or evoked electrical responses in the muscles. Intracellular recordings from 20 fibres in one FDB muscle studied 24 h after nerve section revealed seven fibres with spontaneous MEPPs, but no evoked EPPs or action potentials. Recordings from a similar number of fibres in another muscle studied 48 h after nerve section revealed no signs of innervation. Staining with ZIO in a third muscle studied 72 h after nerve section revealed no nerve terminals.

Structure and function of neuromuscular junctions in Wld<sup>e</sup> muscles
The distal nerve stumps on the axotomized side of 1-month-old Wld<sup>e</sup> mice retained the banded appearance characteristic of intact nerves in all cases examined, up to 15 days after section of the sciatic or tibial nerves. Isolated SOL and FDB muscles contracted visibly in response to stimulation of the distal nerve stump in virtually all these muscles. Freshly dissected muscles on the axotomized side also showed a considerable amount of spontaneous contractile activity, resembling fibrillation. This activity was most conspicuous in FDB muscles 3–8 days after tibial nerve section.

Figure 1A, B shows twitch and tetanic contractions of Wld<sup>e</sup> SOL muscles in response to stimulation of the distal nerve stump, 5 days after sciatic nerve section at the mid-thigh level. Figure 1C–E shows intracellular recordings of spontaneous MEPPs and nerve-evoked responses in FDB muscle fibres, obtained 5 days after tibial nerve section. These responses were typical of the Wld<sup>e</sup> muscles studied within the first few days after axotomy.

Intracellular recordings from many of the fibres in the axotomized Wld<sup>e</sup> FDB muscles also revealed infrequent spontaneous action potentials, accounting for the spontaneous contractile activity of the muscles (Fig. 1D). Spontaneous contractions and the action potentials were inhibited by raising the [Ca<sup>2+</sup>] in the bathing medium from 2 mM to 10 mM; and they were completely blocked in the presence of 0.5 µM d-tubocurarine. The spontaneous action potentials were all apparently triggered from the rising phase of abnormally large MEPPs (Fig. 1E). In unoperated Wld<sup>e</sup> FDB muscle fibres, the mean MEPP amplitude was 2.0 ± 0.6 mV (SEM). By 5 days, the mean MEPP amplitude was almost doubled, to 3.6 ± 0.6 mV. This increase was probably due to an increase in the membrane resistance of the...
Neuromuscular junctions in Wld<sup>s</sup> mice

Fig. 2. (A–D) Motor nerve terminals in Wld<sup>s</sup> muscles stained with ZIO. (A) ZIO-stained terminal in FDB, 5 days after tibial nerve section. (B) FDB terminal 7 days after sciatic nerve section. (C, D) FDB terminals 8 days after tibial nerve section. Note the thinning of the preterminal axons and the retraction of their myelin sheaths in C, and the fragmented appearance of the nerve terminal boutons in D. (E, F) Electron micrographs of neuromuscular junctions and distal nerve stump in FDB muscles of Wld<sup>s</sup> mice 5 days after tibial nerve section. Note the peripheral distribution of synaptic vesicles in E and the accumulation of neurofilaments in F. Calibrations: A–D, 40 μm; E, 2 μm; F, 1 μm.
muscle fibres, as the rise time and half-decay time of the MEPPs were also significantly prolonged (data not shown). The resting membrane potentials of the muscle fibres on the operated side were also less negative than those in contralateral unoperated FDB muscles. By 4–6 days after tibial nerve section, the mean resting potential was $-55.99 \pm 4.62$ mV ($n = 80$ fibres, four muscles), compared with $-65.92 \pm 2.31$ mV ($n = 120$ fibres, six muscles) in controls. Increased membrane resistance and membrane depolarization are well-known characteristics of denervated muscle, so these features—when taken together with the persistent transmitter release from nerve terminals—probably explain the increased excitability of the Wld$^+$ muscle fibres after nerve section. Thus the spontaneous activity of the axotomized Wld$^+$ muscles had a different origin from the classical fibrillation of denervated muscles (cf. Purves and Sakmann, 1974).

The physiological evidence of persistent neuromuscular junctions was corroborated by histological and electron microscopic observations. Figure 2A, B shows examples of motor nerve terminals in ZIO-stained preparations made 5 days after nerve section. These muscles were replete with well-stained terminals. It was difficult to distinguish the appearance of these from terminals in unoperated muscles. At longer times, however, terminals appeared fragmented with fewer boutons, and it appeared that the myelin sheaths had begun to retract from many of the terminals (Fig. 2C, D). Figure 2E, F shows electron micrographs of terminals in FDB muscles, 5 days after tibial nerve section. Synaptic vesicles and mitochondria were still present, although the vesicles appeared to be concentrated near the plasma membrane. A conspicuous feature was accumulation of neurofilamentous material (cf. Jones and Kwanbunbumpen, 1970; Winlow and Usherwood, 1975; Watson et al., 1993). This was not seen in motor terminals from contralateral unoperated muscles. Electron microscopy also confirmed the presence of myelinated axons in the axotomized distal stump, as reported previously (e.g. Tsao et al., 1994).

**Recycling of synaptic vesicles**

In order to test whether motor nerve terminals in Wld$^+$ mice were also capable of recycling synaptic vesicles, in addition to releasing transmitter, we attempted to stain and destain axotomized terminals using the activity-dependent styryl dye, FM1-43 (Beitz et al., 1992; Ribchester et al., 1994a). We found that motor terminals in TS and FDB muscles could be stained with this dye up to 11 days after nerve...
obtained from another six terminals in FDB muscles, up to 7 days after nerve section. Figure 3 shows the destaining of a nerve terminal in a TS preparation that was first loaded with FM1-43 by stimulating the distal nerve stump of an intercostal nerve which had been sectioned 3 days previously. The preparation was washed and then destained by exchanging the bathing medium for one with [K+] elevated to 50 mM ([Na+] ions reduced correspondingly). The terminal destained rapidly over the first 2 min, then fluorescence plateaued above background over the next few min (Fig. 4). Similar results were obtained from another six terminals in FDB muscles, up to 7 days after nerve section.

**Time course of degeneration of terminals in Wld<sup>s</sup> mice**

Motor terminals in Wld<sup>s</sup> mice eventually degenerated after nerve section. Figure 5A, C shows the time course of degeneration of neuromuscular junctions in SOL and FDB muscles, measured from isometric tension and intracellular recordings respectively. By 3 days after nerve section, there was very little difference in the relative tension (indirect/direct) in SOL muscles, and in FDB muscles virtually all fibres responded with action potentials or EPPs on stimulation of the distal nerve stump. By seven days, the tension produced by stimulating the distal stump was about one-third that produced by direct stimulation, while intracellular recordings showed that about half the neuromuscular junctions had ceased to transmit. By 2 weeks, it was difficult to find evidence of functionally transmitting synapses.

Circumstantial evidence for gradual deterioration of synaptic transmission in at least some of the neuromuscular junctions was obtained. First, the proportion of FDB muscle fibres expressing spontaneous MEPPs but failing to respond to nerve stimulation increased up to 7 days after nerve section then declined. The number of completely denervated fibres increased progressively in parallel (Table 1). Secondly, in a few instances, repetitive stimulation produced intermittent failure of transmission, indicating an abnormally low quantal content (Boyd and Martin, 1956). Finally, the latency of evoked responses (action potentials or EPPs) in innervated fibres increased only slightly with time after nerve section. In unoperated contralateral controls, the mean latency was 1.91 ± 0.42 ms (mean ± SEM; n = 120 fibres in six muscles). After 7 days, the latency in those fibres that still responded was 2.99 ± 0.33 ms (n = 46 fibres in seven muscles) and by 11-13 days this figure increased to 3.63 ± 0.09 ms (n = 7 muscle fibres; P < 0.01; t-test). The simplest interpretation of these data is that conduction of action potentials into nerve terminals and motor axon degeneration deteriorated less rapidly than failure of neuromuscular transmission (see also Brown et al., 1994).

**Effect of nerve stump length**

Differences in the length of the distal nerve stump had significant effects on the loss of neuromuscular function (Fig. 5B, D). Thus in SOL muscles, 7 days after section of the sciatic nerve at the sciatic notch, stimulation of the distal stump generated ~70% of the total muscle tension. By contrast, section of the nerve in the popliteal fossa (~2 cm nearer the SOL muscle) resulted in only ~20% of the muscle fibres contributing to the twitch tension after a similar period (Fig. 5B). Similar effects were seen in FDB muscles. Seven days after section of the sciatic nerve at mid-thigh level, ~75% of the FDB muscle fibres still responded with action potentials, EPPs and/or MEPPs, whereas only ~50% of fibres were innervated 7 days after section of the tibial nerve (P < 0.05; t-test), ~12 mm more distal (Fig. 5D). Taking the tension and intracellular data together, the effect of a long nerve stump length was to delay degeneration by 1-2 days per cm of nerve.

**Effect of age**

Perry et al. (1992) showed that persistence of nerve conduction after axotomy in Wld<sup>s</sup> mice declines with age: in 1-month-old mice, compound action potentials declined to only ~80% of their normal amplitude by 5 days after axotomy, whereas in 3- to 6-month-old mice the compound action potentials were reduced to ~20-30% of normal after a similar period. By 1 year of age, compound action potentials in the axotomized distal stumps were lost at about the same rate as in wild-type animals (but only ~30% of the axons appeared histologically 'abnormal' 5 days after nerve section).

We also found evidence of an age-related increase in the rate of degeneration of motor nerve terminals. Thus in three Wld<sup>s</sup> mice aged 4 months and studied 2-3 days after bilateral tibial nerve section, 11 out of 120 muscle fibres in six FDB muscles (that is, only ~9% of fibres) expressed MEPPs, and only one of these innervated fibres responded with an EPP to nerve stimulation. The remainder gave no response. If we assume that the reduction in the compound action potential reported by Perry et al. (1992) is related to the number of axons failing to respond to stimulation, the present data suggest that there were 3- to 30-fold fewer axons with functional motor terminals than in the distal nerve. Thus degeneration of motor nerve terminals in these muscle appeared to precede loss of action potential propagation in axons, as in the young adult Wld<sup>s</sup> mice or wild-type animals.

**Discussion**

Motor nerve terminals degenerate within 24 h of nerve section in normal rodents (Miledi and Slater, 1970; Winlow and Usherwood, 1975). The present data suggest that this property is under genetic as well as epigenetic control. The main findings are first, neuromuscular junctions in Wld<sup>s</sup> mice remain intact and functional many days after terminals in normal mice have degenerated. The persistent junctions are able to recycle synaptic vesicle membrane as well as release transmitter in response to depolarising stimuli. Secondly, as in normal mice, the rate of degeneration depends on the length of the distal nerve stump, but the additional delay is ~48 h per additional cm of distal nerve, at least 5-20 times slower than in normal animals. Thirdly, the capacity of motor terminals to withstand nerve injury is
related to the age of the individual WldS mice: those older than ~3 months lose functional neuromuscular transmission after nerve section much more rapidly than young adult mice of this strain.

**The rate of nerve terminal degeneration**

The rate of nerve terminal degeneration in normal animals can be influenced by at least two important local (epigenetic) variables: the length of the distal nerve stump and temperature. The effects of these variables suggest that axoplasmic transport and components or processes dependent on the energetics of cell metabolism are important.

The effect of nerve stump length on nerve terminal degeneration was carefully studied by Miledi and Slater (1970), who demonstrated that in rat diaphragm, each additional cm of distal nerve stump delayed degeneration by 1–2 h. A similar magnitude of effect was seen with respect to the onset of membrane responses to denervation in muscle fibres (Harris and Thesleff, 1972). By comparison, extraordinary persistence of neuromuscular junctions is seen after nerve sections in the invertebrates, best illustrated by studies of crayfish neuromuscular junctions. Functional transmission may be recorded up to a year after nerve section, and the effect of nerve stump length is to delay degeneration by 7–20 days per cm of distal nerve (Atwood et al., 1973; Chiang and Govind, 1984). The persistent neuromuscular junctions in crayfish are maintained by influences from the surrounding glial cells (Bittner, 1991), but this is unlikely to be a factor in the slow degeneration of terminals in WldS muscles (Perry et al., 1990a; Brown et al., 1991b; Buckmaster et al., 1994; see below).

**Degeneration of terminals is strongly temperature-dependent.** For example, Albuquerque et al. (1978) showed that in hibernating ground squirrels—whose body temperature sinks to ~5°C—motor nerve terminals remain intact and functional in hindlimb muscles up to 30 days after sciatic nerve section. Similarly, Cull-Candy et al. (1982) showed that degeneration of human neuromuscular junctions in organ-cultured preparations of intercostal muscle biopsies occurs more slowly at lower temperatures. They found that in cultures maintained at 23°C, neuromuscular transmission persisted for up to 8 days, whereas transmission deteriorated rapidly in cultured muscles maintained at 36°C. Similar results have been obtained with non-mamma-

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**TABLE 1. Evidence for progressive deterioration of functional transmission in WldS FDB muscles after tibial nerve section**

<table>
<thead>
<tr>
<th>Days after nerve section</th>
<th>% unresponsive</th>
<th>% MEPPs only (&gt;0.1 Hz)</th>
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<tbody>
<tr>
<td>0 (n = 6 muscles; 115 fibres)</td>
<td>0</td>
<td>2.6</td>
</tr>
<tr>
<td>3–5 (n = 6; 115)</td>
<td>10.4</td>
<td>7.8</td>
</tr>
<tr>
<td>6–7 (n = 8; 161)</td>
<td>41.0</td>
<td>20.5</td>
</tr>
<tr>
<td>11–13 (n = 3; 61)</td>
<td>75.0</td>
<td>11.5</td>
</tr>
</tbody>
</table>

The table shows the percentage of the total fibres impaled with either no evoked or spontaneous synaptic activity (% unresponsive) and those showing no evoked responses but displaying spontaneous miniature EPPs at frequencies greater or equal to one event every 10 s (% MEPPs only).

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**Fig. 5. Time course of slow degeneration of functional innervation of WldS muscles and its dependence on nerve stump length.** (A) Data from SOL muscles after section of the sciatic nerve at the mid-thigh level. (B) Comparison of time course of degeneration in SOL muscle after section of the sciatic nerve at the sciatic notch (long stump) and SOL muscle nerve (short stump). (C) Data from intracellular recordings from FDB muscles after section of the tibial nerve, leaving a nerve stump similar in length to A. Each point is from one muscle. Some of the points superimpose. (D) Incidence of functionally innervated fibres in FDB muscles, 7 days after section of the sciatic nerve at the mid-thigh level, compared with tibial nerve section. Data in A, B and D are mean and SEM, based on 3–9 muscles for each point (except the 6 days data in A and B, which are from one animal). There were significantly more transmitting neuromuscular junctions 7 days after sciatic nerve section than after tibial nerve section (D; P < 0.05; t-test). The curve in C was a cubic spline interpolation drawn through the estimated means of the data points.
lian vertebrates. In frogs kept at 23°C terminals persist for ~1 week after nerve section, and much longer if the animals are kept in a cool environment (Langley, 1909; Birks et al., 1960). After 6 days of culture at 14°C, ~30% of fibres still responded to nerve stimulation and showed spontaneous MEPPs (Cherki-Vakil and Meiri, 1990). The same study showed that the frequency of MEPPs inreased and the quantal content of EPPs declined as the terminals slowly degenerated. Some of the slowly degenerating junctions we recorded from in the present study also showed evidence of low quantal contents (data not shown). Cherki-Vakil and Meiri (1990) also noted that during the final stages of degeneration MEPPs could be recorded from some fibres but no evoked responses could be elicited (cf. Table 1). A progressive, 2- to 7-fold decrease in quantal content was also described by Chiang and Govind (1984) in their study of slowly degenerating axons in crayfish muscle. By contrast, the frequency of MEPPs and the quantal content of EPPs increase in muscles that are merely paralysed rather than denervated (Tsujimoto et al., 1990; Plomp et al., 1994).

Thus our present findings suggest that the rate of degeneration of terminals and some of the physiological characteristics of degenerating motor terminals in Wld<sup>d</sup> mice are similar to those in muscles kept at a much lower ambient temperature than normal, or those in naturally ectothermic animals. Our data also suggest that the mechanisms of synaptic vesicle recycling may be quite durable in Wld<sup>d</sup> mice after nerve section. It would be interesting to know whether enhancing the rate of recycling, by chronic stimulation of the axotomized distal nerve stumps, would further accelerate the degeneration of terminals in Wld<sup>d</sup> mice, as reported previously under acute or semi-acute conditions in mammalian and amphibian muscles (Jones and Kwanbunbumpen, 1970; Ceccarelli et al., 1972, 1973; Lynch, 1982).

**The mechanism of nerve terminal degeneration: cytoplastic apoptosis?**

The nature of the trigger for motor nerve terminal degeneration after nerve section is unknown. Two hypotheses are first, that axotomy deprives terminals of essential trophic resources that are normally synthesized in the cell body and transported by fast axonal transport to the terminals (Perisic and Cuenod, 1972; Hudson et al., 1984); and second, that an active signal is generated at the site of injury, and this is propagated into the terminals at a rate that is similar to that of fast axonal transport (Miledi and Slater, 1970).

**Hypothesis 1**

The plausibility of the first hypothesis arises from earlier observations that focal treatment of peripheral nerves with agents that block axoplasmic transport (such as high concentrations of colchicine or low concentrations of batrachotoxin) mimic the effects of nerve section by producing depression of synaptic transmission and nerve terminal degeneration, but without producing any overt signs of axolemmal damage (Albuquerque et al., 1972; Perisic and Cuenod, 1972; Hudson et al., 1984). However, the present findings suggest to us that the validity of this hypothesis is now in some doubt. If terminals depended critically on factors supplied by the cell body, then we should have to assume that in Wld<sup>d</sup> mice the motor terminals had developed some special independence of such factors; that unlike axons and terminals in normal animals, axons in Wld<sup>d</sup> mice carry out protein synthesis; or that the nutritive functions of the cell body are supplanted in Wld<sup>d</sup> mice (but not in normal mice) by supporting cells in the vicinity of the motor terminals. The studies of axons in Wld<sup>d</sup> mice carried out to date have not revealed any significant or distinctive ultrastructural characteristic, such as the presence of ribosomes; and the rate of axoplasmic transport appears to be normal (Smith and Bishy, 1993). The relevant data also suggest that the slow rate of axon degeneration in Wld<sup>d</sup> mice is an intrinsic property of the axons rather than an indirect consequence of some unusual property of supporting glial cells or the myelomonoctytic cells which normally mediate nerve degeneration (Perry and Brown, 1992). For instance, transplanting bone-marrow from normal mice into irradiated Wld<sup>d</sup> mice does not lead to an accelerated Wallerian degeneration after nerve section. Conversely, bone-marrow transplantation from Wld<sup>d</sup> mice into irradiated wild-type mice does not lead to slow Wallerian degeneration after nerve section (Perry et al., 1990a). When neurites growing out from explants of Wld<sup>d</sup> mouse sensory ganglia maintained in culture are severed, the distal neurites degenerate as slowly as they do in vivo, in contrast to the rapid degeneration of axotomized neurites from wild-type explant cultures (Buckmaster et al., 1994). Furthermore, when growth factor is withdrawn from cultures of dissociated Wld<sup>d</sup> neurons the cell bodies degenerate, but the neurites persist for several days (Deckwerth and Johnson, 1994).

**Hypothesis 2**

The second hypothesis, that an active signal for degeneration is propagated into nerve terminals, seems the more attractive of the two. To accommodate our present findings, we need only presume that in Wld<sup>d</sup> mice either the nature of the signal and/or the sensitivity of the degenerative mechanisms in the terminals are somehow defective, and that there are age-dependent changes which restore the signal or the sensitivity of the degenerative mechanisms in the Wld<sup>d</sup> terminals. The gene which is affected in Wld<sup>d</sup> is a single autosomal dominant allele on chromosome 4 (Perry et al., 1990b; Lyon et al., 1993), but the gene product and its precise function have yet to be determined. It seems plausible that one of the enzymes responsible for degradation of principal components of the cytoplasm may be defective. In support of this, Glass et al. (1994) have shown that the Ca<sup>2+</sup> affinity of a Ca-activated neutral protease involved in neurofilament degradation is reduced in Wld<sup>d</sup> mice. However, the rate of neurofilament degradation by Ca-activated proteases is normal under some circumstances (Tsao et al., 1994).

If the degeneration of nerve terminals is an active process, then this implies that the cellular mechanisms required to bring it about are normally present in nerve terminals, but in a latent or repressed state. Thus nerve terminal degeneration may represent a form of programmed cell death, perhaps similar to the degeneration which is triggered by factors in the environment of many types of neurons during normal development, or following deprivation or injury in adults (see also Deckwerth and Johnson, 1994; Lapper et al., 1994). Such 'apoptosis' (Wyllie et al., 1980) normally requires expression of genes that are suppressed in the neuron (Martin et al., 1988; Johnson and Deckwerth, 1993). However, it has recently been shown that anuclear cell fragments (cytoplasts) prepared from cell lines will undergo apoptosis in culture when the cytoplasts are treated with the potent protein kinase inhibitor staurosporine, or by withdrawing essential growth factors from the culture medium (Jacobson et al., 1994). Protein synthesis is not required for the induction of apoptosis in these cytoplasts. Wallerian degeneration of amputated axons or neurites could result from a similar process.

Jacobson et al. (1994) also showed that degeneration of cytoplasts is inhibited if they are made to over-express the bcl-2 gene product. This gene product mitigates cell death in many different types of neurons. For example, over-expression of bcl-2 prevents cell death when neurotrophins are withdrawn from cultures of cranial sensory neurons (Allsop et al., 1993). Bcl-2 and its essential homologue bax...
(bcl-x) are also differentially regulated in the sensory neurons after axotomy of dorsal root ganglia in adults (Boise et al., 1994; Gillardon et al., 1994; Yin et al., 1994). Recently it has also been shown that motoneuron death in vivo is mitigated by the over-expression of bcl-2 in transgenic mice (Dubois-Dauphin et al., 1994; Martinou et al., 1994). The bcl-2 gene, which may protect cells from the harmful effects of free radicals generated by cell metabolism (Hockenbery et al., 1993), is also of potential interest in the context of Wallerian degeneration because it has been reported that the Wld\(^+\) gene product regulates Ca\(^{2+}\) release from intracellular stores (Lam et al., 1994). Ca\(^{2+}\) fluxes across cell membranes increase at the sites of nerve injury, and activation of Ca-sensitive proteases is probably necessary for Wallerian degeneration to occur (Shlaepfer, 1974; Kamakura et al., 1983; Glass et al., 1994).

Although it is unlikely that the bcl-2 gene has a direct role in the slow degeneration of axons in Wld\(^+\) mice (bcl-2 is located on mouse chromosome 1 (Mook et al., 1990)), it would be useful to know whether over-expression of the gene product, or others implicated in control of programmed cell death (Nunez and Clarke, 1994; Reed, 1994), would also mitigate Wallerian degeneration in normal mice; and conversely whether treatment with protein kinase inhibitors, or enhancing production of free radicals, would acutely accelerate Wallerian degeneration of terminals in isolated or organ-cultured preparations from Wld\(^+\) mice. Such data might help to establish whether the rapid deterioration of motor nerve terminals that is a normal consequence of nerve injury, represents another example of `cytoplastic apoptosis` (cf. Jacobson et al., 1994).

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Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>EPP</td>
<td>end-plate potential</td>
</tr>
<tr>
<td>FDB</td>
<td>flexor digitorum brevis</td>
</tr>
<tr>
<td>MEPP</td>
<td>miniature end-plate potential</td>
</tr>
<tr>
<td>SOL</td>
<td>soleus</td>
</tr>
<tr>
<td>TS</td>
<td>triangularis sterni</td>
</tr>
<tr>
<td>Wld(^+)</td>
<td>C57BL/BLD(^+) mouse strain</td>
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<tr>
<td>ZIO</td>
<td>zinc iodide-oximide tetroxide</td>
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References


Neuromuscular junctions in Wld\(^+\) mice 1649
and hemicholinium on synaptic vesicles at the neuromuscular junction. 


