Elimination of Motor Nerve Terminals in Neonatal Mice Expressing a Gene for Slow Wallerian Degeneration (C57Bl/Wld\(^s\))

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Abstract
Degeneration of motor terminals after nerve section occurs much more slowly than normal in young adult mice of the C57Bl/Wld\(^s\) strain. This observation prompted us to re-examine the possible role of degeneration and intrinsic axon withdrawal during neonatal synapse elimination. Polynuclear innervation was assayed by two methods: intracellular recording of end-plate potentials in cut-muscle fibre preparations of isolated hemidiaphragm and soleus muscles; and in silver-stained preparations of triangularis sterni and transversus abdominis muscle fibres. No differences in the rate of synapse elimination were detected in unoperated Wld\(^s\) compared with CBA, C3H/HE and BALB/c mice. At 3 days of age, >80% of fibres were polynuclearly innervated. By 7 days this declined to ~20% of hemidiaphragm, 50% of triangularis sterni and 60% of soleus fibres. Nearly all fibres were mononeuronally innervated by 15 days. The mean number of terminals per triangularis sterni muscle fibre 7 days after birth was 1.55 ± 0.07 in Wld\(^s\) and 1.56 ± 0.09 in wild-type mice. Three to 4 days after sciatic nerve section, near-normal numbers of motor units were evident in isometric tension recordings of the soleus muscle, and intracellular recordings revealed many polynuclearly innervated fibres. Mononeuronally and polynuclearly innervated fibres were also observed in silver-stained preparations of soleus and transversus abdominis muscles made 3–4 days after sciatic or intercostal nerve section. We conclude (i) that the Wld\(^s\) gene has no direct impact on the normal rate of postnatal synapse elimination, (ii) that Wallerian degeneration and synapse elimination must occur by distinct and different mechanisms, and (iii) that muscle fibres are able to sustain polynuclear synaptic inputs even after motor axons have become disconnected from their cell bodies.

Introduction
Adult mammalian skeletal muscles have a characteristic pattern of innervation: each fibre is innervated at a single motor end-plate by a collateral branch of just one motoneuron. This pattern emerges during development from one in which each motor end-plate is convergently innervated by collaterals of several motoneurons (for review see Ribchester and Barry, 1994). In rodents, most of the elimination of this polynuclear innervation occurs postnatally over a period of 2–3 weeks (Redfern, 1970; Brown et al., 1976; Betz et al., 1979; Fladby, 1987). The mechanisms which select the surviving axon collateral and terminal are unknown, but hypotheses include a role for endogenous proteases (Vrbova et al., 1988), competition for limited neurotrophic resources (Purves, 1988), and competition for endogenous proteases (Vrbova et al., 1987; Thompson et al., 1983). Competition may also be based on selective chemical matching of motoneuron and muscle fibre types (Jones et al., 1987; Thompson et al., 1987; Fladby and Jansen, 1990; Gates and Ridge, 1992).

One question which has not been satisfactorily resolved is whether nerve terminals undergoing synapse elimination regress as a result of a process akin to 'degeneration' of terminals, like that seen after nerve injury (Miledi and Slater, 1970; Winlow and Usherwood, 1975; Rosenthal and Tareskevich, 1977). Observations based on quantitative electron microscopy appear to refute this, suggesting that synapse elimination and degeneration are unrelated phenomena (Korneliusen and Jansen, 1976; Riley, 1977; Bixby, 1981). On the other hand, Brown et al. (1976) suggested that some motor axon collaterals undergo a programmed 'intrinsic withdrawal', retracting their terminals and regressing into the parent axon independently of the presence of their competitors, even though this may lead to denervation of fibres (Thompson and Jansen, 1977; Fladby and Jansen, 1987). Although intrinsic withdrawal may not occur in all muscles (Betz et al., 1980), its relationship to nerve 'degeneration' remains unclear. The possibility that Wallerian degeneration is itself a programmed cellular event (Lapper et al., 1994; Ribchester et al., 1995) suggested...
that it was timely to re-examine possible links between programmed nerve degeneration and synapse elimination.

The C57Bl/Wld mouse (formerly known as C57Bl/6Ola) has an autosomal dominant mutation on chromosome 4 which renders distal nerve stumps relatively resistant to Wallerian degeneration after nerve injury (Brown et al., 1991; Lyon et al., 1993; Tsao et al., 1994). One of the earliest signs of axon degeneration in normal mice is loss of motor nerve terminals, usually within 12–24 h (Miledi and Slater, 1970; Winlow and Usherwood, 1975). But in young adult Wld mice motor axons and nerve terminals persist for 3–14 days after nerve injury (Crawford et al., 1995; Ribchester et al., 1995). We reasoned that if synapse elimination takes place by a process related to nerve degeneration, we should expect synapse elimination to occur at a slower rate than normal in the mutant mice. Our results suggest, however, that synapse elimination occurs at a normal rate in neonatal Wld mice, even though the mutant phenotype could be induced during this period. Our findings therefore support the hypothesis that the molecular mechanisms responsible for withdrawal of terminals and axons during synapse elimination are distinct from the mechanisms responsible for Wallerian degeneration. The data also suggest that motor terminals have some autonomy from their parent cell bodies, and that the rate and outcome of neuromuscular synapse elimination is mainly determined by factors in the local environment of the competing terminals.

Preliminary results have been published in abstract form (Mackintosh and Ribchester, 1995; Parson et al., 1995).

Materials and methods

Experiments were carried out using mice drawn from a breeding colony established many years ago in Edinburgh from stock originally obtained from the Harlan–Olac Laboratories (Bicester, UK). The Edinburgh line is formally designated C57Bl/6/EUMM. Independent experiments have established that adults of this strain have slow Wallerian degeneration after peripheral nerve section, similar to that described for the Wld mouse by M. C. Brown and colleagues (Lunn et al., 1989; Ribchester et al., 1995), so we refer to the Edinburgh colony of mice as Wld throughout the present paper. As controls, three strains with normal rates of Wallerian degeneration were studied: BALB/c, CBA and C3HA-E. Mice were killed by stunning and cervical dislocation of cervical vertebrae. Hemidiaphragm, soleus, triangularis sterni and transversus abdominis nerve/muscle preparations were obtained from the Harlan–Olac Laboratories (Bicester, UK). The experiments have established that adults of this strain have slow Wallerian degeneration after peripheral nerve section, similar to that described for the Wld mouse by M. C. Brown and colleagues (Lunn et al., 1989; Ribchester et al., 1995), so we refer to the Edinburgh colony of mice as Wld throughout the present paper. As controls, three strains with normal rates of Wallerian degeneration were studied: BALB/c, CBA and C3HA-E. Mice were killed by stunning and cervical dislocation of cervical vertebrae. Hemidiaphragm, soleus, triangularis sterni and transversus abdominis nerve/muscle preparations were dissected and placed in mammalian saline of the following composition (mM): Na+, 137; K+, 5; Ca2+, 2; Mg2+, 1; Cl–, 122.2, HCO3–, 23.8; H2PO42–, 2; d-glucose, 5; pH 7.2–7.4. Solutions were bubbled to equilibration with 95% O2/5% CO2.

Electrophysiology

Polynuclear innervation was assessed by intracellular recording from cut-muscle fibre preparations of the soleus and hemidiaphragm muscles. Carefully graded stimuli were applied to the muscle nerves using fine-tipped suction electrodes. Mononeuronally innervated fibres were scored as those giving a unitary end-plate potential on graded stimulation. Polynuclearly innervated fibres were indicated by systematic changes in the amplitude of their end-plate potentials in response to graded stimulation (Fig. 1). We did not attempt to score the number of synaptic inputs to each polynuclearly innervated muscle fibre using this method, owing to the difficulty of distinguishing multiple inputs from quantal fluctuations of synaptic potentials (but see Morphology, below). In addition, recordings from muscle fibres in which the responses to graded stimulation were equivocal were not included in the analysis of the results; that is, we excluded those fibres in which the size of random, quantal fluctuations in end-plate potential amplitude made it too difficult to decide whether the fibre was polynuclearly or mononeuronally innervated. At least 20 unambiguous muscle fibres were recorded in each case. Unresponsive, apparently denervated fibres were included in the tally.

Morphology

Some soleus muscles and nerves were fixed in p-formaldehyde/glutaraldehyde in phosphate buffer, postfixed in osmium tetroxide, dehydrated and embedded in Araldite. Transverse thin sections were cut from which estimates of fibre/axon number and area were made using the 'systematic-random' method of Mayhew (1990). Other muscles were fixed in buffered p-formaldehyde and whole mounts were stained with the combined silver/acytcholinesterase method of Namba et al. (1967), as modified by Hopkins and Slack (1981). Morphological analysis was performed on an Apple Macintosh computer using the public domain NIH Image program, developed at the US National Institutes of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov.

Denervations

Unilateral sciatic nerve sections at mid-thigh level were carried out in mice aged 3–4 days. The mice were sedated with halothane then chilled on ice until respiratory movements and vital signs had ceased. After surgery, the mice were gradually warmed up under a lamp. Respiration and other vital signs returned within a few minutes and the mice were then returned to their mothers and littermates. This method of anaesthesia (for which UK Home Office authorization was obtained) was very effective and it did not affect the subsequent feeding behaviour or growth of the mice after recovery. The mice were killed 3–7 days later. Before the animals were killed, the foot on the operated side was tested for withdrawal reflexes by pinching with forceps; they proved unresponsive in every case. The mice always withdrew the unoperated, contralateral limb on pinching. After the animals had been killed, the level of section of the sciatic nerve was confirmed by relocating the site of injury in the thigh. In some mice the intercostal nerves were sectioned on one side instead, axotomizing the motoneurons innervating the triangularis sterni and transversus abdominis muscles.
Isometric tension recordings were made from isolated soleus muscles in three of the operated animals. Motor unit contractions were evoked by carefully grading stimuli to the nerves. This method gave a lower bound estimate of the number of motor units in the neonatal muscles; accurate estimates could not be made because of non-linear summation of motor unit tensions (due to polyneuronal innervation) and the difficulty of resolving all the motor units on the basis of their threshold responses to electrical stimulation.

Statistical data are given as mean ± SEM throughout unless stated otherwise.

Results

Time course of synapse elimination in unoperated Wld\(^\text{a}\) muscles

Electrophysiology

Intracellular recordings from neonatal Wld\(^\text{a}\) muscles are shown in Figure 1. Graded stimulation applied to the muscle nerve revealed many muscle fibres to be polyneuronally innervated, as in normal mice. The time course of synapse elimination in hemidiaphragm muscles comparing Wld\(^\text{a}\) and CBA mice is shown in Figure 2A, and data from Wld\(^\text{a}\) and C3H/HE mouse soleus muscles are shown in Figure 2B. In neither case was there any evidence that synapse elimination occurred more rapidly or more slowly in Wld\(^\text{a}\) muscles compared with the wild-type mice. About half the muscle fibres were mononeuronally innervated by ~6 days in hemidiaphragm muscles and by ~7 days in soleus muscles.

Morphology

The physiological findings were supported by silver/cholinesterase-stained preparations of triangularis sterni muscles from 3- to 16-day-old Wld\(^\text{a}\) and BALB/c mice. Some fibres were clearly mononeuronally innervated and some were equally clearly polyneuronally innervated in these preparations (Fig. 3A, B). Occasionally, evidence of retraction of axons was seen, in the form of 'retraction bulbs', similar to those described by Riley (1977). Significant numbers of Wld\(^\text{a}\) fibres were already mononeuronally innervated 3 days after birth (24.5 ± 4.7%; four muscles, 200 fibres). Figures for the BALB/c control were: 12% mononeuronal and 82 ± 6.9% (four muscles, 200 fibres) polyneuronal at the same ages. The number of inputs to the remaining 6-7% of fibres could not be resolved with certainty. By 7 days approximately half of all fibres were polyneuronally innervated: 47.5 ± 2.5% (four muscles, 200 fibres) of Wld\(^\text{a}\) fibres and 49.0 ± 1.4% (two muscles, 100 fibres) of BALB/c fibres. At this age, the mean number of terminals per muscle fibre was 1.55 ± 0.07 in Wld\(^\text{a}\) and 1.56 ± 0.09 in BALB/c mice (two muscles, 100 fibres in both cases). By 16 days after birth almost all fibres were mononeuronally innervated (~96% of Wld\(^\text{a}\) fibres and 94.5% BALB/c fibres), while the mean number of terminals present per muscle fibre was 1.04 in Wld\(^\text{a}\) and 1.07 in BALB/c (Fig. 4).

Response to nerve injury

We carried out unilateral sections of the sciatic nerve in anaesthetized mice aged 3–4 days in order to test the possibility that the normal rate of synapse elimination in these mice might be due to failure of expression of the Wld\(^\text{a}\) gene perinatally. The outcome of most operations was assessed 3–4 days later (i.e. 6–7 days after birth). In two mice from a control strain (C3H/HE; wild-type with respect to Wallerian degeneration) there was no evidence of any residual innervation after this period of denervation. The distal nerve stumps were translucent in appearance and the muscles did not contract in response either to cutting or to electrical stimulation of the nerve in vitro.

The appearance of nerves and function of muscles in Wld\(^\text{a}\) mice 3–4 days after cutting the sciatic nerve were quite different. As in wild-type mice, the hindlimb on the operated side—particularly the foot—was smaller, more poorly developed and unresponsive to pinching. After killing and dissection, the soleus muscles were overtly pale and atrophic compared with the contralateral, unoperated muscle. But the characteristic banding pattern of intact peripheral nerves—visible through the dissecting microscope—was still apparent, as in controls. Only one of the axotomized distal stumps gave adequate cross-sections for counting axons: this contained 25 myelinated axons, about half the number counted in three contralateral controls (55 ± 5). Transverse sections of the denervated soleus muscles showed they contained similar numbers of muscle fibres, but the cross-sectional area of muscles on the operated side was ~20% less than on the contralateral side (Table 1).

Tension recordings

Stimulation of the distal stump on the operated side evoked strong twitch contractions in the soleus muscles. These muscles also showed a considerable amount of visible, spontaneous contractile activity. By 7 days after nerve section, only weak contractions could be evoked by stimulating the soleus muscle nerve on the operated side. Intracellular recordings revealed only one out of 80 fibres in four muscles giving end-plate potentials. Most measurements were therefore made on mice 3–4 days after neonatal nerve section.

Isometric tension recordings were made from three pairs of soleus muscles. There appeared to be similar numbers of motor units in all these muscles (Fig. 5), but the unreliability of graded stimulation and the degree of twitch tension overlap precluded using this method to reliably estimate the total number of motor units. The time course of the twitch contractions was longer on the operated side, and in two of the three cases the tension was weaker on this side. In the third pair of muscles, surprisingly, soleus twitch tension on the operated side was larger, in spite of its atrophic appearance, compared with the contralateral muscle. In all cases, the direct tension evoked by applying the suction electrode to the surface of the muscle was similar to the contraction...
Synapse elimination in *Wld* mouse muscle

**Intracellular recording**

Intracellular recordings were made from six pairs of muscles. Many of the fibres showed evidence of polynervous innervation (Fig. 5). Data showing the proportions of mononeuronally innervated fibres, polynervously innervated and denervated fibres are given in Figure 6. Also shown are data from five muscles studied 3–4 days after birth, the time when nerve sections were carried out.

The data show that 3–4 days after birth ~80% of muscle fibres were polynervously innervated (compare with Fig. 2). By 6–7 days ~50% of contralateral control soleus muscle fibres were mononeuronally innervated. By contrast, on the operated side ~50% of fibres that failed to respond to nerve stimulation. Of the remaining 50% of fibres that did respond, the majority expressed compound end-plate potentials upon graded nerve stimulation, indicating that they were polynervously innervated. In the light of the tension measurements the proportion of denervated muscle fibres is perhaps surprising, but it was not possible in these experiments to determine whether some of the motor axons failed to respond to stimulation (either due to high stimulus thresholds or to failure of synaptic transmission, which sometimes happens in cut-muscle fibre preparations).

**Silver/cholinesterase staining**

Whole-mount, silver-stained preparations of soleus and transversus abdominis muscles broadly confirmed the electrophysiological data.
Synapse elimination in Wld<sup>d</sup> mouse muscle

In Wld<sup>d</sup> mice 3–4 days after nerve section there were many polyn neur-ally innervated muscle fibres, with multiple axons converging onto single motor end-plates. The end-plates on the axotomized side had a more immature, ‘blebbly’ appearance compared with the unoperated contralateral controls, however (Fig. 3C, D; compare for example with Balice-Gordon et al., 1993), as if nerve section had arrested maturation of the end-plates. Their appearance was similar to that of terminals in paralysed muscles of neonatal mice injected with botulinum toxin (Brown et al., 1982).

We did not attempt to quantify the numbers of denervated and innervated muscle fibres in these preparations, due to the small size and overlapping appearance of the muscle fibres in the light microscope.

Discussion

The main findings of the present study are (i) that neonatal synapse elimination occurs normally in mice with a genetic defect that slows nerve degeneration after axotomy, and (ii) that neonatal nerve section in these mice initially preserves the polyn neuronal innervation of many of the muscle fibres.

Time course of synapse elimination in mouse skeletal muscle

Previous studies have recorded the time course of synapse elimination in mouse muscles in terms of the decline in motor unit size based on tension measurements, or using imaging methods (Fladby, 1987; Fladby and Jansen, 1987; Balice-Gordon and Lichtman, 1993; Balice-Gordon et al., 1993). Our data confirm that synapse elimination in mouse muscles occurs somewhat earlier than in neonatal rats. In the rat soleus muscle, for example, all muscle fibres are polyn neurally innervated up to ~10 days after birth (Brown et al., 1976), whereas our present data show that only ~20% of muscle fibres remain polyn neurally innervated at this age in mice (Fig. 2B). We saw a few mononeuronally innervated fibres as early as 3 days after birth. The rate of emergence of mononeuronally innervated fibres appears to be similar in the mouse and rat, however. Thus the present data show polyn neuronal innervation declining from 80 to ~10% of fibres over the course of 5–7 days, similar to the time course typically seen in rat and rabbit muscles (Brown et al., 1976; Rosenthal and Tareskevich, 1977; Betz et al., 1979; Soha et al., 1987).

Relationship between synapse elimination and Wallerian degeneration

If synapse elimination and Wallerian degeneration were related—that is, if both processes employed common cellular mechanisms—we might have expected the time of onset or the rate of neuromuscular synapse elimination to be measurably slower in Wld<sup>d</sup> mice than normal. We saw no evidence of this. For instance, after nerve section all terminals degenerated within 24–48 h in wild type mice, whereas ~50% of fibres were still innervated 72–96 h after nerve section in Wld<sup>d</sup> mice. We argue that this difference is big enough that we should have detected a slowing of synapse elimination in Wld<sup>d</sup> mice if the degenerative gene played any role in this process. Our data therefore support earlier suggestions, based on electron microscopic descriptions of immature nerve terminals, that synapse elimination and Wallerian degeneration are independent and distinct cellular phenomena.
equivalent in their cytochemical characteristics, so that collaterals (or motoneuron inputs until 50% of the muscle fibres were denervated, three-and-a-half-fold, this generated an average number of about four motoneurons) showing similar vulnerability to nerve injury tend to retain a polyneuronal innervation whereas, experimentally, we observed ~80% of innervated fibres retaining polyneuronal innervation whereas, experimentally, we observed experimentally. For example, when we simulated a mononeuronally innervated fibres in axotomized Wld^e mice was more resistant to chemical injury than terminals in wild-type mice, for example by treating them with staurosporine, which can trigger programmed cell degeneration even in the absence of cell nuclei (Jacobson et al., 1994).

Perspective of polyneuronal innervation

Our tension data suggest that significant numbers of motor units remained after nerve section, whereas the intracellular data suggest that ~50% of the muscle fibres became completely denervated within 3 days of nerve section. The ratio of polyneuronally innervated to mononeuronally innervated fibres in axotomized Wld^e mice was similar to that in muscle at the time of the operation, however: i.e. ~80% of the innervated fibres were polyneuronally innervated whereas only ~50% of contralateral muscle fibres were polyneuronally innervated. A plausible explanation for this derives from the known effects of muscle inactivity on synapse elimination. Polyneuronal innervation is preserved in paralysed muscles (Thompson et al., 1979; Taxt, 1983; Barry and Ribchester, 1995). In the Wld^e mice, nerve section may have produced an effect similar to paralysis in a wild-type animal because the terminals attached to distal stump axons, although intact, were of course incapable of mediating activity from the central nervous system.

Computer simulations (not shown), based on initially random innervation patterns with random removal of motoneuronal connections, confirmed intuition by failing to generate any pattern resembling that observed experimentally. For example, when we simulated a random initial innervation pattern of the soleus muscle by 17 motoneurons (Fladby, 1987), each with an average expansion of three-and-a-half-fold, this generated an average number of about four motoneuron inputs per muscle fibre. When we then randomly removed motoneuron inputs until 50% of the muscle fibres were denervated, only ~5-10% of the remaining innervated muscle fibres were predicted to retain a polyneuronal innervation whereas, experimentally, we observed ~80% of innervated fibres retaining polyneuronal innervation in the axotomized Wld^e soleus muscles. At present we are therefore left with two appealing alternative explanations for the coexistence of completely denervated and polyneuronally innervated muscle fibres 3 days after neonatal nerve section: either all the collateral branches of a motoneuron are equivalent in their cytochemical characteristics, so that collaterals (or motoneurons) showing similar vulnerability to nerve injury tend to innervate the same muscle fibres and degenerate after injury at the same rate; or perhaps after nerve injury some sort of retrograde signal feeds back onto the motor terminals innervating the fibre and triggers their synchronous rejection. Indirect support for a role of the muscle fibre in synapse elimination is provided by observations that it takes place after changes in the organization of postsynaptic molecules (Rich and Lichtman, 1989; Balice-Gordon and Lichtman, 1994), and that the release of neurotransmitter onto immature muscle cells in culture leads to inhibition of transmitter release by other synapses on the same cells, probably via a muscle cell-derived intermediate (Dan and Poo, 1995). The possible nature of the retrograde signal is obscure, but it could arise from a decline in the level of a target-derived neurotrophic factor (Lohof et al., 1995), from products of membrane breakdown such as arachidonic acid or its derivatives (Harish and Poo, 1992), or from the production of free-radicals such as nitric oxide (Wang et al., 1995).

The source of signals which trigger synapse elimination and axon withdrawal need not necessarily be the muscle fibre, however. For example, Thompson and colleagues have shown that terminal Schwann cells regulate sprouting and regeneration of motor axons after nerve injury in adults, and that the survival of Schwann cells is obligatory for axon regeneration after nerve section in neonates (Son and Thompson, 1995a,b; Trachtenberg and Thompson, 1996; Son et al., 1996). More data are required before any firm conclusions can be made about the primacy of motor nerve terminals, muscle fibres or the terminal Schwann cells in the mechanism of synapse elimination. The slow rate of degeneration of motor nerve terminals in Wld^e muscles suggests that such studies might fruitfully be conducted in vitro, for example by organ culture of suitable neuromuscular preparations.

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