Delayed synaptic degeneration in the CNS of Wld<sup>s</sup> mice after cortical lesion


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Therapies that might delay degeneration of synapses offer an appealing strategy for treatment of neurodegenerative diseases, including Alzheimer’s disease and related dementias, prion diseases, schizophrenia and amyotrophic lateral sclerosis. Analysis of mouse mutants provides one possible avenue towards identifying relevant mechanisms. Here, we used quantitative and serial section electron microscopy to find out whether the onset and time course of pre-synaptic nerve terminal degeneration is delayed in the striatum of Wallerian degeneration slow (Wld<sup>s</sup>) mutant mice. Synaptic degeneration was observed within 48 h of cortical ablation in wild-type mice but was delayed by approximately 1 week in Wld<sup>s</sup> mice. However, the morphological characteristics of degenerating nerve terminals in wild-type and Wld<sup>s</sup> mice were indistinguishable, in contrast to the differences reported previously in studies of the PNS. Surprisingly, the delayed onset of synaptic degeneration was accompanied by an increased incidence of complex synaptic morphologies on post-synaptic spines in the denervated Wld<sup>S</sup> striatum indicating an enhanced plastic response at both injured and uninjured synapses. The data suggest that targeting Wallerian-like mechanisms of synaptic degeneration could lead to the development of new therapies for the treatment of CNS disorders where synapse loss is a primary feature.

Keywords: Synapse; neurodegeneration; neuroprotection; brain; Alzheimer’s disease; electron microscopy

Abbreviations: GFAP = glial fibrillary acidic protein; LCP = large complex/perforated; Wld<sup>s</sup> = Wallerian degeneration slow

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Introduction

Synapses appear to be primary pathological targets in Alzheimer’s disease and related dementias (Scheff and Price, 1993; Zhou et al., 1998; Selkoe, 2002; Scheff and Price, 2003), with synapse loss being the major correlate of cognitive impairment (Terry et al., 1991). There are currently two main strategies directed towards the goal of effective therapy for such diseases: delaying synaptic degeneration (Gillingwater and Ribchester, 2003; Coleman et al., 2004) or promoting synaptic repair (Schnell et al., 1994; Li et al., 1997). The present study is concerned with the first of these two approaches.

Synapse loss in neurodegenerative disease is thought to occur predominantly in otherwise viable neurons (Coleman et al., 2004), suggesting that degeneration within synaptic compartments is regulated differently from degeneration of axons and cell bodies (Gillingwater and Ribchester, 2001, 2003). This hypothesis is supported by studies of conditions such as prion disease (Cunningham et al., 2003), schizophrenia (Harrison, 1999; Frankle et al., 2003) and amyotrophic lateral sclerosis (Sasaki and Iwata, 1996), where synapses are also considered to be the most vulnerable. In addition, alterations in synaptic homeostasis and nerve terminal loss have been reported after traumatic brain injury (Erb and Povlishock, 1991; Sullivan et al., 1998) and following exposure to recreational drugs such as methamphetamine (Cadet et al., 2003). Thus, the mechanisms that delay degeneration in synaptic compartments may be of critical importance for the development of therapeutics to treat a wide variety of neurodegenerative conditions throughout the CNS (Coleman et al., 2004).

The Wallerian degeneration slow (Wld<sup>f</sup>) gene is known to protect axons in both CNS and PNS from injury-,...
neurotoxin- and disease-induced neurodegenerative stimuli (Lunn et al., 1989; Gillingwater and Ribchester, 2001; Wang et al., 2001, 2002). These properties have already been shown to mitigate disease progression in mouse models of transient global cerebral ischaemia (Gillingwater et al., 2004), Parkinson’s disease (Sajadi et al., 2004), motoneuron disease (Ferri et al., 2003; Fischer et al., 2005; but see Velde et al., 2004), gracile axonal dystrophy (Mi et al., 2005) and myelin-related axonopathies (Samsam et al., 2003). The proven ability to deliver the Wld<sup>e</sup> gene to wild-type cells and confer neuroprotection across different species offers the possibility of developing therapeutics for some of these diseases in humans (Wang et al., 2001; Adalbert et al., 2005). Moreover, the lack of any detectable non-neuroprotective related phenotype in mice and rats expressing Wld<sup>e</sup> suggests that global application of Wld<sup>e</sup>-based neuroprotection could potentially rescue degenerating compartments within neurons whilst having no detrimental effect on the surrounding, unaffected neuronal population (Coleman and Ribchester, 2004; Adalbert et al., 2005).

Synapse degeneration in the PNS is delayed by the Wld<sup>e</sup> gene (Ribchester et al., 1995; Gillingwater et al., 2002, 2003), but it is not clear how synapses in the CNS of Wld<sup>e</sup> mice respond to stimuli that normally trigger their degeneration. Axonal protection has been well documented in the CNS of Wld<sup>e</sup> mice (Perry et al., 1991; Ludwin and Bisby, 1992), but anatomical evidence for delayed synaptic degeneration is so far indirect, based on immunocytochemical (Cesa et al., 2005) and behavioural (Sajadi et al., 2004) studies. Here, we directly tested the ability of the Wld<sup>e</sup> gene to delay synaptic degeneration in the CNS, using electron microscopy to quantify the degeneration of synaptic terminals following unilateral cortical lesions. We show that the Wld<sup>e</sup> gene significantly delays nerve terminal degeneration in the striatum following ablation of the ipsilateral cerebral cortex. However, degenerating pre-synaptic nerve terminals in Wld<sup>e</sup> mice showed similar morphological characteristics to those in wild-type mice after a similar cortical lesion. Surprisingly, we also found that the delay in nerve terminal degeneration in Wld<sup>e</sup> mice was accompanied by an increase in the rate of appearance of large, complex synaptic morphologies at intact synapses. Such changes are indicative of an intrinsic plastic response in the striatum following loss of afferent inputs and have previously been implicated in synaptic plasticity after traumatic head injury (McKinney et al., 1997; Santhakumar et al., 2001). Together, these findings suggest that a more complete understanding of the mechanisms of Wld<sup>e</sup>-mediated synaptic protection will open up new avenues for the treatment of neurodegeneration in humans, either following trauma or in Alzheimer’s and other neurodegenerative diseases.

**Material and methods**

**Mice**

Natural mutant C57Bl6/Wld<sup>e</sup> (Wld<sup>e</sup>) mice and C57Bl6 (wild-type) mice aged 2 months were obtained from Harlan Olac Laboratories (Bicester, UK) and housed within the animal care facilities in Edinburgh. Wld<sup>e</sup>-CFP mice, expressing cyan fluorescent protein in subsets of neurons, were bred in Edinburgh as described previously (Gillingwater et al., 2002).

**Wld<sup>e</sup> protein immunocytochemistry**

Mice were killed by cervical dislocation and brains were rapidly removed, immersed in artificial CSF and 100-μm vibratome slices were cut and fixed for 30 min in 4% paraformaldehyde (Sigma). Slices were incubated overnight in blocking solution containing 4% bovine serum albumin (Sigma) and 0.5% Triton X-100 (Sigma) made up in 1× phosphate-buffered saline (PBS). Wld-18 antibody (Samsam et al., 2003; Gillingwater et al., 2004) was applied overnight (1:500 dilution in serum blocking solution), washed and incubated with a TRITC-conjugated anti-rabbit secondary antibody (DAKO; 1:20 dilution in PBS). Slices were finally washed before mounting in Mowiol. Staining was visualized on a laser scanning confocal microscope (BioRad Radiance 2000, Hemel Hempsted, UK) and Z-series were merged using Lasersharp (BioRad) software.

**Cortical lesions**

All operations were performed under licence from the UK Home Office. Mice were anaesthetized by intraperitoneal (i.p.) injection of Ketanest (100 mg/kg) and Rompun (5 mg/kg) before clamping the head in a Kopf stereotaxic frame. The head was shaved with scissors before making an incision through the skin at the midline. Four holes were drilled on the left side of skull; the first just off midline close to bregma, the second in line with the first but at the level of lambda, the third further caudal on the lateral side just above the temporalis muscle and the fourth anterolateral hole in line with the first and third holes. The skull was cut in lines connecting all holes except the most caudal border, and was then reflected. A suction pipette was used to remove all visible cortex down to the level of the corpus callousum, which was clearly discernible down a dissecting microscope (Fig. 1A). Gel foam (Ethicon) was placed into the site of lesion and the skull-flap was placed back. The overlying skin was then sutured and the mouse placed on a heated blanket until it had recovered fully from the anaesthetic. Mice were maintained in standard animal house conditions and were checked daily for any signs of distress or discomfort.

One to 10 days later, anaesthetized mice (i.p. injection of Ketanest (100 mg/kg) and Rompun (5 mg/kg) were killed by perfusion fixation with 0.1 M phosphate buffer containing 4% paraformaldehyde and 2.5% glutaraldehyde, before removing the brain and immersing it in fixative for a further 12 h. Brains were then washed in 0.1 M phosphate buffer before cutting free-floating 70-μm thick coronal sections on a vibratome.

**GFAP immunocytochemistry**

Vibratome sections were collected in 0.05 M PBS, pH 7.4, containing 0.3% Triton-X100 (PBS-TX100). Sections were incubated for 24 h in rabbit anti-GFAP (glial fibrillary acidic protein) (1:2000; DAKO Cytomation, Denmark) followed by 1 h in biotinylated goat anti-rabbit secondary. Sections were then processed using the Vectastain ABC peroxidase method (Vector Laboratories Ltd; as per the manufacturer’s instructions) using DAB (3,3-diaminobenzidine) as chromogen. The sections were mounted on gelatine-coated slides, dried, dehydrated and cover-slipped in distyrene, tricresyl phosphate and xylene (DPX).
Note that expressing cyan fluorescent protein (CFP; Gillingwater et al., 1998) in cortical layer V neurons from a mouse brain that underwent unilateral cortical ablation 8 days previously resulted in strong expression of CFP in cortical neurons, and led to delayed GFAP upregulation in striatal neurons after cortical lesion. (A) Upper line diagrams illustrate dorsal and sagittal views of a typical cortical lesion, leaving the corpus callosum (cc) and striatum (st) intact. The vertical line shows the position from which the lower high power image was taken. The montage shown was produced from a 70 μm vibratome section from a wild-type mouse brain that underwent unilateral cortical ablation 8 days previously. (B) Immunocytochemical stain for Wild1 protein (red) in cortical layer V neurons from a Wild1 mouse also endogenously expressing cyan fluorescent protein (CFP; Gillingwater et al., 2002). Note that Wild1 protein is strongly expressed in the nucleus of most neurons. (C and D) GFAP staining in striatum of wild-type mice (C) and Wild1 mice (D) 4 days after cortical lesion. Extensive GFAP-positive staining of astrocytes was present in wild-type mouse neuropil, but was almost totally absent in Wild1 mice. Scale bars = 1 mm (A), 40 μm (B), 200 μm (C and D).

Electron microscopy of striatal synapses

The region of striatum selected for analysis was between 0.70 and 1.06 mm posterior to bregma, directly under the corpus callosum at the level of the ventral border of the lateral ventricle (Franklin and Paxinos, 1997). Vibratome sections containing this region were post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 45 min. Following dehydration through an ascending series of ethanol solutions and propylene oxide, all sections were embedded on glass slides in Durecupan resin. Regions of striatum (~1 mm × 1 mm) to be used for assessment of synaptic structure were then cut out from a randomly selected section using a scalpel and glued onto a resin block for sectioning. Ultrathin sections (~60 nm) were cut and collected on formvar-coated grids (Agar Scientific, UK), stained with uranyl acetate and lead citrate in an LKB Ultrostainer and then quantitatively assessed in a Philips CM12 transmission electron microscope (TEM). Negatives taken in the microscope were scanned onto an Apple Macintosh G5 computer using an Epson 4870 Photo flat-bed scanner at 600 dpi, and subsequently processed using Adobe Photosho.

For serial section analysis, images from between 5 and 15 consecutive sections through a complete synaptic profile were prepared as described above. For 3D reconstruction and surface rendering, micrograph stacks were transferred to a Unix workstation (Sun Microsystems). A custom programme (Reconstruct) was used to construct 3D-voxel images. The programme ‘MAPaint’ (both programmes written and developed by the MRC Human Genetics Unit, Edinburgh; http://genex.hgu.mrc.ac.uk) was used to reconstruct 3D volumes. These were defined by examining each 2D image and manually delineating the border of each feature. The 3D volumes were then saved to independent files, calibrated and surface-rendered using Imaris software (Bitplane, Zurich, Switzerland).

Synapse quantification

Ultrastructural characteristics were used to classify synapses as either degenerating or large complex/perforated (LCP) synapses. Section assessment was started at the level of the corpus callosum before progressing away from this structure until the edge of the section was reached. The section was then moved across by one screen’s-width before progressing back towards the corpus callosum, and so on. Ultrathin sections from comparable regions of ipsilateral striatum were placed on coded grids and scored in the EM for two periods of 15 min each by two independent observers. Both observers were unaware of the identities of the sections for this analysis and the subsequent unbiased methodology; the codes were broken only after the data analysis was complete. Raw data were collated using Microsoft Excel and statistical tests were performed using GraphPad Prism software.

For unbiased estimation of synaptic density, Wild1 and wild-type mice were compared at 3 days post-lesion using the Disector method (Sterio, 1984; Ingham et al., 1998). Several pairs of serial sections were used in order to collect enough Disectors and these were separated by at least 10 sections to ensure that the same synapses could not be sampled twice. Negatives were imaged as described above and analysed on a PC computer using Adobe Photosho. An unbiased counting frame of known area (72.885 μm²) was constructed as a layer and positioned in the exact same region over the two images of each Disector. This sometimes required rotation of one of the images. Synapses were counted if they occurred in the reference section but not the look-up section. Three categories of synapse as defined in the results were counted: asymmetric tops; degenerating synaptic tops; and large complex asymmetric tops (counted if discontinuity in synaptic specialization was present in reference but not look-up section). The latter category was considered a subdivision of the asymmetric category as all large complex synapses were asymmetric. The numerical densities of each category were estimated per cubic micrometre and the percentage of the total number of asymmetric synapses (degenerating plus normal asymmetric synapses) was calculated for each category.

Results

Analysis of synaptic degeneration in mouse models of CNS neurodegenerative disease is often complicated by
the presence of somatic/dendritic cell death in regions of interest (Raff et al., 2002; Tsai et al., 2004). Quantifying nerve terminal degeneration in the corticostriatal system avoids this problem, as the cell bodies of lesioned neurons (cortical layer V neurons) are not located in the same region as their synaptic terminals (in striatum). This model system therefore allowed us to examine synaptic degeneration in isolation from pathological events in other regions of the neuron.

In order to isolate cell bodies of corticostriatal neurons, and thus denervate the ipsilateral striatum, a large lesion of the left hemisphere of neocortex was performed using aspiration under general anaesthesia (Fig. 1A). All mice recovered fully within an hour of surgery and showed no discernible behavioural deficits following cortical lesion. The extent of the lesion was mapped in each mouse to ensure comparability between animals. Three out of 32 mice with cortical lesions were excluded from quantitative assessment after post-mortem analysis showed that either the lesion had extended beyond the level of the corpus callosum, or that perfusion fixation had failed to fully penetrate the entire brain.

Immunocytochemical staining with an antibody specifically raised against the \textit{Wld} protein confirmed that \textit{Wld} is strongly expressed in the nuclei of cortical neurons that project to the striatum (Fig. 1B; Parent and Hazrati, 1995). Previous studies examining hippocampal neuropil after unilateral aspiration lesions of the entorhinal cortex showed upregulation of GFAP by 2–4 days in wild-type mice, but with a delay of at least 10 days in \textit{Wld} mice (Steward and Trimmer, 1997). GFAP is upregulated in astrocytes in response to nerve injury, and is commonly used as a marker for gross neuronal degeneration (Burbach et al., 2004). We observed a similar delay in the onset of GFAP immunoreactivity in the \textit{Wld} mouse corticostriatal pathway after cortical ablation. By 4 days after the lesion, GFAP-positive cells were numerous in wild-type mouse striatal neuropil (Fig. 1C), but were absent in comparable \textit{Wld} tissue (Fig. 1D), and only appeared after ~8 days (data not shown).

We used TEM to examine and measure the degeneration of individual striatal synaptic terminals and their axon collaterals after cortical lesion, in sections cut from denervated regions of striatum. Normal synaptic terminals were located by identifying vesicle-filled boutons, separated from their post-synaptic target by a synaptic cleft (Fig. 2A and B). In contrast, degenerating synaptic profiles in \textit{Wld} mice (both 8 days post-lesion). (G and H) Bar charts showing the number of degenerating synaptic profiles (counted in 15-min observation periods) in wild-type (E) and \textit{Wld} (F) mouse striatum for up to 10 days after cortical ablation (mean ± SEM). Scale bars = 0.4 μm (A); 0.6 μm (C and E); 0.5 μm (B and D); 0.7 μm (F).
collaterals were identified by their dark, electron-dense cytoplasm. Some of these contained disrupted synaptic vesicles and mitochondria. These features are consistent with other studies of degenerating synapses after cortical lesions (Fig. 2C–F; Kemp and Powell, 1971; Somogyi et al., 1981).

Virtually all degenerating synaptic terminals were in asymmetrical contact with dendritic spines—presumably on medium spiny neurons (McNeill et al., 2003)—showing distinct post-synaptic densities, consistent with the glutamatergic nature of the corticostriatal projection (Bernard et al., 1997). In addition, we noted an apparent increase in the incidence of complex synaptic morphologies (LCF synapses). We return to these below.

We first quantified the onset of nerve terminal degeneration after cortical lesion in both WldS and wild-type mice using a timed-counting protocol. One day after cortical lesion, few degenerating nerve terminal profiles were found in either strain of mouse (degeneration index = 0.8 ± 0.5 in wild-type; 0.8 ± 0.3 in WldS; index calculated as mean number of degenerating profiles in 15 min ± SEM; n = 8 counts, N = 2 mice per strain/time-point). By 2 days post-lesion, the degeneration index in wild-type mice had increased to 14.5 ± 2.3, and remained high at 3 days (17.3 ± 2.0), 4 days (8.5 ± 1.3) and 6 days (11.0 ± 1.4) after cortical ablation. Nerve terminal degeneration appeared to be completed by 10 days post-lesion, by which time the degeneration index had diminished to 1.0 ± 0.3 (Fig. 2G). In contrast, nerve terminal degeneration was virtually absent in WldS mice for up to 4 days after the lesion (Fig. 2H). Substantial increases, comparable with the levels observed in wild-type mice, were not observed until between 8 (degeneration index = 5.9 ± 0.9) and 10 (7.8 ± 2.2) days post-lesion. Overall, these data suggest that nerve terminal degeneration was significantly delayed in WldS mice by ~7 days compared with wild-type mice (P < 0.05 at all time-points 2–6 days post-lesion; P > 0.05 at 8 days post-lesion; Mann–Whitney U-test, two-tailed). Interestingly, the rate of increase in the number of degenerating nerve terminal profiles was also slower in WldS mice compared with wild-types (Fig. 2G and H).

Since the timed-counting protocol did not take into account the possibility that changes in synaptic volume could lead to different detection rates between samples, we re-examined our findings using unbiased design-based stereology. We used a physical Disector technique (see Material and methods) to measure the density of degenerating nerve terminals in a single wild-type and WldS mouse 3 days post-lesion. As with the timed-counting protocol, degenerating nerve terminal boutons were readily identifiable in wild-type striatum (141 tops in 174 Disectors) with an estimated numerical density of 0.197 per μm³, but were almost entirely absent in WldS striatum (1 top in 194 Disectors, density of 0.001 per μm³). Thus, there was a >100-fold increase in the number of degenerating synaptic boutons per square micrometre in wild-type striatum compared with WldS. In addition, we also quantified degenerating nerve terminals as a percentage of the total number of asymmetrical synapses in the region being analysed, using the same material. Again, the proportion of degenerating nerve terminals in the wild-type mouse at 3 days post-lesion (20.8%) was larger than in the equivalent WldS mouse (0.126%).

Next, we examined the morphological appearance of the degenerating synapses in more detail. This examination was motivated by our previous findings that synapse loss at neuromuscular junctions in skeletal muscle following lesions of peripheral nerve is not only delayed but morphologically distinct in WldS compared with wild-type mice. These differences are apparent at both light and electron microscope levels (Gillingwater et al., 2002, 2003). We were therefore surprised to observe that, in spite of the delayed onset of synaptic degeneration in the striatum, the morphological appearance of the degenerating synapses was similar in WldS and wild-type mice. In both strains of mice, degenerating pre-synaptic nerve terminals showed dark, electron-dense cytoplasm (Fig. 2C–F). This suggests that synapse loss occurs by a similar mechanism in both cases. Importantly, previous studies have shown that striatal neurons themselves do not degenerate after loss of their cortical inputs (Somogyi et al., 1981; Chen and Hillman, 1990), suggesting that all degenerative pre-synaptic profiles we observed arose from cortical projection neurons.

Non-synaptic, electron-dense profiles were also readily identifiable throughout the striatum of wild-type and WldS mice, confirming that degeneration-induced darkening of neuronal cytoplasm is not synapse-specific and can occur in other, presumably axonal, compartments (data not shown; Chen and Hillman, 1990). Serial section analysis of identified degenerating striatal synapses confirmed that degenerating synaptic terminals remained attached to their degenerating axon collaterals. Of 11 serially sectioned synapses investigated from both wild-type and WldS mice, 9 showed degenerative (i.e. darkened and disrupted cytoplasm) morphology throughout the pre-synaptic nerve terminal and its incoming axon collateral, with intact plasma membranes connecting the two compartments. Interestingly, however, two synapses showed unequivocal signs of degeneration in the pre-synaptic nerve terminal, but there were no signs of a degenerative morphology in their incoming pre-terminal axon (Fig. 3). One of these synapses was from a wild-type mouse (3A–C) and the other was from a WldS mouse (3D). These observations suggest that axotomized synapses degenerate before axons in lesioned brains of both strains of mouse, as in the PNS (Miledi and Slater, 1970; Gillingwater et al., 2002). We volume-reconstructed and surface-rendered one synapse in order to verify continuity of the degenerating pre-synaptic terminal and healthy axon collateral (Fig. 3A–C). In the other serially sectioned example, the plane of section allowed us to observe the gradual transition from degenerating cytoplasmic morphology in the nerve terminal to normal (clear, non-electron-dense) cytoplasmic morphology in the pre-terminal axon (green arrows in Fig. 3D, panel 1). We never
observed non-degenerating nerve terminals supplied by a degenerating axon collateral, suggesting that neurodegeneration is initiated at pre-synaptic nerve terminals, from where it spreads retrogradely into the axon.

Next, we asked whether protracted nerve terminal degeneration in Wld<sup>e</sup> mice affects the degeneration-induced plastic changes at unlesioned synapses that remained in the striatum following incomplete cortical ablation. Lesion of the cortico- and/or nigrostriatal pathway leads to an increase in the number of asymmetrical synapses with complex or perforated post-synaptic densities (LCP synapses: Ingham et al., 1998; Meshul et al., 2000). LCP synaptic profiles show multiple, post-synaptic densities, clearly separated and with a total length in excess of 0.5 μm (Fig. 4A and B; cf. Fig. 2A and B). Such changes in synaptic morphology are thought to represent compensatory responses of remaining synapses that follow loss of other excitatory inputs to dendritic spines (Greenough et al., 1978; Ganeshina et al., 2004).

We noted an apparent increase in the number of LCP-like synapses in these lesioned Wld<sup>e</sup> mouse brains. We therefore quantified the incidence of LCP synapses in the same material used for counts of degenerating synaptic profiles.

The basal number of LCP synapses in unoperated Wld<sup>e</sup> (8.4 ± 0.9; mean ± SEM) and wild-type (9.3 ± 0.8) striatum was not significantly different (P > 0.05; Mann–Whitney, two-tailed; n = 12 counts, N = 3 mice for each strain; Fig. 4C and D). The number of LCP synapses in wild-type striatum was not significantly increased above the levels observed in unoperated controls until 8 days after surgery (Fig. 4C; P < 0.05; n = 8 counts, N = 2 mice for each lesion time-point). However, a significant increase in the number of LCP synapses in Wld<sup>e</sup> striatum was detected as early as 1 day post-lesion (P < 0.02; Fig. 4D). These levels remained elevated for the entire 10-day period studied (but dipped below statistical significance at 2 days post-lesion; P > 0.05). Thus, overall, the rate of onset of lesion-induced plasticity in striatum was markedly increased in Wld<sup>e</sup> mice compared with wild-type mice.

To quantify these observations further, we first re-applied the physical Disector techniques to measure the density of LCP nerve terminals at 3 days post-lesion. This was necessary to take into account the possibility of volumetric changes in synapses between samples (see above). LCP terminal boutons were more prevalent in Wld<sup>e</sup> striatum (104 tops in 194 Disectors; estimated numerical density, 0.132 per μm<sup>3</sup>) than in wild-type striatum at 3 days post-lesion (34 tops in 174 Disectors, density of 0.047 per μm<sup>3</sup>). Thus, there was almost a 3-fold increase in the number of LCP synaptic profiles per unit area in Wld<sup>e</sup> striatum compared with wild-type.

Next, we quantified LCP synapses as a percentage of the total number of asymmetrical synapses in the region of interest. As with both the timed and density counts, there was a greater proportion of LCP synapses in the Wld<sup>e</sup> mouse striatum at 3 days post-lesion (13.18%) compared with the wild-type mouse striatum (5.01%).
Finally, to test the hypothesis that axotomized synapses themselves may have undergone plastic changes in their morphology, we measured the frequency of LCP synapses that also showed a degenerating morphology (see Fig. 2D). This was done by counting a minimum of 30 randomly selected synapses from wild-type mice at 3–4 days post-lesion and Wld^s mice at 8–10 days post-lesion, when the number of degenerating profiles was maximal in either case (Fig. 4C and D). Remarkably, there were significantly more degenerating LCP synapses in the lesioned Wld^s mice (Fig. 5; P < 0.05; Mann–Whitney, one-tailed), suggesting that at least some of the axotomized terminals underwent reactive remodelling, even though they were disconnected from their cell bodies.

**Discussion**

The present study shows, on the basis of rigorous morphological criteria, that the Wld^s gene delays degeneration of axotomized synaptic terminals in the CNS. In addition, the data show that several morphological characteristics of the degenerating central synapses differ qualitatively from those described previously in studies of peripheral synaptic degeneration after injury. Surprisingly, striatal synapses that persist after cortical lesions in Wld^s mice also show an enhanced plastic response. Together, these findings demonstrate the ability to delay the onset of neurodegeneration in synaptic compartments by blocking Wallerian-like degeneration pathways. Understanding the mechanism of the neuroprotective properties of the Wld^s gene may therefore prove to be a productive strategy in the development of therapeutics.
for neurodegenerative conditions in humans where synapses constitute primary pathological targets (Coleman et al., 2004).

The data demonstrate that pre-synaptic nerve terminals in the CNS of Wld\(^s\) mice are structurally protected from degeneration for more than a week after a lesion removing their cell soma. We previously reported that neuronal cell bodies in the striatum and hippocampus are protected following transient global cerebral ischaemia in Wld\(^s\) mice (Gillingwater et al., 2004). However, in that study, we were unable to distinguish direct from indirect effects of ischaemia on degeneration of either synapses, axons and/or cell bodies. The present data suggest that the somatic protection we observed following transient global ischaemia probably occurred indirectly, as a result of delayed degeneration of synaptic terminals as well as axons. This would be consistent with other data suggesting that somatic compartments themselves are not directly protected by the Wld\(^s\) gene (Deckwerth and Johnson, 1994). Thus, future studies aimed at preventing neuronal soma loss following cerebrovascular insults, as well as other neurodegenerative disorders where neuronal soma are vulnerable, should note the potential contribution of synapse-specific degeneration to the neurodegeneration process (Ishimaru et al., 2001; Jourdain et al., 2002).

The present data extend several other previous studies that have demonstrated neuroprotection in the CNS of Wld\(^s\) mice (Pery et al., 1990; Ludwin and Bisby, 1992; Steward and Trimmer, 1997; Sajadi et al., 2004; Cesa et al., 2005). For instance, Cesa et al. (2005) demonstrated that the pre-synaptic marker VGlut2 is retained in the molecular layer of the cerebellum in Wld\(^s\) mice for 11 days longer than in wild-type rats after severing the inferior cerebellar peduncle. In addition, the behavioural and biochemical findings reported by Sajadi et al. (2004) suggested that axotomized synapses in the lesioned nigrostriatal pathway were protected, as indicated by continued secretion of dopamine for an extended period in Wld\(^s\) mice. We have shown here direct evidence for delayed structural breakdown of axotomized synaptic terminals as the most likely basis for these behavioural, biochemical and immunocytochemical findings. Interestingly, there were also abnormalities in transmitter release and behaviour reported in the study by Sajadi et al. (2004). Data from the present study show that delayed nerve terminal degeneration following de-afferentation of the Wld\(^s\) mouse striatum results in a more rapid plastic response in striatum (see below). It is possible that some of the abnormalities reported by Sajadi et al. (2004) may have resulted from the type of compensatory changes (LCP synapses) we report here.

We could not discern any differences in the ultrastructural appearance of Wld\(^s\) and wild-type degenerating synaptic boutons after cortical lesion, suggesting that common degenerative mechanisms are activated at synapses in both strains of mice, albeit delayed when Wld\(^s\) is present. At first sight, this appears in contrast to our observations of axotomized neuromuscular synaptic connections (Gillingwater et al., 2002, 2003). For instance, electron microscopy of degenerating synapses at young adult Wld\(^s\) neuromuscular junctions showed pre-synaptic nerve terminals undergoing retraction from post-synaptic membranes, but the synaptic boutons that remained in contact with the end-plate during this process showed good preservation of cytoplasmic density, synaptic vesicles and mitochondria (Gillingwater et al., 2003). Moreover, we never observed any of the distinctive morphological characteristics associated with wild-type Wallerian degeneration at any time-point during synapse loss. Electrophysiological data indicated that these partially occupied neuromuscular junctions showed normal signs of functional synaptic transmission, including spontaneous miniature synaptic potentials and nerve-stimulation-evoked end-plate potentials. In this respect, axotomy-induced synapse loss in young adult Wld\(^s\) mice resembles neuromuscular synapse elimination during normal post-natal development (Gillingwater and Ribchester, 2003; Gillingwater et al., 2003). Such similarities suggest that the distinct synapse withdrawal mechanism elicited at neuromuscular junctions in young adult Wld\(^s\) mice may be due to the retention of developmental mechanisms into early adulthood, and that these may be re-triggered by axotomy. This hypothesis is supported by the finding that delayed synaptic degeneration was absent in old Wld\(^s\) mice (Gillingwater et al., 2002). The delay in onset of synaptic degeneration in the CNS shown here is similar to that observed in the PNS of young adult Wld\(^s\) mice, but the process of synapse loss once initiated is similar to that observed in old Wld\(^s\) or wild-type mice (Gillingwater et al., 2002). The data also show that more LCP synapses are induced within the first 24 h of a corticostriatal lesion in Wld\(^s\) mice than their wild-type counterparts. Paradoxically, this suggests that intrinsic striatal plastic responses are more rapidly induced in Wld\(^s\) than wild-type mice, whereas studies on the PNS suggest that reactive changes, including nerve sprouting by intact axons, occur more slowly after nerve lesions in Wld\(^s\) mice (Brown et al., 1991; Subang et al., 1997; Carroll and Frohnhert, 1998; T. Gillingwater and R. Ribchester, unpublished data). These subtle differences in the response of axotomized synapses in the CNS and PNS of Wld\(^s\) mice could possibly be related to the role of the ubiquitin–proteasome system in different neuronal compartments. Proteasomal activity, for instance, is known to play an important role in synapse-specific plasticity (Upadhya et al., 2003). Perhaps the Ube4b component of the chimeric Wld\(^s\) gene therefore plays a more significant role in the ubiquitin–proteasome signalling pathway at this location than has previously been appreciated (see below; Coleman and Ribchester, 2004; Laser et al., 2005).

One surprising finding from the current study was that some persistent axotomized synapses in Wld\(^s\) mice underwent morphological plastic changes, specifically by adopting LCP morphological characteristics. These findings imply that morphological plasticity at synapses does not require any input from the cell soma. As it is virtually impossible to envisage such morphological plasticity without protein
synthesis, the data provide further support to the idea of protein synthesis and degradation controlled locally within neurons, at the level of individual synapses (Alvarez et al., 2000; Martin, 2004; Upadhya et al., 2005). It would therefore be interesting to know whether protein synthesis inhibitors would block the appearance of LCP synapses after cortical lesion in WldΔ mice. However, our findings suggest that such plastic changes may be just as sensitive to damaged neural circuits in disease states as they are to those modified patterns of neural activity that are responsible for modifying synaptic strength in order to confer long-term information storage (Sutton and Schuman, 2005).

Interestingly, several studies have reported increased levels of synaptic plasticity after traumatic head injury (McKinney et al., 1997; Santhakumar et al., 2001). This synaptic hyperexcitability is thought to underlie the development of post-traumatic epilepsy associated with head injuries (McKinney et al., 1997; Albersen and Janigro, 2003). However, we did not observe any seizures in either WldΔ or wild-type mice after cortical ablation, so it is not possible for us to say whether the delayed degeneration phenotype and/or increased rate of onset of LCP plasticity had any effect on mouse behaviour. Nevertheless, it has been demonstrated previously that the evolution of motor and cognitive impairment is delayed in WldΔ mice after traumatic brain injury (Fox and Faden, 1998). We do not yet know how widespread this enhanced response to plasticity-inducing stimuli is in WldΔ mice. It would therefore be of interest to investigate whether other examples of synaptic plasticity, not necessarily involved in the response to injury (i.e. LTP in hippocampus), can be more readily induced in WldΔ mice than in wild-type counterparts.

Although the present study extends knowledge of the limits of the WldΔ phenotype, it remains unclear how the WldΔ gene delays degeneration in synaptic terminals. The WldΔ mutation evidently arose spontaneously in C57Bl6 mice supplied by Harlan-Olac. The mutation is a tandem triplication of an 85 kb genomic region on chromosome 4, which created a chimeric gene at the boundary of the repeat unit. This hybrid gene comprises the N-terminal sequence (encoding 70 amino acids) of the gene for the E4 ubiquitin factor Ube4b, fused with the entire open reading frame for the NAD synthesizing enzyme nicotinamide mononucleotide adenylyltransferase (Nmnat; Mack et al., 2001). A recent in vitro study suggested that the WldΔ gene confers neuroprotection via increased nuclear Nmnat activity in neurons (Araki et al., 2004). This study is only partly supported by another in vitro study (Wang et al., 2005). Moreover, the findings cannot account for the entire in vivo neuroprotective phenotype as other data implicate the Ube4b component of the chimeric mutant gene and the ubiquitin–proteasome signalling pathway (Coleman and Ribchester, 2004; Laser et al., 2005). Our recent analysis suggests that both components of the chimeric gene may be necessary for expression of the delayed axosynaptic degeneration phenotype (Gillingwater et al., 2006). Consistent with this, the finding that WldΔ protein is localized almost exclusively to neuronal nuclei (Mack et al., 2001; Fang et al., 2005) implies that the mutant protein exerts its action indirectly, by regulating downstream neuroprotective signalling pathways or protein molecules (Coleman, 2005). The finding that WldΔ neuroprotection can be successfully transferred across species, with no apparent side-effects, also suggests that it is acting on a specific, conserved step in both synaptic and axonal compartments (Coleman and Ribchester, 2004; Adalbert et al., 2005; Gillingwater et al., 2006). Several recent studies have provided insights into the molecular pathways that are present in synaptic terminals and are capable of regulating synaptic dysfunction following the application of neurodegenerative stimuli (LaFontaine et al., 2002; Gilman et al., 2003). For example, molecules such as p53 have been shown to be powerful mediators of synaptic degeneration (Gilman et al., 2003).

In conclusion, the present study establishes certain limits on the degree of synaptic protection conferred by expression of the WldΔ gene in brain, complementing and extending previous analyses of synaptic degeneration in the PNS. Convergent mechanistic studies must take account of the differences if the utility of the WldΔ gene in preserving axons and their connections is to be developed and ultimately translated into effective treatments for neurodegenerative diseases in humans.

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References


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Wang MS, Davis AA, Culver DG, Glass JD. Wld\(^{s}\) mice are resistant to paclitaxel (taxol) neuropathy. Ann Neurol 2002; 52: 442–7.
