Quantitative and qualitative analysis of Wallerian degeneration using restricted axonal labelling in YFP-H mice

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Abstract

We investigated the usefulness of YFP-H transgenic mice [Neuron 28 (2000) 41] which express yellow fluorescent protein (YFP) in a restricted subset of neurons to study Wallerian degeneration in the PNS. Quantification of YFP positive axons and myelin basic protein (MBP) immunocytochemistry revealed that YFP was randomly distributed to approximately 3% of myelinated motor and sensory fibres. Axotomy-induced Wallerian degeneration appeared as fragmentation of fluorescent signals in individual YFP positive axons with a morphology and timing similar to Wallerian degeneration observed by more traditional methods. In YFP-H transgenic mice co-expressing a high dosage of Wld S, a chimeric gene that protects from Wallerian degeneration [Nat Neurosci. 4 (2001) 1199], axonal fragmentation in distal tibial nerves after sciatic nerve axotomy was approximately 10 times delayed. Considerable retardations of Wallerian degeneration using the same transgenic expression system were also observed in cultures of nerve explants, enabling in vitro real-time imaging of axonal fragmentation. Remarkably, single YFP-labelled axons could be traced in peripheral nerves for unusually long distances of up to 2.9 cm exploiting confocal fluorescence imaging. Altogether transgenic YFP-H mice prove to be a valuable tool to study mechanisms of Wallerian degeneration in vivo and in vitro.

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1. Introduction

Wallerian degeneration is a spontaneous degenerative process of the distal portions of peripheral or central nerve axons that are separated from the parent cell body (Waller, 1850). Not only do traumatic disorders such as spinal cord injury result in this form of degeneration but it is now broadly accepted that Wallerian degeneration is mechanistically related to axon loss in many neurodegenerative disorders such as amyotrophic lateral sclerosis, multiple sclerosis and toxic neuropathy (Bjartmar et al., 1999; Coleman and Perry, 2002; Griffin et al., 1996). Wallerian degeneration has usually been described in common neuropathology textbooks as a focal phenomenon just focusing on a microscopically small part of the degenerating fibre, often neglecting the fact that axons can reach lengths of many centimetres to meters and that topographic factors of Wallerian degeneration along axons play an important role in many axon disorders (Cavanagh, 1979; King, 1999a; Spencer and Schaumburg, 1976). There is relatively little and contradictory published information regarding the behaviour of the axon in its entirety after disconnection from its supporting neuronal cell body mainly due to limits of axon visualisation over longer distances. To the best of our knowledge, only very few and moreover exceedingly complicated classical histological techniques are available to date for reliable visualisation of pathological processes in single axons over substantial nerve distances, and these are often particularly labour-intensive and susceptible to artefacts due to preparation and processing (King,
Jackson Laboratories (Bar Harbor, USA) and maintained as YFP-H)2Jrs, stock number: 003782) was obtained from the

2.1. Crossbreeding and genotyping of transgenic mice

whether selective neuronal YFP expression in transgenic YFP-H mice permits continuous long-range and long-term tracing of individual axon courses in peripheral nerve whole-mount preparations in conjunction with the optical sectioning capability of the confocal laser scanning microscope. No expression is detectable in non-neuronal cells such as Schwann cells. Thus YFP, as a strong and specific vital marker for a small percentage of axons in peripheral nerves that are brightly and homogeneously fluorescent all the way to the terminals. The excised prefixed spinal cord root or peripheral nerve segments were placed on a culture dish coated with Durcupan resin embedding.

2.2. Intracardial perfusion

2.3. Dissection of spinal cord roots

2.4. Dissection of peripheral nerve segments

For the assessment of the YFP-labelled axon population in PNS, 10 mm segments of sciatic nerves were excised from intracardially perfused YFP-H mice, postfixed for a further 12 h in 4% paraformaldehyde in 0.1 M PBS and either subsequently embedded in paraffin or prepared for wholemount fluorescent preparation. For visualisation of Wallerian degeneration after different post lesion times using confocal microscopy or conventional light and electron microscopy, distal tibial nerve segments (~8 mm) from either intracardially perfused mice or mice killed by cervical dislocation were removed and treated for wholemount fluorescent preparation and for Durcupan resin embedding.

2.5. Wholemount fluorescent preparations

The excised prefixed spinal cord root or peripheral nerve segments were placed on a culture dish coated with

1999b; Somogyi et al., 1979). Against this background, the availability of a method for long-range tracing of axons undergoing pathological processes would improve our understanding of axodegenerative mechanisms. Yellow fluorescent protein (YFP) is a derivative of green fluorescent protein (GFP), a versatile reporter molecule which has found use in many neuroscience applications (Chalfie et al., 1994). Transgenic mice are now available that express YFP or related fluorophores such as cyan fluorescent protein (CFP) at high levels selectively in various neuronal subsets under the control of regulatory elements derived from the mouse Thy1 gene (Feng et al., 2000). In contrast to mice expressing the CFP fluorophore in a majority of PNS neurites, transgenic YFP-H mice obtained from the Jackson Laboratories (Bar Harbor, USA) show only a few axons in peripheral nerves that are brightly and homogeneously fluorescent all the way to the terminals. No expression is detectable in non-neuronal cells such as Schwann cells. Thus YFP, as a strong and specific vital marker for a small percentage of axons in peripheral nerves that are brightly and homogeneously fluorescent all the way to the terminals.
Mounting Medium (Vector Laboratories) and stored dark segments were mounted then on glass slides in Vectashield and then washed extensively in fresh 0.1 M PBS. The X-100 (Sigma) in 0.1 M PBS for 5–10 min at room temperature and then washed extensively in fresh 0.1 M PBS. The segments were mounted then on glass slides in Vectashield Mounting Medium (Vector Laboratories) and stored dark at 4°C overnight until used for confocal imaging. In all experiments, the YFP fluorophore was stable and resistant to fading during storage enabling data collection over time periods of at least several months.

2.6. Myelin basic protein immunocytochemistry on paraffin embedded tissue

Transverse sciatic nerve paraffin sections attached to poly-L-lysine coated slides were dewaxed, washed several times in 0.05 M TBS (Tris-buffered saline), rinsed in citrate buffer overnight (60°C) and incubated with 0.1% Triton X-100 (Sigma) in 0.05 M TBS. After blocking with 5% BSA (Sigma) in 0.05 M TBS sections were incubated overnight at 4°C with rabbit anti-MBP antibodies (dilution 1:800 in 0.8% BSA/TBS; gift from Peter Brophy, Edinburgh). Thereafter, sections were treated for 1 h at room temperature with TRITC (tetramethylrhodamine-5-isothiocyanate)-conjugated donkey anti-rabbit secondary antibodies (dilution 1:1000 in 0.8% BSA/TBS, Jackson Immunoresearch Laboratories). Nuclear counterstaining was performed using TO-PRO-3 iodide (Molecular Probes) and paraffin sections were mounted in Vectashield Mounting Medium.

2.7. Laser scanning confocal microscopy

Confocal imaging was performed using a Biorad Radiance 2000 laser scanning system (Hemel Hempsted, UK) on an unique Nikon Eclipse microscope equipment with water and oil immersion objectives. Wholemount fluorescent preparations of nerve or spinal cord root segments, transverse paraffin nerve sections and teased fibre preparations from YFP-H mice were scanned in the z-series (for YFP excitation/543 nm (for TRITC excitation)/637 nm (for TO-PRO-3 iodide excitation) laser optics and the z-series obtained were merged using LaserSharp 2000 software.

2.8. Quantification of peripheral YFP positive axons and consecutive morphometry

The numbers of YFP positive fibres in the processed ventral/dorsal spinal cord roots and sciatic nerves were counted and the calculated mean values compared for statistical analysis. Axonal diameters including the MBP labelled myelin sheath of all YFP positive axons were measured in three representative transverse paraffin sciatic nerve sections applying morphometry software (Meta Imaging, Universal Imaging Corp.).

2.9. Sciatic nerve lesions

Six- to 10-week-old YFP-H mice were anaesthetised by intraperitoneal injection of Ketanest (5 mg/kg; Parke Davis) and Rompun (100 mg/kg; Bayer). Right sciatic nerves were transected close to the Foramen intrapiriforme and the wound closed with a single suture. After 36 h to 12.5 days the mice were intracardially perfused and nerve segments dissected as described above.

2.10. Durcupan resin embedding of distal tibial nerve segments

After intracardial perfusion of YFP-H mice, prefixed distal tibial nerve segments were fixed for further 12 h in 0.1 M phosphate buffer containing 3.9% glutaraldehyde. Preparations were then washed in 0.1 M phosphate buffer before postfixing in an aqueous 1% osmium tetroxide solution for 1 h and dehydration through an ascending series of ethanol solutions including an uranyl acetate en bloc staining step. Before infiltration with Durcupan resin (Fluka) propylene oxide was used as intermediate. Durcupan tissue blocks were cured for 48 h at 65°C.

2.11. Conventional light and electron microscopy

Five hundred nanometers thick Durcupan semithin sections (transversal and longitudinal tibial nerve sections) were stained with toluidine blue and photomicrographed using a Zeiss Axioskop microscope connected to a LM digital camera system (Universal Imaging Corporation). Silver thin Durcupan sections of ~80 nm thickness suspended on Formvar-coated copper grids were stained with 1% aqueous uranyl acetate (20 min) and Reynolds’s lead citrate (7 min). Sections were examined with a Zeiss EM 902 electron microscope at 80 kV acceleration voltage and pictures were taken with an EM digital camera system (McView, analySIS® docs 3.2, Soft Imaging Systems).

2.12. Assessment of axonal preservation

The percentage of surviving myelinated axons at different time points after sciatic nerve lesion was calculated in digital pictures of transverse Durcupan sections (~500 counted myelinated axons in randomly chosen areas) and in confocal z-series photographs obtained from the longitudinally embedded YFP-H wholemount preparations (~30 counted YFP-labelled axons). In Durcupan specimens criteria for survival were normal myelin sheaths, uniform axoplasm and intact mitochondria. In YFP-H wholemount preparations criteria for survival were unfragmented YFP positive fibres traversing the whole segment length of a few
millimeters. Therefore, fibres that showed clear interruptions and swellings in the segment were counted as degenerated. Statistical analysis was performed using SPSS for Windows.

2.13. Teased fibres with subsequent myelin basic protein immunocytochemistry

Teased fibres from lesioned nerve segments were obtained by careful dissection with fine acupuncture needles (Seirin) and attached on TESPA (3-aminopropytriethoxysilane; Sigma–Aldrich) coated slides. For teased fibre immunocytochemistry the preparations were treated for 20 min with acetic acid at −20°C, encircled with PAP-Pen (Daido Sangyo Co.) and permeabilised with 0.1% Triton X-100 in 0.1 M PBS. After a blocking step with 5% fish skin gelatine (Sigma–Aldrich) diluted in PBS teased fibres were incubated overnight with primary antibodies directed against MBP (dilution 1:800 in blocking solution; gift from Peter Brophy, Edinburgh). After washing on the next day, the specimens were incubated for 2 h in a 1:1000 dilution (in blocking solution) of donkey anti-rabbit secondary antibodies conjugated to the fluorescent label TRITC (Jackson ImmunoResearch Laboratories) and mounted in Vectashield Mounting Medium. Imaging documentation was performed by confocal microscopy.

2.14. Confocal tracing of individual axons in long whole-mounted YFP-H nerves

The entire nerve distance from the proximal sciatic to the distal tibial nerve was exposed, carefully excised with a minimum of attached adipose tissue and fixed under slight tension for 1 h with 4% paraformaldehyde in 0.1 M PBS. The ~3 cm long nerve segments were incubated in Tris-buffer containing 0.1% protease (Sigma–Aldrich, EC 3.4.24.31) for 10 min at 37°C and pinned out under tension (10–20% stretch) with fine insect needles in a culture dish containing 0.1 M PBS. After careful removal of the perineurium under a dissection microscope the branch of the common fibular nerve was identified within the sciatic nerve and carefully removed under a dissection microscope and using insect pins. The entire nerve distance from the proximal sciatic to the distal tibial nerve was exposed, carefully excised with a minimum of attached adipose tissue and fixed under slight tension for 1 h with 4% paraformaldehyde in 0.1 M PBS. The ~3 cm long nerve segments were incubated in Tris-buffer containing 0.1% protease (Sigma–Aldrich, EC 3.4.24.31) for 10 min at 37°C and pinned out under tension (10–20% stretch) with fine insect needles in a culture dish containing 0.1 M PBS. After careful removal of the perineurium under a dissection microscope the branch of the common fibular nerve was identified within the sciatic nerve and carefully removed under a dissection microscope and using insect pins so that the remaining nerve segment contained a small group of YFP-labelled axons (~20) running continuously from the proximal sciatic to distal tibial nerve. Next the processed nerve segments were treated as described for wholemount fluorescent preparation. For tracing of individual YFP-labelled axons a single confocal optical layer containing a sharp image of the chosen fibre was captured at the proximal site of the wholemount nerve segment. Moving a constant length distally using a joystick controlled precision motor coupled to the stage of the confocal system and focusing the laser beam in the z-axis the next consecutive sharp single layer shot from the chosen axon was obtained and the process repeated until the most distal point of the nerve segment was reached. A composite picture was assembled containing the intra-nerve course of an individual YFP-labelled axon. Image processing was performed using LaserSharp 2000 software and Adobe Photoshop.

2.15. Nerve explant culture

Unlesioned tibial nerves (ca. 1 cm) were removed quickly from mice that had been killed by cervical dislocation and transferred into a sterile dish containing L-15 CO₂ medium (Invitrogen). As much non-neuronal tissue as possible was removed under a dissection microscope and using insect pins the nerve was placed under slight tension (10–20% stretch) onto a sterile 3 cm dish that had previously been coated with transparent Sylgard (Dupont). Dishes had been repeatedly sterilised with 70% ethanol and overnight baking at 60°C. Throughout this procedure, the nerve was kept moist with sterile PBS. Nerves were then cultured in 1.5 ml of OptiMEM 1 (Invitrogen) in a humid atmosphere at 30°C, 5% CO₂ for the times specified, as previously described (Tsao et al., 1999). Degeneration was assessed using a Zeiss Axiosvert S100 fluorescent microscope and photographed using a Kodak DC120 Zoom Digital Camera linked to a Macintosh G3 Powerbook running FUIX Photograb-300Z (Ver. 2.01) software.

2.16. Animal experiments

All animal experiments were carried out under appropriate German licences: Tierschutzgenehmigung K 13, 11/00 and Anzeige K30/99.

3. Results

3.1. YFP labels a representative small percentage of myelinated PNS axons

In order to classify the fluorescent axon population in YFP-H mice we first examined quantitatively wholemount fluorescent preparations of L4 ventral and dorsal roots. YFP labelled both motor and sensory axons in approximately equal frequency (Fig. 1A and B). In L4 ventral roots we counted 24.5 ± 6.3 (N = 6) and in dorsal roots 30.5 ± 8.4 (N = 6) YFP positive fibres (no significant difference in Student’s t-test, data not shown). Next we analysed the number, distribution, myelination and axonal diameter of YFP-labelled axons in sciatic nerves. Myelin basic protein (MBP) immunocytochemistry on paraffin cross-sections in conjunction with confocal microscopy revealed that microscopically resolvable YFP signals were located exclusively in myelinated axons (Fig. 1C and D). There was no clustering as YFP-labelled axons were randomly distributed throughout the section. The continuous fluorescent signals of YFP positive axons could be clearly illustrated in wholemount preparations of longitudinally mounted peripheral nerves (Fig. 1E). We found 28.5 ± 8.3 (N = 3) YFP positive myelinated axons per sciatic nerve, corresponding to a
percentage of $3.1 \pm 0.9\%$ of the myelinated axon population that was countable in MBP labelled sciatic nerve sections. The diameter spectrum of myelinated YFP positive axons matched very well the spectrum of all myelinated axons in our sciatic nerve samples ranging from $\sim 1$ to $11 \mu m$ (data not shown). In both groups axons with diameters between 3 and $6 \mu m$ made the majority with an average percentage of approximately $70\%$. Thus, the YFP-labelled axon subpopulation represents the majority of myelinated PNS nerve fibres and can be viewed therefore as a simplified but valid model system for the biological behaviour of the whole myelinated axon population.

3.2. YFP-labelled axons fragment during Wallerian degeneration with a time course similar to disintegration of the axoplasm

The time course of axotomy-induced Wallerian degeneration in distal tibial nerves of YFP-H mice was demonstrated using confocal microscopy of wholemount preparations and
Fig. 2. Fragmentation of YFP-labelled axons correlates qualitatively with Wallerian degeneration assessed by conventional light and electron microscopy. (A–F) Confocal scans through whole mount preparations of YFP-H distal tibial nerves at different time points (0 h–52 h) following sciatic nerve axotomy. (G–L) Corresponding photographs of Toluidin-blue stained longitudinal semithin sections at the same time points after axotomy. (M–R) Transversal electron micrographs of equivalent distal tibial nerves at the same time points after axotomy. Notice beginning YFP axon fragmentation (arrows) after 36 h following axotomy (B) that was accompanied by occasional axon breakdown (asterisk), macrophage influx ('#') and oedema in light (H) and electron microscopy (N). Strongly increasing YFP axon fragmentation over the next hours in (C–F), morphologically confirmed in (I–L) by light microscopy and in (O–R) by electron microscopy. Complete axon degeneration at 52 h post axotomy in YFP confocal (F), light (L) and electron microscopy (R). Scale bar: (A–F) 100 μm; magnification: (G–L) 1000×; magnification: (M–R) 4400×.
Fig. 3. Quantitative correlation of preservation rates assessed by counting intact axons in fluorescent YFP wholemount preparations ( ■ ) and in conventional Durcupan resin sections using LM/EM ( ▴ ). Note that there was no significant difference ( Student’s t -test) between both preservation rate assessments at all investigated time points.

compared to conventional light microscopy of semithin sections and electron microscopy from the same nerve (Fig. 2). Photographs of control samples (Fig. 2A, G and M) show well preserved axons obtained with all three described morphological techniques. Following proximal sciatic nerve transection first signs of degeneration in distal tibial nerve were detected after 36 h (Fig. 2B, H and N) with occasional axons broken into fragments of varying length and thickness interspersed with complete interruptions with no visible fluorescence (Fig. 2B, arrows). Semithin sections of longitudinally embedded corresponding nerve segments revealed also a small number of disintegrated axons displaying ovoid body formation (Fig. 2H, asterisk), macrophage influx ( ’#’) and nerve oedema. This partial axon degeneration after 36 h was additionally confirmed by electron microscopy (Fig. 2N) exposing break-up of axoplasm and mitochondrial swelling in a few axons (asterisk). Over the next hours the number of structurally preserved axons decreased sharply and by 52 h after axotomy every YFP-labelled tibial nerve axon in all investigated YFP-H mice shows distinct fragmentation (Fig. 2F). However, in light and electron microscopy occasional axonal protection was still detectable at this time owing to the higher number of axons accessible to morphological analysis (Fig. 2L and R).

Morphological quantification and statistical testing comparing axonal preservation data revealed a good correlation between curves obtained from counting axons in wholemount fluorescent preparations and in conventional sections (Fig. 3). There were no significant differences between the counting data sets for each timepoint applying Student’s t -test although theoretically divergence between counting results of a large more heterogeneous axon group and a minor YFP-labelled axon subset might be expected (data not shown).

3.3. Fragmentation of YFP-labelled axons corresponds to myelin ovoid formation

In order to show that fragmentation of YFP-labelled axons is due to axon breakdown and not to any YFP protein segregation, confocal imaging of teased fibres from YFP-H tibial nerve segments with additional MBP immunolabelling was performed (Fig. 4). In tibial nerves 42.5 h after disconnection, we were able to isolate individual fibres without any features of axonal disintegration that were normally myelinated (Fig. 4A). Sometimes narrowings in the course of the YFP positive fibre could be detected probably representing nodes of Ranvier (Fig. 4B). In contrast, we also obtained individual teased fibres that showed either mild or extensive YFP fragmentation (Fig. 4C and D). Interestingly, YFP positive fragments were surrounded by MBP indicating that the observed structures correspond to ovoid bodies that are known to occur in the course of Wallerian degeneration (Gillingwater and Ribchester, 2001; Hughes and Perry, 2000). Thus, the morphology of YFP fragmentation fits with descriptions of Wallerian degeneration assessed by more traditional methods.

3.4. YFP axonal fragmentation is delayed by the WldS gene

If fragmentation of YFP positive axons corresponds to Wallerian degeneration it should be delayed by the WldS
gene, which has previously been shown to slow down Wallerian degeneration by a factor of 10 (Lunn et al., 1989; Mack et al., 2001). Triple heterozygote mice carrying single alleles of the original and transgenic \( Wld^S \) mutations plus YFP-H were subjected to sciatic nerve transection. These mice were used instead of \( Wld^S \) homozygotes for reasons of convenience, as double homozygotes had already been generated (unpublished) so triple heterozygotes could be produced in a single mating. As they express a similar level of \( Wld^S \) protein to \( Wld^S \) homozygotes (Gillingwater et al., submitted), and as expression level correlates closely with strength of phenotype (Mack et al., 2001), there is good reason to expect that similar results would be obtained with \( Wld^S \) mice. Remarkably, in these mice fragmentation had not started before 12.5 days after transection (Fig. 5A), showing that Wallerian degeneration was strongly delayed and that turnover of YFP is sufficiently slow to allow extended observations over several weeks. On the other hand, 5 days after transection in control YFP-H mice, whole-mounted tibial nerves showed absolute disintegration of all YFP positive axons (Fig. 5B) and by 10 days almost no YFP signal was detectable (Fig. 5C). Probably the few visible YFP remnants represent digested axonal fragments located in Schwann cells or macrophages.

3.5. Repeated observation of Wallerian degeneration in YFP-H nerve explants

Fixation was used in the above procedures to stop degeneration at a defined timepoint and to permit confocal
analysis on a later date. However, YFP-labelled axons, and thus Wallerian degeneration, can also be observed in unfixed tissue, suggesting that it may be possible to observe Wallerian degeneration in nerve explants maintained in culture. Significantly, this would enable repeated real-time observations of structural changes in single axons undergoing Wallerian degeneration, observations that have not previously been possible with any technique. Any repeated observation of a single nerve explant has so far been confined to functional analysis (Tsao et al., 1999).

We cultured nerve explants from YFP-H mice and observed axon fragmentation that is morphologically indistinguishable from nerves that have undergone Wallerian degeneration in vivo (Fig. 6A, axon fragmentation after 72h in culture). The time to the appearance of the first axon break (52.8 ± 17.5h, N = 7) was similar to, although considerably more variable than in vivo (Fig. 6C). This may be due to variations in the length of the removed tibial nerve, the harshness of the dissection, cleaning and pinning procedures, the many differences between the environment in vitro and in vivo, or the fact that the nerve is cut at both ends. To further validate that this is a model for Wallerian degeneration, we subjected YFP-H nerves from mice additionally expressing WldS protein (triple heterozygote mice) to the same procedure and observed drastically delayed axon fragmentation (Fig. 6B, axonal breakdown not before 7 days in culture). On average the time to the appearance of the first axon break in these nerves was significantly longer (179.7 ± 78.3h, N = 4; P = 0.0021, Fig. 7C), confirming that the mechanism of YFP-H axon fragmentation in nerve...
We started our investigations observing degeneration dynamics in better temporal resolution. In our transgenic triple heterozygote mice YFP axonal fragmentation is delayed approximately tenfold compared to YFP-H mice without expression of WldS protein opening the way for further investigations on the mechanism with a classification of the fluorescent axon population in the PNS of YFP-H mice since the response of individual axons to nerve injury appears to depend on properties like axonal diameter, myelination and conduction of motor or sensory information (Bisby and Chen, 1990; Bisby et al., 1995; Lubinska, 1977, 1982). Previous investigations provide only vague information on these determinants of Wallerian degeneration suggesting that “many” sensory but only “few” motor axons were YFP-labelled (Feng et al., 2000). Our results show that YFP-H mice carry approximately up to 3% fluorescent myelinated axons in peripheral nerves that are equally distributed between motor and sensory system and that can be visualised by confocal laser scanning microscopy. Because this axon subpopulation resembles the entire myelinated axon population of a peripheral nerve in important fibre characteristics it may represent the latter in its biological behaviour. This presumption prompted experiments to study axotomy-induced degeneration in peripheral nerves of YFP-H mice. We found that the YFP-labelled axon subpopulation serves as a simplified but valid model system to analyse Wallerian degeneration in a peripheral nerve, just as CFP expressed in all axons can be used to study degeneration where just a few axons are present (Gillingwater et al., 2002). On the other hand, we showed that the characteristic YFP positive fragmentation of fluorescent axons undergoing Wallerian degeneration correspond to axonal disintegration and consecutive ovoid body formation which can be viewed as classical morphological hallmarks in the cascade of axon degeneration (Gillingwater and Ribchester, 2001; Hughes and Perry, 2000). Concerning the formation of ovoid bodies, also termed “myelin balls”, “digestive chambers” or “ellipsoids”, it is supposed that once the lesioned axon has begun to break down the myelin sheath twines itself around the axonal fragments and therefore segregates the nerve into digestive compartments that later on are invaded and digested by myelomonocytic cells (Beuche and Friede, 1984). The finding that YFP fragmentation reflects axons undergoing Wallerian degeneration, including formation of myelin ovoids, made us ask to what extent the WldS gene might delay the YFP fragmentation process in these individual axons. The murine mutant C57BL/Ad (Lunn et al., 1989) and the transgenic slow Wallerian degeneration (Gillingwater and Ribchester, 2001; Gillingwater et al., 2002; Mack et al., 2001). Naturally occurring Wallerian degeneration in disconnected distal axon segments of wild-type mice proceeds relatively fast within 24–48 h (Miledi and Slater, 1970; Waller, 1850), so delaying the degeneration process should prove to be advantageous for observing degeneration dynamics in better temporal resolution. In our transgenic triple heterozygote mice YFP axonal fragmentation is delayed approximately tenfold compared to YFP-H mice without expression of WldS protein opening the way for further investigations on the mechanism
of axon breakdown in C57BL/6 mice. Furthermore we demonstrated with this result the potential speed and simplicity of efficient screening for candidate mutants with deviated courses of Wallerian degeneration or mutants displaying axon disorders. Diverse mutants can now be crossed to YFP-H mice or genome-wide mutagenesis could be applied on this strain in order to determine the effect on Wallerian degeneration. Although we present confocal images of fixed and processed YFP-H peripheral nerves, axon fragmentation can be assessed rapidly in unfixed tissue by conventional fluorescence microscopy (unpublished observation). This is not possible with time-consuming light or electron microscopy.

In addition to validating the use of YFP-H peripheral nerve axons to analyse Wallerian degeneration in vivo, we also show that such nerves can be used to follow a related degeneration process in vitro that is similar in morphology, timing and genetic regulation. This method is unique in allowing repeated observation of structural changes in an axon undergoing Wallerian-like degeneration and can now be used in real-time studies to address long-standing issues in Wallerian degeneration, such as whether degeneration progresses along the nerve and whether fragmentation begins at nodes or internodes. The system could also be used to test the effect on Wallerian degeneration of substances added to the medium, and is thus potentially adaptable for high-throughput screening of chemical libraries. Similarly, it may be possible to use this method to screen for other genetic mutations that alter the rate of Wallerian degeneration, for example in genome-wide mutagenesis experiments. There are, however, inevitable experimental limitations of the explant cultures, such as the nerve being cut at both ends, the dissection procedure, and the artificial medium and oxygenation conditions, leading to greater variation in degeneration time and a shorter preservation of axons by the WldS mutation compared to in vivo. The absence of Triton/X vectashield enhancement and necessary use of Syngard dishes and an inverted microscope limits the number of axons that can be clearly visualised in a single nerve usually to two or three. Nevertheless, this technique can now provide the first structural information to complement electrophysiological analysis of nerves degenerating in vitro.

Substantial lengths of single fibres (1–1.5 cm) have been traditionally examined using teased-fibre preparations (Boulton and Cavanagh, 1979; King, 1999b) although this is extremely labour-intensive, prone to mechanical artefacts and has a limited sample size. This approach also has been applied to the examination of pathological alterations in fibres undergoing axonal degeneration (Boulton and Cavanagh, 1979; Ghribi and Allt, 1979; Knowles, 1978; Lubinska, 1977; Mastalgia et al., 1976; Oldfors, 1981). Serial longitudinal sectioning of resin embedded nerve tissue and tedious subsequent three-dimensional reconstruction could also in theory be used to investigate the course and structure of individual fibres (Fraher, 1978a,b; Friede and Martinez, 1970; Kidd and Heath, 1988; Tuisku and Hildebrand, 1992), but mostly it is impossible to preserve the orientation of nerves with sufficient precision to show even two complete internodes. Further, more easily applicable axon tracing techniques with confocal fluorescence imaging do not utilise axoplasmic tracking for axon tracing (Reynolds and Heath, 1995; Reynolds et al., 1994) and are therefore not suitable for examination of intrinsic axonal phenomena. Furthermore they label indiscriminately all fibres and also supporting cells in peripheral nerves, leading to disturbing optical overlays when following individual axons (Cashway et al., 1996). Other modern long-range axon tracing techniques require technically difficult injections of tracers or the delivery of exogenous cofactors or substrates for transgenes to generate visible axons that are mainly incorporated only for a limited time (Callahan et al., 1998; Miwa et al., 2001). Besides confocal laser scanning, magnetic resonance microscopy has been used for direct visualisation of axons (Wright et al., 2002). Although this method is non-invasive and non-destructive unselective imaging and insufficient resolution make this technique inappropriate for precise axon tracing. Furthermore, the equipment is unavailable to most labs.

Confocal imaging has opened up the possibility of visualisation of fine details of line shaped structures over substantial lengths in thick specimens and the single one-field-of-view images produced are ready available for subsequent two- or three-dimensional computer reconstruction and analysis (Keller-Peck et al., 2001; King and Delaney, 1994). Notably, the method avoids disruption of adjacent cellular and extra-cellular relationships. An ideal combination for axonal tracing would be the union of confocal imaging and selective long-term expression of microscopically detectable markers in a few individual axons within a nerve. YFP-H mice carrying only ∼1% fluorescent axons in peripheral nerves present an excellent tool to achieve this aim.

In this study, we overcome the problem of axon tracing in PNS by documenting for the first time the advantages of utilising YFP-H mice in conjunction with confocal microscopy to study peripheral axon morphology in individual PNS axons over unique distances that are only limited by the length of the axon itself. So we managed to present the intra-nerve course of an individual ∼2.9 cm long YFP-labelled mouse axon with visualisation of its inherent axoplasmic structure. This method should now be applicable to the controversial issue of whether Wallerian degeneration after physical injury and also generally in nerve pathology proceeds simultaneously, spreads in a somotofugal or in a somatopetal direction along the peripheral nerve stump (Donat and Wisniewski, 1973; Dyck et al., 1993; Lubinska, 1977; Lunn et al., 1990; Malbouisson et al., 1984). Progress on this issue is held up in part by the absence of any method to analyse Wallerian degeneration along the whole length of a single axon such that all conclusions have to be inferred indirectly from measurements at single points. Similarly, “dying back” degeneration of motor or sensory axons is often inferred from observations on neuromuscular junctions, muscle spindles...
and ventral spinal cord roots with little or no observation of a gradient of axon degeneration in peripheral nerves (Ferre et al., 1999; Miura et al., 1993; Oda et al., 1992; Schmalbruch et al., 1991). Against this background, our long-range tracing approach of individual axons enables future studies that will facilitate our understanding of the spatiotemporal evolution of axon pathology.

In summary, firstly, we have validated expression of YFP in a small representative subset of axons in a peripheral nerve for dependable investigation of Wallerian degeneration. We have shown that cultured isolated nerve segments of YFP-H mice allow repeated structural observations of Wallerian degeneration. Thirdly, we have established a new method for long-range tracing of myelinated YFP-labelled axons in PNS both in normal condition and in degeneration state.

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