**RIP2-mediated LKB1 deletion causes axon degeneration in the spinal cord and hind-limb paralysis**

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**SUMMARY**

Axon degeneration is observed in neurodegenerative diseases and neuroinflammatory disorders, such as Alzheimer’s disease, Parkinson’s disease and multiple sclerosis. The molecular basis of this process remains largely unknown. Here, we show that mice deleted for the tumour suppressor LKB1 (also called STK11) in the spinal cord, some parts of the brain and in the endocrine pancreas (LKB1KO mice) develop hind-limb dysfunction and axon degeneration at about 7 weeks. Demyelination and macrophage infiltration are observed in the white matter of these mice, predominantly in the bilateral and anterior funiculi of the thoracic segment of the spinal cord, suggesting damage to the ascending sensory signalling pathway owing to LKB1 deletion in the brain. Microtubule structures were also affected in the degenerated foci, with diminished neurofilament and tubulin expression. Deletion of both PRKAA1 genes, whose products AMPKα1 and AMPKα2 are also downstream targets of LKB1, with the same strategy was without effect. We thus define LKB1 as an intrinsic suppressor of axon degeneration and a possible target for strategies that can reverse this process.

**INTRODUCTION**

Neuronal polarization, involving the initiation of immature neurites, the specification and formation of axon and dendrites, and the formation of synaptic contacts, is essential for signal transmission in the central nervous system (CNS) (Arimura and Kaibuchi, 2007; Dotti et al., 1988). Proper microtubule organization is crucial for this process (Lefcourt and Bentley, 1989; Hirokawa and Takemura, 2005; Kimura et al., 2005; de Anda et al., 2005). Thus, disruption of microtubule structure, or of the formation of the myelin sheath that surrounds the axons, are associated with several pathologies, including axon degeneration (Coleman and Perry, 2002; Perrin et al., 2005) and rare myelin protein mutations (e.g. Pelizaeus-Merzbacher disease) (Garbern, 2005; Zhao et al., 2001). Axon degeneration is also found in various neurodegenerative disorders such as multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), Alzheimer’s disease (AD), Parkinson’s disease (PD) and others (Coleman and Perry, 2002; Perrin et al., 2005).

Liver kinase B1 (LKB1; also called STK11) is a protein kinase and potent tumour suppressor. Mutations in the LKB1 gene result in Peutz-Jeghers syndrome (PJS) (Boueau et al., 2003). LKB1 is a partial mammalian homologue of three kinases in Saccharomyces cerevisiae, Elm1, Pak1 and Tos3; these kinases phosphorylate SNF1, the yeast homologue of mammalian AMP-activated protein kinase (AMPK) (Sutherland et al., 2003). LKB1 is one of three upstream kinases for AMPK in mammalian cells (Hawley et al., 2003; Woods et al., 2003) and also phosphorylates 12 further kinases in the AMPK subfamily (Lizcano et al., 2004). LKB1 is implicated in the control of cell polarity, and homologues of LKB1 in Drosophila melanogaster (dLKB1) and Caenorhabditis elegans (PAR-4) control epithelial cell polarity (Jansen et al., 2009). AMPK has been proposed as a further probable mediator of the effects of LKB1 on cell polarity (Zhang et al., 2006). In neurons, LKB1 is thought to control neuron polarity by affecting axon differentiation (Shelly et al., 2007; Barnes et al., 2007). The latter effects are thought to be mediated via the inactivation of a signal transduction cascade that activates late-onset sporadic Alzheimer’s disease (SAD)-A/B kinases, also called Brsk1/2 (Barnes et al., 2007). In C. elegans, the homologue of the LKB1 binding partner STRAD, termed STRD-1, binds together with LKB1 to form a tightly associated functional complex with the C. elegans SAD-A/B kinase SAD-1 to organize synaptic proteins and establish neuron polarity (Kim et al., 2010). SAD-A/B kinase is thought to act by phosphorylating tau, a microtubule stabilization protein (Kishi et al., 2005), at Ser262.

Despite the above evidence, a direct demonstration that LKB1 is involved in these processes in vivo in adult mammals is lacking. Cre expression under rat insulin 2 promoter (RIP2) has been found in mid and ventral brain and spinal cord. Using mice lacking LKB1 in these regions (Wicksteed et al., 2010), we demonstrate here that LKB1 in certain brain areas is required for axon stability and normal hind-limb locomotor control.

**RESULTS**

**Mice lacking LKB1 owing to the RIP2-Cre transgene develop hind-limb paralysis**

To generate mice lacking LKB1 in the mid and ventral brain, and in the spinal cord from embryonic day 11.5 (E11.5) (Gannon et al., 2000; Wicksteed et al., 2010), we crossed mice bearing floxed LKB1 alleles with RIP2-Cre mice (Sun et al., 2010b). We have previously shown that these mice (BLKB1KO) are hyperinsulinemic and mildly hypophagic due respectively to deletion of LKB1 in the...
pancreatic β-cell and in a small population of hypothalamic neurons (Sun et al., 2010b). In addition, as reported below, βLKB1KO mice developed dysfunction of both hind limbs at approximately 7 and 8 for females and males, respectively. This was characterized by clumsy and autonomous twitching of both hind limbs (Fig. 1A,B). At 1-2 weeks after the initial onset of an observable abnormality, both legs of βLKB1KO mice became paralyzed. However, as assessed by toe pinching, basal muscle reflexes in the hind limbs of βLKB1KO mice still existed at this time point, which is indicative of unaffected muscle function. Moreover, both front limbs of βLKB1KO mice maintained mobility (Fig. 1B), and the mice were still able to gain access to food and water 2 weeks after the initial onset of symptoms. The latter findings suggested that the upper section (cervical) of the spinal cord was less affected than the lower section by LKB1 deletion. Furthermore, before paralysis onset, βLKB1KO mice showed similar mobility to wild-type control mice. However, after the onset of hind-limb dysfunction, βLKB1KO mice displayed significantly reduced mobility as characterized by an unwillingness to move and by crouching, both of which are indications of possible trauma. In addition to the above changes,
BLKB1KO mice displayed complete loss of control of their tails 1-2 weeks after the initial onset of the hind-limb dysfunction (Fig. 1A). A large reduction in body weight increases was also observed up to 2 weeks after paralysis, after which point BLKB1KO mice displayed rapid weight loss and loss of urinary control (data not shown). In all cases, the animals died 4-5 weeks after the initial onset of paralysis (Fig. 1C).

Recent studies on RIP2-Cre expression in adult mice by Wicksteed and his colleagues showed extensive expression of Cre in the mid and ventral brain (Wicksteed et al., 2010). In the present study, Cre and LKB1 expression were also studied in the spinal cord: analysis of LKB1 expression in the cervical, thoracic and lumbar regions of the spinal cord of BLKB1KO mice by quantitative reverse transcription PCR (qRT-PCR) using primers flanking exon 3 and 6, and western (immuno)blotting analysis using anti-LKB1 antibody raised against the C-terminal of LKB1, demonstrated that RIP2-Cre-mediated recombination occurred substantially in the thoracic area, and to a lesser extent in the cervical and lumbosacral areas (supplementary material Fig. S1A,B). Correspondingly, immunohistochemical analysis of the spinal cord from BLKB1KO mice revealed lowered numbers of cells that were intensely stained for LKB1 in the grey matter of thoracic regions (supplementary material Fig. S1C) compared with wild-type controls.

BLKB1KO mice develop axon degeneration in the white matter of the thoracic segment of the spinal cord

To try to understand the molecular and cellular basis of the above changes, post-mortem analysis of different organs extracted from BLKB1KO mice was performed 1 or 7 days after the initial onset of hind-limb dysfunction. The tissues analyzed included: whole brain (cerebral cortex, hypothalamus and cerebellum), heart, lung, liver, spleen, kidney, skeletal muscle of hind limb, pelvis, sciatic nerve and spinal cord. Haematoxylin and eosin (H&E) staining was used for general morphological analysis and luxol fast blue (LFB) was used to detect myelin. This analysis revealed several large foci of axon degeneration in the white matter of the thoracic region of BLKB1KO mouse spinal cord 7 days after the initial onset of hind-limb disability (Fig. 2Ad,Ai), whereas fewer and smaller foci only were found 1 day after initial onset (Fig. 2Ac,Ah). In the area where degeneration occurred, axon disintegration and demyelination were evident by using LFB staining, with a loss of dark blue myelin staining and the formation of large digestion chambers caused by myelin vacuolation (Fig. 2Af,Ab,Al,Bb,Bd,Bf,Bh). Notably, brightly eosinophilic acellular spheroids were present in some of the vacuolated chambers when the sections were stained with H&E (Fig. 2Ac,Bd, arrows), suggesting possible necrosis or swollen axons (Fig. 2Bd,Bh) (Fujimura et al., 2009). At 7 days after the onset of hind-limb dysfunction in BLKB1KO mice, a complete loss of myelin staining and the disappearance of axons was observed in the thoracic region of the spinal cord (Fig. 2Ad,Ai,C,D). A striking increase in the overall number of cell nuclei was also evident at the foci of degeneration of BLKB1KO mice 1 day after the initial onset of hind-limb dysfunction, which is suggestive of inflammation and macrophage infiltration, and this increase continued as degeneration progressed (Fig. 2Ac,Ad,Ab,Ai and Fig. 4A). In addition to the large foci of axon degeneration, several smaller foci with fewer and smaller digestion chambers and vacuolated axons were found in the thoracic area of the spinal cord (Fig. 2Ac,Aj). In these areas, axons were not completely disintegrated, but still retained a partial myelin sheath, which was detached from the axons.

BLKB1KO mice display impaired sensory signalling pathways in the thoracic segment of the spinal cord

Transverse sections of this area in BLKB1KO mouse spinal cord 1 day after the initial onset of hind-limb dysfunction revealed the degeneration foci mainly in the lateral and anterior funiculi, including in the spinothalamic and spino-cerebellar tracts of the spinal cord, indicating damage primarily to the ascending sensory signalling pathways (Fig. 2Aa,Bc,Be,Bg). By contrast, changes to the descending motor systems were less evident, suggesting that alterations in these pathways, which are likely to underlie the hind-limb paralysis, might occur either in higher brain regions in which RIP2-Cre-mediated deletion is also likely to occur, or were sufficiently diffuse in the thoracic region of the spinal cord to escape from histological detection. Examination of BLKB1KO mice before paralysis also revealed several sites of mild demyelination in the thoracic region of the spinal cord (Fig. 2Ab,Ag,C,D), a phenotype similar to that seen in the cervical and lumbosacral areas of the spinal cord in BLKB1KO mice 7 days after the onset of symptoms (Fig. 2C,D; supplementary material Fig. S2).

A limited degree of LKB1 deletion was previously found in the ventromedial hypothalamus of BLKB1KO mice (Sun et al., 2010b), consistent with earlier studies using RIP2-Cre deleter mice bred with a lacZ reporter strain (Gannon et al., 2000; Choudhury et al., 2005; Lin et al., 2004). However, close examination of the hypothalamus and other parts of the brain, including the forebrain, cortex and cerebellum, did not reveal any evidence for the presence of degenerating axons in these regions of BLKB1KO mice before or after hind-limb paralysis (supplementary material Fig. S3).

BLKB1KO mice display disintegration of neurofilaments and microtubules in the spinal cord

To examine the effects of LKB1 deletion on the formation of microtubules and neurofilaments within spinal cord axons, the degenerated areas were stained with markers for neurofilaments [neuron filament 165kD (NF165kD; 2H3); Fig. 3A], tau (Fig. 3A) and β-tubulin III (data not shown). Affected sections of BLKB1KO mouse spinal cord displayed diminished NF165kD, tau and β-tubulin III staining in an age-dependent manner. Thus, disorganization, disintegration and partial loss of neurofilaments and microtubules in the foci of spinal cord were apparent in mice 1 day after the initial onset of hind-limb dysfunction, and almost total loss of signals was evident 1 week from the initial onset, indicating severe axon degeneration (Fig. 3A). Several neurofilament-containing spheroids (arrows in Fig. 3A) were also evident in the degenerated areas. These findings were confirmed by western (immuno)blotting analysis of protein extracts from thoracic regions of the spinal cord from BLKB1KO mice. Reduced NF165kD and tau immunoreactivity was also evident in spinal cord sections from BLKB1KO mice 1 day after the initial onset of paralysis (Fig. 3B).

BLKB1KO mice develop increased macrophage invasion after the initial onset of hind-limb dysfunction

Macrophage invasion is commonly observed during axon degeneration (Zhang and Guth, 1997; Buss et al., 2004). In view of
the increase in apparent cell number (Fig. 4A), but diminished number of axons (Fig. 2C), in foci in the spinal cord of \( \beta \)LKB1KO mice, we next explored the possibility that inflammatory macrophage infiltration might be involved in the present model. Consistent with this view, progressive macrophage invasion in areas of axon degeneration was revealed by periodic acid Schiff (PAS) staining for glycogen and other carbohydrate-containing macromolecules in active macrophages (Fig. 4B) (Sobolev, 1959). This was further confirmed by F4/80 immunofluorescence staining for these cells (Leenen et al., 1994) (Fig. 4C). However, neither the thoracic areas of the spinal cord of \( \beta \)LKB1KO mice before the onset of hind-limb dysfunction, nor the cervical or lumbar segments from these mice after paralysis, showed any positivity for PAS or F4/80 staining (data not shown).

Deletion of AMPK\( \alpha_1 \) and AMPK\( \alpha_2 \) catalytic subunits does not affect spinal cord morphology or motor function

Given the role of AMPK in mediating the effects of LKB1 in the control of cell polarity in other systems (see the Introduction), we further investigated the role of this enzyme in mediating the spinal cord degeneration observed in \( \beta \)LKB1KO mice. In contrast to animals deleted for \( LKB1 \) in the spinal cord and other regions using the RIP2-Cre deleter strain, mice generated using the same transgene to delete both AMPK\( \alpha_1 \) and AMPK\( \alpha_2 \) subunits in the same cell types (\( \beta \)AMPKdKO mice) (Sun et al., 2010a) did not display any locomotor abnormalities (Fig. 5A) nor altered survival curves (not shown) compared with heterozygous or wild-type animals. Moreover, close examination of the thoracic and other segments of the spinal cord from \( \beta \)AMPKdKO mice did not reveal
any changes in axon or myelin formation (Fig. 5B). Thus, LKB1 seems to control axon stability in the spinal cord independently of AMPK.

DISCUSSION

RIP2-Cre-mediated LKB1 deletion leads to hind-limb paralysis by affecting axon stability

We recently noted, in performing a metabolic characterization of mice deleted for LKB1 in the endocrine pancreas and a restricted set of CNS neurons using a RIP2-Cre transgene (Sun et al., 2010b), that older animals became paralyzed. The principal aim of the present study was thus to dissect the pathology behind this change and, in doing so, to determine the role of LKB1 in regulating neuronal polarity and survival in the CNS in vivo.

Although mice null for LKB1 throughout the body die before E11.5, the use of an Emx1-Cre deleter strain to allow deletion in pyramidal neuron progenitors demonstrated that LKB1 is required for the polarization of cultured neurons from the neonatal hippocampus and cortex (Barnes et al., 2007; Shelly et al., 2007). We therefore reasoned that LKB1 might play a similar role in axon development and, importantly, in signal transmission along the spinal cord. Given the crucial role of the spinal cord for the normal control of motor function, we further reasoned that deletion of LKB1 in neurons that extend their axons along the spinal cord and in neurons in the spinal cord might allow us to study the importance of LKB1 function in neurons in the adult animal.

We therefore generated a mouse model in which LKB1 was deleted by RIP2-driven Cre expression. In addition to near complete loss of LKB1 in pancreatic β-cells, resulting in a substantial increase in pancreatic β-cell mass and hyperinsulinism (Sun et al., 2010b), this approach also led to the elimination of LKB1 from a subset of neurons within the CNS (see Results and below), including those in the mid and ventral brain (Wicksteed et al., 2010) and spinal cord (this study). We would stress that it is unlikely that increased circulating insulin levels (Sun et al., 2010b) drive the dramatic alterations that we observed in CNS neuron survival within the spinal cord. Thus, similar elevations in circulating insulin occur in a number of other transgenic mouse models as a result of β-cell hyperplasia (Remedi et al., 2006; Hennige et al., 2003; Mori et al., 2009b), with no reported effects on CNS function, motor control or mortality.

The striking finding of this study is that RIP2-Cre-mediated LKB1 deletion in a restricted set of neurons leads to severe hind-limb dysfunction and paralysis in young adult mice and eventually to premature death. The white matter of thoracic spinal cord of βLKB1KO mice displayed morphological changes, displaying some of the characteristics of axon degeneration: large foci of disrupted, vacuolized and demyelinated axons with disorganized microtubules. The above changes were accompanied by macrophage infiltration, a feature that is very similar to that found in humans (Becerra et al., 1995; Buss et al., 2004) and rats (Zhang and Guth, 1997; Buss and Schwab, 2003) after spinal cord injury, and in P301L tau-expressing transgenic mice, in which the microtubules of spinal cords were also disrupted (Lewis et al., 2000; Lin et al., 2005). To characterize the neurons in which LKB1 was possibly deleted, transverse sections of thoracic spinal cord from βLKB1KO mice were closely assessed and the affected foci demonstrated widespread areas of axon degeneration in spinal tracts containing axons involved in predominantly ascending sensory pathways. Although deletion of LKB1 in βLKB1KO mice was also seen in spinal cord, given that most of the neuron cell bodies of the degenerated axons in this location reside in the brain,
we propose that deletion of LKB1 in these neurons in the brain leads to interruption of cell polarity and axon degeneration in the spinal cord, which eventually causes hind-limb paralysis. Notably, examination of neuron cell morphologies in the forebrain cortex, hypothalamus and cerebellum, regions that have been shown by others to express Cre (Wicksteed et al., 2010), did not reveal any obvious axon degeneration phenotype. This observation might, however, reflect the expression of Cre in a limited population of neurons in these areas, such that axon degeneration, if it occurred at all, was difficult to observe. What molecular mechanism underlies the effects of LKB1 deletion in neurons that form axons along the spinal cord? Deletion of LKB1 in pyramidal neurons from neonatal cortex shows disruption of axon formation, with decreased SAD-A/B phosphorylation and the phosphorylation of its downstream target tau (Barnes et al., 2007). Although it is difficult to monitor phosphorylation levels of SAD-A/B or tau in RIP2-Cre neurons owing to the limited distribution of these neurons in the wide area of brain and low expression of Cre in these neurons, the work of Barnes and his colleagues suggest that an LKB1-SAD-tau (phosphorylated on Ser262) signalling pathway is important in controlling neuronal polarity in spinal cord. In addition, transgenic mice overexpressing human tau protein containing the FTDP-17 mutation (P301L) display behavioural abnormalities with symptoms of Wallerian degeneration in the spinal cord but with increased tau (S262) phosphorylation (Lewis et al., 2000; Lin et al., 2005). Interestingly, Biernat and Mandelkow (Biernat and Mandelkow, 1999) reported that hypophosphorylation of tau (S262), resulting from mutation of the KXGS domain, inhibited outgrowth of extensions in Sf9 cells. Conversely, hyperphosphorylation of tau at S262 destabilized microtubules and axons (Biernat et al., 1993; Biernat and Mandelkow, 1999). Finally, Kishi and colleagues observed reduced tau (S262) phosphorylation and reduced axon extension in neurons from hippocampus and cortex of SAD-A/B-null mice (Kishi et al., 2005). Thus, the maintenance of an appropriate but limited degree of tau phosphorylation is likely to be essential for normal neuronal polarization and stability.
Nonetheless, direct evidence for this contention would require the simultaneous inactivation of SAD-A/B in RIP2-Cre neurons, and thus lies outside the scope of the present study.

Might the decreased phosphorylation and activity of other targets for LKB1 be involved in neuronal degeneration? Importantly, deletion of both AMPK catalytic (α) subunits in the same cells as those affected by LKB1 deletion failed to lead to neuronal degeneration (this study). Likewise, deletion of tuberous sclerosis complex 1 (TCS1), a downstream target of AMPK and involved in the control of the mammalian target of rapamycin (mTOR) and in regulating neuron polarity (Buckmaster et al., 2009; Biernat et al., 2002; Terabayashi et al., 2007), has been reported to lie downstream of LKB1 (Wang et al., 2007). However, although MARK2 knock-out mice displayed dwarfish, no obvious behavioural deficiencies were reported (Bessone et al., 1999). The latter observation again argues against the involvement of an LKB1-MARK2-tau signalling pathway in controlling axon development in the spinal cord of βLKB1KO mice.

We demonstrate here that LKB1 is essential to maintain normal polarity and function of a crucial subset of neurons in the brain. Mice deleted for LKB1 in these cells might provide a useful animal model for studying axon degeneration and certain neurodegenerative disorders in humans.

METHODS

Generation of mutant mice lacking LKB1, or AMPKα1 and AMPKα2, in the spinal cord

Mice null for LKB1 (βLKB1KO) (Sun et al., 2010b) and PRKAA1 (AMPKα1 and α2; βAMPKdKO) (Sun et al., 2010a) in the spinal cord, pancreatic β-cell and a restricted group of hypothalamic neurons were generated as previously described. Neither LKB1 heterozygous (LKB1β1/+) nor RIP2-Cre-recombinase-positive (wild-type) mice displayed symptoms of paralysis or abnormal spinal cord morphology and were used as controls for βLKB1KO mice throughout.

Mouse maintenance and diet

Mice were housed at two to five animals per cage in a pathogen-free facility with a 12-hour light-dark cycle. Animals were fed ad libitum with a standard mouse chow diet (Research Diet, New Brunswick, NJ). βLKB1KO mice were kept alive for 2 weeks after the initial onset of hind-limb dysfunction. Subsequently, mice were culled by cervical dislocation. All in vivo procedures were approved by the UK Home Office according to the Animals (Scientific Procedures) Act 1986 and were performed at the Central Biomedical Service, Imperial College London, UK.

Body weight and foot print (paralysis) measurements

Fed mice were weighed 1 and 2 weeks after the first onset of paralysis. Weight increases after paralysis were calculated. To measure locomotion by following foot prints, both fore-paws were immersed in red ink and hind-paws were immersed in blue ink before mice were allowed to walk freely on white filter paper (Whatman).

RNA extraction and qRT-PCR

Total cellular RNA from the cervical, thoracic and lumbar regions of mouse spinal cord and hypothalamus was obtained using TRIzol reagent (Invitrogen, Paisley, UK) and RNA was further purified.
against DNA contamination with a DNA-free kit (Applied Biosystems, Warrington, UK). Total RNA (1.5-2 µg) was then reverse transcribed into cDNA with a high-capacity reverse transcription kit (Applied Biosystems, Warrington, UK) according to the manufacturer’s instructions. To detect the deletion of LKB1 exons 3-6, two pairs of primers within exon 1 (LKB1 fwd: 5’-AGGTGAAGGAGGTGCTGG-3’) and 8 (LKB1 rev: 5’-TCTGGCCCTTGTTGGATA-3’) were designed. To detect deletion of the allele encoding AMPKα1, the primers used were: Ampkα1-knockout_forward: 5’-CACCCTACATACATAAA-3’; Ampkα1-knockout_reverse: 5’-AAACACTCGTTGCCT-3’. The size of product generated from the wild-type allele was 588 bp, whereas that from the AMPKα1-knockout allele was 408 bp. To detect the deletion of the allele encoding AMPKα2, the primers used were: Ampkα2-knockout_forward: 5’-GTCTGCGTGGATGACTG-3’; Ampkα2-knockout_reverse: 5’-AGCTGGTCTTGAGGGTCA-3’. The size of wild-type-allele-derived product was 588 bp and that of the AMPKα2-knockout was 375 bp.

**Western (immuno)blot analysis**

Mouse spinal cord was snap frozen in liquid nitrogen before lysis in ice-cold buffer [in mM: 50 Tris- HCl (pH 7.4, 4°C), 150 NaCl, 1 NaF, 1 Na2VO3, 1 ethylenediaminetetraacetic acid, 1 phenylmethylsulfonyl fluoride, 1% Nonidet P40, 0.25% sodium deoxycholate, and protease inhibitor cocktail (Roche, Burgess Hill, UK)]. Protein (50 µg) extracted from cervical, thoracic and lumbar regions of the spinal cord were loaded onto SDS-PAGE gels (10% Tris-acrylamide gel) for analysis.

**Immunohistochemistry, and H&E, LFB and PAS staining**

Different segments of the spinal cord were fixed in 10% neutral formalin overnight and embedded in paraffin wax before sectioning into slices of ~7 µm [for immunofluorescent and diaminobenzidine (DAB) staining] or 12 µm (for LFB and PAS staining). Slides were submersed sequentially in 100% [vol/vol] xylene followed by decreasing concentrations of industrial methylated spirits to remove paraffin wax. Antigen epitopes were then retrieved (de-crosslinked) in Tris-EDTA-0.05% [vol/vol] Tween buffer (pH 9.0) or 10 mM sodium citrate buffer (pH 6.0). Slides were subsequently blocked in 5% [vol/vol] goat serum in Tris-buffered saline with 0.05% [vol/vol] Tween (TBS-T) for 20 minutes at room temperature and then incubated overnight at 4°C in a mixture of primary antibodies at the concentrations indicated. After being washed in TBS-T three times for 5 minutes each, slices blotted with primary antibodies were either visualized with Alexa-Fluor-568- or -488-conjugated IgG (1:500; Invitrogen, Paisley, UK) under fluorescence microscopy using an Olympus IX-81 microscope (10× objective lens; data collection with cell®R software) or further incubated with biotinylated secondary antibodies (universal anti-mouse/anti-rabbit antibody) and subsequently revealed with peroxidase substrate using DAB (Vector Laboratories, Peterborough, UK).

For H&E staining, rehydrated slices were submerged in haematoxylin for 5 minutes. After being washed in water, slices were dipped in 1% [vol/vol] hydrogen chloride in 70% methanol [vol/vol] five times, followed by staining with 1% eosin [wt/vol] for 30 seconds.

For LFB staining, de-waxed sections were immersed in 0.1% LFB solution [wt/vol] (Sigma-Aldrich, Dorset, UK) at 60°C overnight.
Disease Models & Mechanisms

LK1 and axon degeneration

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COMPETING INTERESTS

The authors declare no competing or financial interests.

AUTHOR CONTRIBUTIONS

G.S. performed experiments and drafted the manuscript; R.R. and I.L. designed the experiments and edited the manuscript; G.A.R. designed the research and wrote the manuscript.

SUPPLEMENTARY MATERIAL

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