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Cell Injury, Repair, Aging and Apoptosis

Two Closely Related Ubiquitin C-Terminal Hydrolase Isozymes Function as Reciprocal Modulators of Germ Cell Apoptosis in Cryptorchid Testis

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The experimentally induced cryptorchid mouse model is useful for elucidating the *in vivo* molecular mechanism of germ cell apoptosis. Apoptosis, in general, is thought to be partly regulated by the ubiquitin-proteasome system. Here, we analyzed the function of two closely related members of the ubiquitin C-terminal hydrolase (UCH) family in testicular germ cell apoptosis experimentally induced by cryptorchidism. The two enzymes, UCH-L1 and UCH-L3, deubiquitinate ubiquitin-protein conjugates and control the cellular balance of ubiquitin. The testes of gracile axonal dystrophy (*gad*) mice, which lack UCH-L1, were resistant to cryptorchid stress-related injury and had reduced ubiquitin levels. The level of both anti-apoptotic (Bcl-2 family and XIAP) and prosurvival (pCREB and BDNF) proteins was significantly higher in *gad* mice after cryptorchid stress. In contrast, *Uchl3* knockout mice showed profound testicular atrophy and apoptotic germ cell loss after cryptorchid injury. Ubiquitin level was not significantly different between wild-type and *Uchl3* knockout mice, whereas the levels of Nedd8 and the apoptotic proteins p53, Bax, and caspase3 were elevated in *Uchl3* knockout mice. These results demonstrate that UCH-L1 and UCH-L3 function differentially to regulate the cellular levels of anti-apoptotic, prosurvival, and apoptotic proteins during testicular germ cell apoptosis. *(Am J Pathol 2004, 165:1367–1374)*

In the ubiquitin-proteasome system, the levels of poly- and monoubiquitin are strictly controlled by the balance of two groups of specific enzymes: ubiquitinating enzymes (E1, E2, and E3) and deubiquitinating enzymes (DUBs).[1,2] DUBs are subdivided into ubiquitin C-terminal hydrolases (UCHs) and ubiquitin-specific proteases (UBPs).[3,4] The genes for at least four UCHs, UCH-L1 and UCH-L3, UCH-L4, and UCH-L5, have been identified in mice.[5,6] Among them, UCH-L1 and UCH-L3 predominate; these isozymes have 52% amino acid identity and share significant structural similarity;[7] however, the distribution of these two isozymes is quite distinct in that UCH-L3 mRNA is expressed ubiquitously whereas UCH-L1 mRNA is selectively expressed in the testis/ovary and neuronal cells.[7–10] Despite the high-sequence homology, the *in vitro* hydrolytic activities of these two enzymes differ significantly. The activity (Kcat) of UCH-L3 is more than 200-fold higher than UCH-L1 when a fluorogenic ubiquitin substrate is used.[11] In addition to its relatively weak hydrolase activity, UCH-L1 exhibits dimerization-dependent ubiquityl ligase activity.[11] In contrast, UCH-L3 has little or no ligase activity compared with UCH-L1.[11] It was recently suggested that UCH-L1 has anti-proliferative activity in tumor cells, and that its expression is induced in response to tumor growth.[12] Furthermore, UCH-L1 associates with monoubiquitin and prolongs ubiquitin half-life in neurons.[13] Other work demonstrated that UCH-L3 binds to Nedd8 and subsequently processes its C-terminus.[14] Nedd8 is a small ubiquitin-like protein that shares with ubiquitin the ability to be conjugated to a lysine residue in a substrate protein.[15] Covalent conjugation of proteins by Nedd8 is an important form of the posttranscriptional modification and plays a critical role in many cellular processes.[16] These conju-

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gates are regulated by a large number of deconjugating enzymes. This activity is unique to UCH-L3 because UCH-L1 is relatively weak to cleave the C terminus of Nedd8.[14–16] Collectively, these data suggest that the two mouse isozymes, UCH-L1 and UCH-L3, have distinct but overlapping functions. In addition, we recently found that *gad* mice, which lack UCH-L1 expression, show reduced retinal cell apoptosis in response to ischemia, suggesting that UCH-L1 may promote apoptosis.[17]

Our previous work focused on the possibility that UCH-L1 and UCH-L3 exhibit functional diversity during spermatogenesis. We showed that both UCH-L1 and UCH-L3 are strongly but reciprocally expressed in the testis during spermatogenesis,[18] suggesting that each isozyme may have a distinct function in the testis. To elucidate the pathophysiological roles of these two isozymes in the testis, our present work examines the extent of heat-induced stress using experimentally induced cryptorchidism in *Uchl3* knockout[7] and *gad* mice.[8] Normally, the testes are maintained in the scrotum at a temperature lower than that of the abdomen. Exposure of a testis to higher body temperature via experimentally induced cryptorchidism results in rapid degeneration of testicular germ cells.[19–22] Recent studies show that testicular germ cell degeneration in cryptorchid testes occurs via apoptosis, and that protein and lipid oxidation, along with p53 promote germ cell death.[23–25] The ubiquitin-proteasome system is required for the subsequent degradation of the damaged testicular germ cells.[26–28] Here, we show that both UCH-L1 and UCH-L3 have reciprocal functions in testicular germ cells during cryptorchid-induced apoptosis. Our data show that the absence of UCH-L1 causes resistance to cryptorchid-induced testicular germ cell apoptosis, and that the knockout of UCH-L3 promotes germ cell apoptosis after cryptorchid injury.

Materials and Methods

Animals

We used 8-week-old *Uchl3* knockout (C57BL/6J)[7,18] and *gad*[8,18,29] (CBA/RFM) male mice. *Uchl3* knockout mice were generated by the standard method using homologously recombinant ES cells, and the knockout line was back-crossed several times to C57BL/6J mice.[7] The *gad* mouse is an autosomal recessive mutant that was obtained by crossing CBA and RFM mice.[8] The *gad* line was maintained by intercrossing for more than 20 generations.[8,29] Both strains were maintained at our institute. Animal care and handling were in accordance with institutional regulations for animal care and were approved by the Animal Investigation Committee of the National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo, Japan.

Unilateral Experimental Cryptorchidism

Unilateral cryptorchidism was experimentally induced under pentobarbital anesthesia (Abbott Laboratories, North Chicago, IL).[20,22] Briefly, a midline abdominal incision was made, and the left testis was displaced from scrotum and fixed to the upper abdominal wall. The right testis remained in the scrotum as an intact control within the same animal. At 0, 4, 7, and 14 days after the operation, four control and four cryptorchid testes were harvested to determine testis weight.

Histological and Immunohistochemical Assessment of Testes

Testes were embedded in paraffin wax after fixation in 4% paraformaldehyde, sectioned at 4-μm thickness, and stained with hematoxylin and eosin.[29] Light microscopy was used for routine observations. For immunohistochemical staining, the sections were incubated with 10% goat serum for 1 hour at room temperature, followed by incubation overnight at 4°C with a rabbit polyclonal antibody against ubiquitin (1:500; DakoCytomation, Glostrup, Denmark) or Nedd8 (1:500; Alexis Biochemicals, San Diego, CA) diluted in phosphate-buffered saline (PBS) containing 1% bovine serum albumin. Sections were then incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:200; Jackson ImmunoResearch, West Grove, PA) for 1 hour at room temperature and examined by confocal laser-scanning microscopy (Olympus, Tokyo, Japan).

Apoptotic cells in testicular tissues were identified by terminal deoxynucleotidyl transferase (TdT)-mediated nick-end labeling (TUNEL) using the DeadEnd Fluorimetric TUNEL system kit (Promega, Madison, WI) and the anti-PARP p85 fragment pAb (Promega) according to the manufacturer's instructions.

Quantitative Analysis of Apoptotic Germ Cells

The number of apoptotic cells was determined by counting the positively stained nuclei in 30 circular seminiferous tubule cross-sections per testis section.[23,29] The proportion of seminiferous tubules containing apoptotic germ cells was calculated by dividing the number of seminiferous tubules containing apoptotic cells by the total number of seminiferous tubules. The incidence of apoptotic cells per apoptotic cell-containing seminiferous tubule was categorized into three groups, defined as 1 to 5, 6 to 10, and >11 positive cells.

Western Blotting

Western blots were performed as previously reported.[8,18,29] Total protein (5 μg/lane) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 15% gels (Perfect NT Gel; DRC, Japan). Proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA) and blocked with 5% nonfat milk in TBS-T [50 mmol/L Tris base, pH 7.5, 150 mmol/L NaCl, 0.1% (w/v) Tween-20]. The membranes were incubated individually with one or more primary antibodies to UCH-L1 and UCH-L3 (1:1000; peptide antibodies[18]), Bcl-2, Bcl-xL, Bax, p53, and caspase-3, (1:1000; all from Cell Signaling Technology, Beverly, MA), phosphorylated cyclic AMP response element-binding protein (pCREB, 1:500; Upstate Biotech-

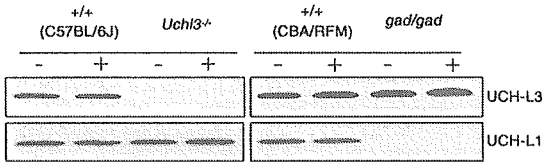

Figure 1. Western blotting analyses of both UCH-L3 and UCH-L1 in the testes of *gad* and *Uchl3* knockout mice, respectively, on day 4 after cryptorchid injury. Scrotal and cryptorchid testes did not differ significantly with respect to protein expression (−, scrotal testes; +, cryptorchid testes).

nology, Waltham, MA), brain-derived neurotrophic factor (BDNF, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA), XIAP (1:500; Transduction Laboratories, Franklin Lakes, NJ), polyubiquitin (1:1000, clone FK-2; Medical & Biological Laboratories, Nagoya, Japan), monoubiquitin (1:1000, u5379; Sigma-Aldrich, St. Louis, MO), and Nedd8 (1:1000; Alexis Biochemicals, San Diego, CA). Blots were further incubated with peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (1:5000; Pierce, Rockford, IL) for 1 hour at room temperature. Immunoreactions were visualized using the SuperSignal West Dura extended duration substrate (Pierce) and analyzed with a Chemi-Imager (Alpha Innotech, San Leandro, CA). Each protein level was relatively quantificated after analysis with a ChemiImager using AlphaEase software.

Statistical Analysis

The mean and SD were calculated for all data (presented as mean ± SD). One-way analysis of variance was used for all statistical analyses.

Results

Level of Two UCH Isozymes in Scrotal and Cryptorchid Testes from Uchl3 Knockout and Gad Mice

We first confirmed the lack of UCH-L3 protein in the testes from *Uchl3* knockout mice by Western blotting (Figure 1). Similarly, we did not detect UCH-L1 protein in the testes of *gad* mice (Figure 1), as we previously observed.[13] Thus, in a biochemical sense, *gad* mice are analogous to *Uchl1*-null mice.[8,13] Compensatory level of UCH-L3 and UCH-L1 in *gad* and *Uchl3* knockout mice, respectively, was not observed (Figure 1; compare UCH-L3/UCH-L1 level with that of wild-type control mice). Experimental cryptorchidism did not affect UCH-L3 level in *gad* or wild-type control mice. Similarly, cryptorchidism did not affect UCH-L1 level in *Uchl3* knockout and wild-type control mice (Figure 1). Quantitative reverse transcriptase-polymerase chain reaction analysis showed that transcription from the *Uchl3* and *Uchl1* in both scrotal and cryptorchid testes from *gad* and *Uchl3* knockout mice was not significantly different from that measured in the corresponding wild-type control mice (data not shown). These results suggest that the level of UCH-L3 is regulated independently of UCH-L1 in the mouse testis, and that cryptorchid injury does not affect the level of either protein.

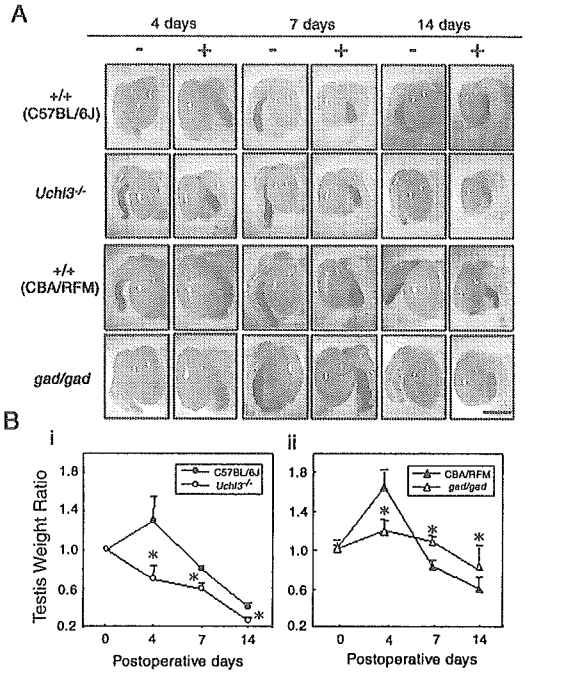

Figure 2. Comparison of testicular sizes and weights after experimental cryptorchidism. **A:** Gross images of changes in testicular size throughout time in two wild-type (C57BL/6J and CBA/RFM), *Uchl3* knockout, and *gad* mice (−, scrotal testes; +, cryptorchid testes). **B:** Ratio of cryptorchid to scrotal testis weight on days 0, 4, 7, and 14 after injury. i: Throughout time, the ratio for *Uchl3* knockout mice (**open circles**) differed significantly compared with wild-type mice (**filled circles**). ii: The ratio for *gad* mice (**open triangles**) did not differ significantly throughout time compared with wild-type mice (**closed triangles**). ($n = 4$; *, $P < 0.05$). Scale bar, 5 mm. Original magnifications, ×40.

Changes in Testicular Weight and Structure in Cryptorchid Uchl3 Knockout and Gad Mice

Unilateral cryptorchidism was surgically induced in *Uchl3* knockout and *gad* mice, and testes were evaluated on days 0, 4, 7, and 14 after the operation (Figure 2). Nonoperated (scrotal) testes served as controls for the evaluation of testicular weight and histochemistry. Cryptorchid testes from *Uchl3* knockout mice appeared smaller than the nonoperated controls at each time point, whereas the size of the cryptorchid testes from *gad* mice was similar to the controls (Figure 2A). Figure 2B shows the time course of the ratio of testicular weight of cryptorchid testes to scrotal testes. In wild-type mice (C57BL/6J and CBA/RFM), the ratio transiently increased 4 days after cryptorchid injury, most likely a consequence of inflammation-induced fluid accumulation[22,23] and biochemical changes observed. The ratio for these mice subsequently decreased below 1.0 by day 7. The ratio remained ~1.0 in *gad* mice (range,1.15 ~ 0.85), whereas it decreased significantly in *Uchl3* knockout mice compared with wild-type mice (Figure 2B). These results demonstrate that testes from *Uchl3* knockout and *gad* mice differ in their response to experimental cryptorchidism.

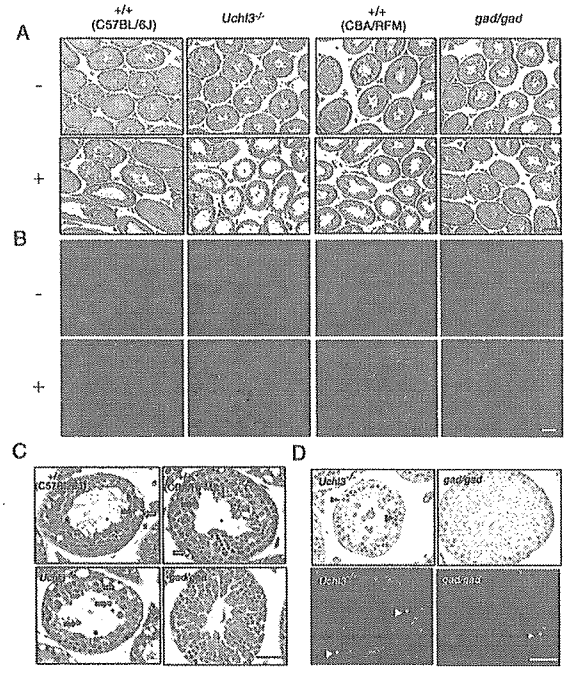

Figure 3. Histology and TUNEL staining of testicular cross sections after experimental cryptorchidism. **A:** Morphological analysis of seminiferous tubules on day 7 after cryptorchid injury. Note the germ cell loss and atrophy in cryptorchid testes compared with uninjured controls. (−, scrotal testes; +, cryptorchid testes). **B:** TUNEL staining of testicular cross-sections on day 7 after cryptorchid injury. Green fluorescence, TUNEL-positive cells; red fluorescence, nuclei stained with propidium iodide. **C:** Magnified cryptorchid testes sections. Pyknotic bodies (**filled arrows**) and Sertoli cell vacuolization (**open arrows**) were evident in cryptorchid testes of *Uchl3* knockout and the two wild-type (C57BL/6J and CBA/RFM) mice on day 7 after injury. **D:** PARP analysis to detect apoptotic germ cells in cryptorchid testes of *Uchl3* knockout and *gad* mice on day 7 after injury. The detection of apoptotic germ cells (**arrowheads, top**) by PARP analysis was consistent with that of apoptotic germ cells (**arrowheads, bottom**) by TUNEL analysis. Scale bar, 50 μm. Original magnifications: **A** and **B** ×100; **C** and **D** ×200.

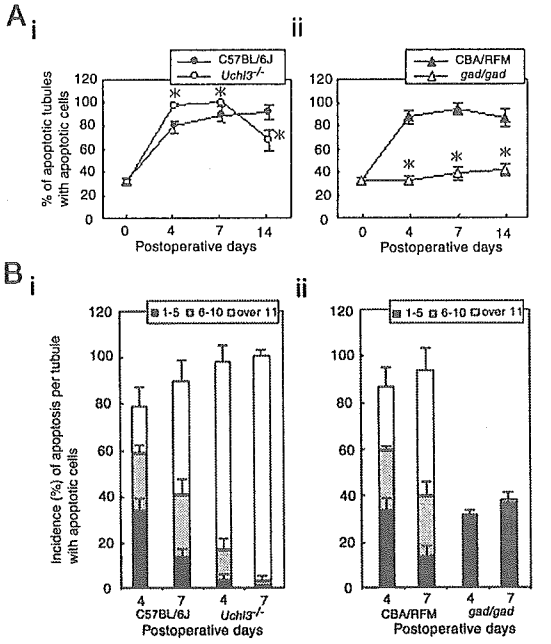

Figure 4. Quantitation of testicular germ cell apoptosis in testes after experimental cryptorchidism. **A:** The percentage of seminiferous tubules containing apoptotic germ cells in cryptorchid testes on days 0, 4, 7, and 14 after injury. **i:** The increase in the percentage of tubules containing apoptotic cells in the cryptorchid testes of *Uchl3* knockout mice is statistically significant compared with wild-type mice on days 4, 7, and 14. Each value represents the mean ± SD; *, $P < 0.05$. **ii:** The percentage of apoptotic tubules in cryptorchid testes of *gad* mice is significantly different on days 4, 7, and 14 after injury. Each value represents the mean ± SD; *, $P < 0.01$. **B:** Incidence of apoptosis per seminiferous tubule with apoptotic germ cells on days 4 and 7 after injury. The incidence of seminiferous tubules containing >11 apoptotic germ cells is significantly increased ($P < 0.05$) in cryptorchid testes of *Uchl3* knockout mice compared with wild-type mice. **i:** Comparison with *Uchl3* knockout mice. **ii:** Comparison with *gad* mice. Each value represents the mean ± SD.

Testicular Germ Cell Apoptosis in Cryptorchid Uchl3 Knockout and Gad Mice

To explore the mechanism underlying the observed differences between *Uchl3* knockout and *gad* cryptorchid testes, we prepared histological cross-sections on day 7 after testicular injury (Figure 3, A and C). The presence of nuclear pyknosis, multinucleated giant cells, and Sertoli cell vacuolization with germ cell loss in the germinal epithelium is indicative of cryptorchid testes.[22,23] These hallmarks of testicular injury were the most remarkable characteristics of cryptorchid testes from *Uchl3* knockout mice, demonstrating profound testicular atrophy and germ cell loss compared with wild-type mice (Figure 3, A and C). In contrast, no nuclear pyknosis, cellular shrinkage, or germ cell loss was observed in cryptorchid testes from *gad* mice. Spermatocytes and early spermatids comprised the majority of affected cell types in cryptorchid testes (Figure 3, A and C).

Germ cell apoptosis was further examined by TUNEL and PARP assays in tissue sections from postoperative day 7 mice (Figure 3, B and D). All but the *gad* cryptorchid testes showed a time-dependent increase in germ cell apoptosis during experimental cryptorchidism; germ cell apoptosis was always found in tubules that had germ cell loss on days 4, 7, and 14 (Figure 3, B and D, and Figure 4). Compared to wild-type mice, the cryptorchid testes of *Uchl3* knockout mice showed a marked increase in apoptotic germ cells in response to testicular injury, whereas *gad* mice lacked cryptorchid-induced germ cell apoptosis (Figure 3B and Figure 4). By postoperative days 4 and 7, the percentage of seminiferous tubules containing apoptotic germ cells increased with statistical significance ($n = 4$) ($P < 0.05$) in cryptorchid testes of *Uchl3* knockout mice as compared with wild-type mice (Figure 4A). In addition, cryptorchid testes of *Uchl3* knockout mice showed a high incidence of seminiferous tubules containing >11 apoptotic germ cells on days 4 and 7 days as compared with wild-type mice (Figure 4B); however, germ cell apoptosis did not increase in cryptorchid testes of *gad* mice during postoperative days 4 to 14 ($P < 0.01$) (Figure 4, A and B).

Cellular Mono- and Polyubiquitin Level in Cryptorchid Uchl3 Knockout and Gad Mice

Ubiquitin is required for energy-dependent degradation of structurally altered proteins.[26] We previously reported that UCH-L1 binds ubiquitin and stabilizes ubiquitin turnover in neurons, and that the level of monoubiquitin is decreased in *gad* mice.[13] In a model of ischemic insult in the retina, ubiquitin induction was unexpectedly lower and ischemic

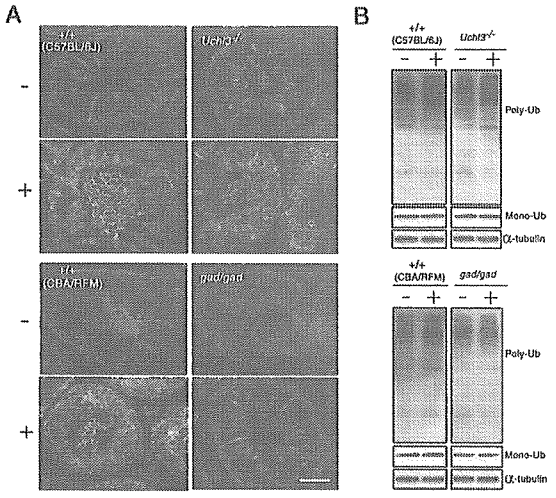

Figure 5. Immunohistochemical and Western blotting analyses of mono- and polyubiquitin in testes on day 7 after experimental cryptorchidism. **A:** Ubiquitin induction was not different between cryptorchid testes from *Uchl3* knockout and wild-type mice, whereas cryptorchid-induced ubiquitin induction in *gad* mice was reduced. Green fluorescence, ubiquitin-positive cells; red fluorescence, nuclei stained with propidium iodide. **B:** Polyubiquitin level in *Uchl3* knockout mice and the two wild-type (C57BL/6J and CBA/RFM) mice substantially increased after injury, whereas that in *gad* mice did not change significantly. Monoubiquitin level did not change after injury. Representative images from four independent experiments are shown (−, scrotal testes; +, cryptorchid testes). Scale bar, 50 μm. Original magnifications, ×200.

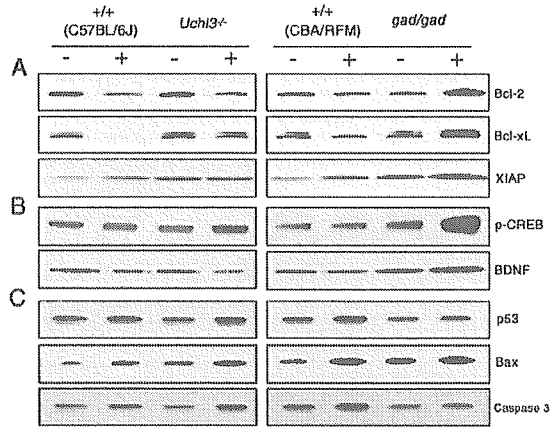

Figure 6. Western blotting analysis of anti-apoptotic, prosurvival, and apoptotic proteins in testes after experimental cryptorchidism. Total protein (5 μg per lane) was prepared from scrotal and cryptorchid testes on day 4 after cryptorchid injury. The expression levels of anti-apoptotic (**A**), prosurvival (**B**), and apoptotic (**C**) proteins in the cryptorchid testes of *Uchl3* knockout, *gad*, and the two wild-type (C57BL/6J and CBA/RFM) mice were significantly different compared with control mice. Representative images from four independent experiments are shown (−, scrotal testes; +, cryptorchid testes).

damage was weaker in the retina of *gad* mice (compared with wild-type mice) after ischemic insult.[17] To determine whether the increase in germ cell apoptosis in cryptorchid testes is associated with ubiquitin induction, we performed immunohistochemical analysis of testes from postoperative day 7 mice. Ubiquitin immunoreactivity increased substantially in cryptorchid testes from *Uchl3* knockout mice and the two wild-type mice, whereas those from *gad* mice showed only minor ubiquitin induction (Figure 5A). The scrotal testes of *Uchl3* knockout and *gad* mice did not show significant differences in ubiquitin induction compared with corresponding controls (Figure 5A). Interestingly, most of the increased ubiquitin induction was detected in spermatocytes and spermatids, consistent with the data on germ cell apoptosis after cryptorchid injury (Figure 3D and Figure 5A). Cryptorchid-induced polyubiquitin levels in the testes from *Uchl3* knockout and the two wild-type mice also increased substantially after injury, whereas the cryptorchid testes of *gad* mice showed no significant difference compared with scrotal testes (Figure 5B); however, the expression levels of monoubiquitin did not change significantly in any of the mice after cryptorchid injury.

Level of Anti-Apoptotic and Apoptotic Proteins in Cryptorchid Uchl3 Knockout and Gad Mice

We previously showed that anti-apoptotic proteins such as Bcl-2 and prosurvival proteins including phosphorylated cyclic AMP response element-binding protein (pCREB) are up-regulated in degenerated retina of *gad* mice.[17] These proteins are degraded by ubiquitination-mediated proteolysis.[30] We examined the expression of the Bcl-2 family proteins, XIAP, pCREB, and caspases to determine their role in testicular germ cell apoptosis after experimental cryptorchidism 4 days after injury in *Uchl3* knockout and *gad* mice. The level of anti-apoptotic proteins such as Bcl-2, Bcl-xL, and XIAP was up-regulated (323.8 ± 57.5, 262.3 ± 22.1, and 209.9 ± 11.7, respectively, as compared with wild type, 100) in the cryptorchid testes of *gad* mice compared with wild-type mice (Figure 6A). Additionally, pCREB, which is normally degraded in a ubiquitination-mediated manner,[30] was apparently highly up-regulated (259.0 ± 22.6, as compared with wild type, 100) in the cryptorchid testes of *gad* mice (Figure 6B). It has been demonstrated that pCREB activates genes that up-regulate trophic factors including BDNF.[31,32] Consistent with pCREB up-regulation, BDNF level also increased (203.0 ± 19.6, as compared with wild type, 100) in cryptorchid testes of *gad* mice (Figure 6B). Level was variable for anti-apoptotic, prosurvival, and apoptotic proteins in the cryptorchid testes of *Uchl3* knockout mice. The level of pCREB, p53, Bax, and caspase3 was slightly increased (169.9 ± 15.2, 152.6 ± 12.9, and 157.3 ± 14.0, respectively, as compared with scrotal testes, 100) in cryptorchid testes of *Uchl3* knockout mice compared with scrotal testes (Figure 6, B and C). Wild-type control mice had a similar expression level pattern except for pCREB. Because p53 acts as an upstream activator of Bax expression,[33] the observed Bax up-regulation after cryptorchid injury was consistent with the elevated p53 level in *Uchl3* knockout and wild-type control mice (Figure 6C). In contrast, BDNF was down-regulated (74.3 ± 7.7 as compared with wild type, 100) in cryptorchid testes of *Uchl3* knockout mice (Figure 6B). The down-regulation of BDNF combined with the up-regulation of pCREB suggests that BDNF might be regulated by another pathway that involves UCH-L3 but not pCREB.[34] Compared with scrotal testes, the expression of anti-apoptotic proteins decreased or was unchanged in cryptorchid testes of *Uchl3* knockout mice (Figure 6A).

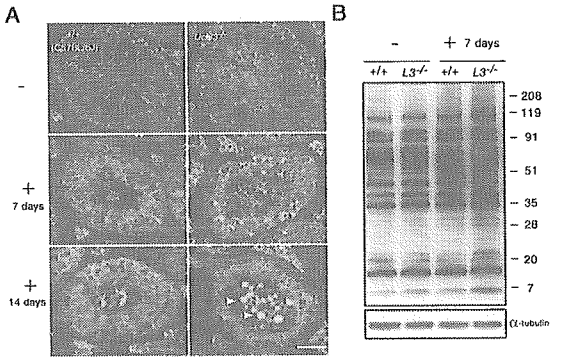

Figure 7. Immunohistochemical and Western blotting analyses of Nedd8 in testes from *Uchl3* knockout mice on days 7 and 14 after experimental cryptorchidism. **A:** Nedd8 induction in *Uchl3* knockout mice increased in both scrotal and cryptorchid testes. The shedding germ cells (**arrowheads**) in the cryptorchid testes of *Uchl3* knockout mice showed strong Nedd8 induction (−, scrotal testes; +7 days and + 4 days, cryptorchid testes). Green fluorescence, Nedd8-positive cells; red fluorescence, nuclei stained with propidium iodide. **B:** On day 7, the expression levels of Nedd8-conjugated proteins in *Uchl3* knockout mice were higher than in wild-type mice. Representative images of four independent experiments are shown. Scale bar, 50 μm. Original magnifications, ×200.

Nedd8 Level in Cryptorchid Uchl3 Knockout Mice

The varied expression levels of ubiquitin, anti-apoptotic, and apoptotic proteins in cryptorchid testes did not adequately explain the relatively exacerbated testicular atrophy and germ cell loss in *Uchl3* knockout mice compared with wild-type mice. We explored the underlying mechanism of this observation using the fact that UCH-L3 cleaves Nedd8.[14,16] We tested whether any change in Nedd8 expression correlated with greater testicular atrophy and germ cell loss in *Uchl3* knockout mice. Nedd8 immunoreactivity was highly detected in scrotal and cryptorchid testes from *Uchl3* knockout mice compared with wild-type mice (Figure 7A). The increased Nedd8 induction was mainly observed in spermatocytes and spermatids, and its expression pattern was similar to that of UCH-L3 during spermatogenesis.[18] These results suggest that Nedd8 may interact closely with UCH-L3 during testicular atrophy and germ cell loss. The cryptorchid testes of *Uchl3* knockout mice showed time-dependent and rapid Nedd8 induction compared with wild-type mice throughout the period 7 to 14 days after injury (Figure 7A). Moreover, the cryptorchid testes of *Uchl3* knockout mice showed strong Nedd8 induction in luminal shedding germ cells on day 14. An immunoblot of scrotal and cryptorchid testes proteins on day 7 confirmed the higher expression levels of Nedd8-conjugated proteins in *Uchl3* knockout mice as compared with wild-type mice (Figure 7B).

Discussion

During spermatogenesis, apoptosis controls germ cell numbers and eliminates defective germ cells to facilitate testicular homeostasis.[35–37] Recent studies indicate that ubiquitination targets proteins for degradation and modulates the turnover of various classes of short-lived signaling proteins.[28,38] Germ cell apoptosis after cryptorchid stress involves genes for various factors, such as Bcl-2 family proteins, p53, and caspases;[39–44] however, the impact of the ubiquitin system on the regulatory mechanisms of germ cell apoptosis is not fully understood. In a previous study, we used *gad* mice, which lack UCH-L1 expression, to show that neural cell apoptosis is suppressed after ischemic retinal injury *in vivo*.[17] These results suggest that UCH-L1 is involved in apoptosis-inducing pathways after stress. UCH-L1 and UCH-L3 are highly similar in sequence; however, UCH-L3 is expressed ubiquitously,[7] whereas UCH-L1 is selectively expressed in neurons and testes/ovaries.[8,9] We recently demonstrated that the expression of these UCH isozymes is differentially and developmentally regulated during spermatogenesis, and that UCH-L1 and UCH-L3 likely have distinct functions during different developmental phases.[18]

To understand the pathophysiological roles of UCH-L1 and UCH-L3 *in vivo*, two mutant mice, *Uchl3* knockout and *gad* mice, were examined after cryptorchid injury. The cryptorchid testes of the two mutant mice had fundamental differences after injury, in that testes of *Uchl3* knockout mice showed profound apoptosis-mediated germ cell loss, whereas *gad* mice were relatively resistant to injury (Figures 3 and 4). In addition, cryptorchid testes of *Uchl3* knockout mice showed greater testicular atrophy and germ cell loss than wild-type mice.

There are several proposed mechanisms for germ cell loss after experimental cryptorchidism.[21–23,45] The tumor suppressor protein, p53, is highly expressed in the testis and regulates both cell proliferation and apoptosis.[23,28,37] A role for p53 in experimental cryptorchidism has been demonstrated convincingly. The higher temperature of the testis caused by cryptorchidism induces p53-mediated apoptosis in the testis, and p53 overexpression results in increased germ cell apoptosis and decreased spermatozoa production.[23,46] In addition to p53, the Bcl-2 family and IAP (inhibitor of apoptosis protein) family are other major classes of intracellular apoptosis regulators.[47,48] The Bcl-2 family can be divided into anti-apoptotic members, such as Bcl-2, Bcl-xL, and Bcl-w, and proapoptotic members, such as Bax and Bak.[49] It has been suggested that the ratio of proapoptotic to anti-apoptotic Bcl-2 family members is important in determining whether a cell will undergo apoptosis.[49] A major function of the Bcl-2 family members appears to be the regulation of mitochondrial events, such as the release of proapoptotic factors.[50] The IAP family inhibits apoptosis primarily by inactivating and degrading proapoptotic proteins.[51] XIAP, a member of IAP family, can bind to and inhibit the proteinase activity of cellular caspase-3 and caspase-9, and thereby block the apoptotic process.[44,52,53]

With regard to cryptorchid injury, the balance between the expression of apoptosis-inducing and apoptosis-protecting proteins constitutes one possible mechanism underlying the observed germ cell apoptosis and protection from apoptosis in *Uchl3* knockout and *gad* mice, respectively. In *gad* mice, cryptorchid injury caused a large increase in the anti-apoptotic proteins Bcl-2, Bcl-xL, and XIAP, consistent with our previous report using retina.[17]

In addition, the expression levels of the prosurvival proteins pCREB and BDNF also increased in *gad* mice. Consistent with these results, caspase-3 expression was suppressed in *gad* mice. Cryptorchid testes of *Uchl3* knockout mice showed slightly increased expression of the apoptotic proteins p53, Bax, and caspase-3 after injury, although similar increases were also observed in wild-type control mice. In total, these results suggest that UCH-L1 plays a role in balancing the expression of apoptosis-inducing and apoptosis-protecting proteins. In contrast, UCH-L3 seems to resist germ cell apoptosis after cryptorchid injury.

Recent studies demonstrate that many molecules in the cellular apoptosis machinery, such as p53,[39,41] Bcl-2 family,[42,43,54] XIAP,[52] and caspase[44] members, are targets for ubiquitination.[28] This suggests that ubiquitination is one of the major mechanisms by which apoptotic cell death is regulated. UCH-L1 has been suggested to associate with monoubiquitin,[13] and the monoubiquitin pool is reduced in *gad* mice relative to wild-type mice. Protection from cryptorchid injury was reported in testes of mice expressing a mutant K48R ubiquitin,[22] suggesting that ubiquitin plays a critical role in processing or modulating testicular insults. Normally, damaged proteins are polyubiquitinated and degraded via the ubiquitin-proteasome system; however, if damaged proteins are not degraded as easily when monoubiquitin is either depleted or mutated, then germ cell death could be delayed.[17,22] Our results with the *gad* mouse suggest that ubiquitin induction plays a critical role in regulating cell death during cryptorchid injury-mediated germ cell apoptosis.

Uchl3 knockout mice exhibit severe retinal degeneration, suggesting that the UCH-L3-mediated ubiquitin pathway is involved in retinal homeostasis.[55] In the cryptorchid testes of *Uchl3* knockout mice, however, the profound testicular weight reduction and germ cell apoptosis after injury cannot be explained by ubiquitin induction alone. Our present results show that *Uchl3* knockout and wild-type mice have similar ubiquitin expression level in the testes, suggesting that UCH-L3 has another nonhydrolase activity in the ubiquitin-proteasome system. UCH-L3 also binds and cleaves the C-terminus of the ubiquitin-like protein, Nedd8.[14,56] This activity is unique to UCH-L3 because UCH-L1 does not cleave Nedd8. Thus, UCH-L3 appears to have dual affinities for ubiquitin and Nedd8. Our present results show that Nedd8 is strongly induced in scrotal testes of *Uchl3* knockout mice compared with those of wild-type mice (Figure 7). Cryptorchid testes of both *Uchl3* knockout and wild-type mice showed Nedd8 induction after injury, although the induction was higher in *Uchl3* knockout mice. These observations suggest that UCH-L3 may function as a deneddylating enzyme[16] *in vivo*, although further studies are necessary to clarify whether UCH-L3 interacts with Nedd8 during spermatogenesis.

In the present study, we demonstrate apparent reciprocal functions for the two deubiquitinating enzymes, UCH-L1 and UCH-L3, with respect to mediating injury after experimental cryptorchidism (Figure 8). Our results advance our understanding of the role of the ubiquitin-proteasome system in regulating apoptosis, and provide a unique opportunity for effective therapeutic intervention.

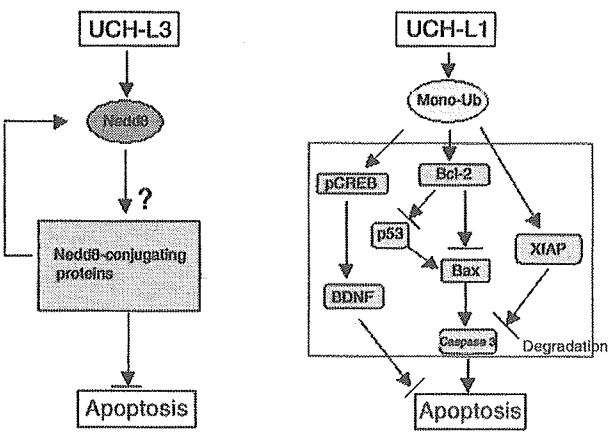

Figure 8. Differential function of the two UCH isozymes in response to experimental cryptorchidism. UCH-L3 has specificity for Nedd8. Cryptorchid injury results in protein damage and the accumulation of Nedd8-conjugated proteins. The accumulation of Nedd8-conjugated proteins in *Uchl3* knockout mice may contribute to profound germ cell loss via apoptosis. Hence, UCH-L3 might function as an anti-apoptotic regulator. UCH-L1 is involved in the maintenance of monoubiquitin levels. A deficiency in monoubiquitin results in delayed polyubiquitination and the accumulation of short-lived proteins after cryptorchid injury. Hence, UCH-L1 may function as a regulator of apoptosis.

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The slow Wallerian degeneration gene, *Wld*^{S}, inhibits axonal spheroid pathology in gracile axonal dystrophy mice

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Summary

Axonal dystrophy is the hallmark of axon pathology in many neurodegenerative disorders of the CNS, including Alzheimer's disease, Parkinson's disease and stroke. Axons can also form larger swellings, or spheroids, as in multiple sclerosis and traumatic brain injury. Some spheroids are terminal endbulbs of axon stumps, but swellings may also occur on unbroken axons and their role in axon loss remains uncertain. Similarly, it is not known whether spheroids and axonal dystrophy in so many different CNS disorders arise by a common mechanism. These surprising gaps in current knowledge result largely from the lack of experimental methods to manipulate axon pathology. The slow Wallerian degeneration gene, *Wld*^{S}, delays Wallerian degeneration after injury, and also delays 'dying-back' in peripheral nervous system disorders, revealing a mechanistic link between two forms of axon degeneration traditionally considered distinct. We now report that *Wld*^{S} also inhibits axonal spheroid pathology in gracile axonal dystrophy (*gad*) mice. Both gracile nucleus ($P < 0.001$) and cervical gracile fascicle ($P = 0.001$) contained significantly fewer spheroids in *gad/Wld*^{S} mice, and secondary signs of axon pathology such as myelin loss were also reduced. Motor nerve terminals at neuromuscular junctions continued to degenerate in *gad/Wld*^{S} mice, consistent with previous observations that *Wld*^{S} has a weaker effect on synapses than on axons, and probably contributing to the fact that *Wld*^{S} did not alleviate *gad* symptoms. *Wld*^{S} acts downstream of the initial pathogenic events to block *gad* pathology, suggesting that its effect on axonal swelling need not be specific to this disease. We conclude that axon degeneration mechanisms are more closely related than previously thought and that a link exists in *gad* between spheroid pathology and Wallerian degeneration that could hold for other disorders.

Keywords: axon; axonal spheroid; gracile axonal dystrophy; ubiquitin; Wallerian degeneration

Abbreviations: APP = amyloid precursor protein; *gad* = gracile axonal dystrophy; GFAP = glial fibrillary acidic protein; H & E = haematoxylin and eosin; NMJ = neuromuscular junction; PFA = paraformaldehyde; PNS = peripheral nervous system; *Wld*^{S} = slow Wallerian degeneration gene, mutation or mice; Wld^S = slow Wallerian degeneration protein; YFP = yellow fluorescent protein

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Introduction

Axonal dystrophy and spheroids are hallmarks of CNS axon pathology. Axonal spheroids are focal 10–50 μm diameter swellings, which are sometimes, but not always, terminal endbulbs, and are filled with disorganized neurofilaments,

tubules, organelles or multi-lamellar inclusions. Dystrophic axons are usually smaller swellings often associated with continuity of the axon. One or both of these aberrant axon morphologies is found in a wide range of CNS neurodegenerative disorders, including stroke (Dewar *et al.*, 1999), myelin disorders (Griffiths *et al.*, 1998), tauopathies (Lewis *et al.*, 2000; Probst *et al.*, 2000), amyotrophic lateral sclerosis (Tu *et al.*, 1996; Oosthuyse *et al.*, 2001; Howland *et al.*, 2002), traumatic brain injury (Cheng and Povlishock, 1988), Alzheimer's disease (Brendza *et al.*, 2003), Parkinson's disease (Galvin *et al.*, 1999), Creutzfeldt–Jakob disease (Liberski and Budka, 1999), HIV dementia (Raja *et al.*, 1997; Adle-Biassette *et al.*, 1999), hereditary spastic paraplegia (Ferreirinha *et al.*, 2004) and Niemann–Pick disease (Bu *et al.*, 2002). They also occur during normal ageing and secondarily in some serious illnesses (Sung *et al.*, 1981). In contrast, peripheral nervous system (PNS) axons undergo 'Wallerian-like' or 'dying-back' degeneration, even in diseases where CNS axons form swellings (Miura *et al.*, 1993; Lewis *et al.*, 2000; Oosthuyse *et al.*, 2001), although swellings do also occur in some rare PNS disorders (Miike *et al.*, 1986; Bomont *et al.*, 2000).

The roles of axonal swellings in disease are poorly understood, as illustrated by the following examples. First, in multiple sclerosis, many large spheroids are terminal endbulbs of transected axons but there are also a few 'en passant' swellings of similar shape and dimension (Trapp *et al.*, 1998) and many small dystrophic swellings (Ferguson *et al.*, 1997; Kornek *et al.*, 2000, 2001). It remains unclear whether these different types of swelling have common or different origins. Secondly, it is not clear whether disease-specific mechanisms lead to a common final pathway of axonal dystrophy, as in Alzheimer's disease, stroke and multiple sclerosis, and if so how they do this. Thirdly, it is not known why swellings predominate in distal axons in some diseases, such as gracile axonal dystrophy (*gad*) (Yamazaki *et al.*, 1988; Mukoyama *et al.*, 1989), caused by loss of ubiquitin C-terminal hydrolase l1 (*Uch-l1*) (Saigoh *et al.*, 1999), while in other diseases they occur in proximal axons, as in amyotrophic lateral sclerosis (Tu *et al.*, 1996) and tauopathy (Probst *et al.*, 2000). Finally, a better understanding is needed of the relationship between axon swelling and impaired axonal transport. Amyloid precursor protein (APP) accumulates in axonal swellings and spheroids in stroke (Dewar *et al.*, 1999), traumatic brain injury (Gentleman *et al.*, 1993), multiple sclerosis (Ferguson *et al.*, 1997), Creutzfeld–Jakob disease (Liberski and Budka, 1999), HIV dementia (Raja *et al.*, 1997; Adle-Biassette *et al.*, 1999) and *gad* (Ichihara *et al.*, 1995), indicating that axonal transport is impaired. However, it is not known whether axon swelling in these disorders is simply a consequence of impaired axonal transport, or whether it causes the transport defect, or both. These and other important questions remain unanswered largely because experimental methods to manipulate axonal swelling have not been available.

A mutant mouse gene, Wld^S, blocks a rate-limiting step common to Wallerian degeneration and diverse PNS axon disorders, including dysmyelination (Samsam *et al.*, 2003), motor neuronopathy (Ferri *et al.*, 2003) and Taxol toxicity (Wang *et al.*, 2002). Recently, Wld^S was reported to be effective in acute CNS lesions modelling stroke (Gillingwater *et al.*, 2004) and Parkinson's disease (Sajadi *et al.*, 2004) but its effect in a chronic CNS disease has not been reported. Wld^S is a chimeric gene (Conforti *et al.*, 2000) formed by a stable triplication (Coleman *et al.*, 1998; Mi *et al.*, 2003) encoding the N-terminus of multiubiquitylation factor Ube4b fused in-frame to nicotinamide mononucleotide adenylyltransferase (Nmnat1) plus a short novel sequence (Mack *et al.*, 2001). Nmnat1 appears to be sufficient to confer the phenotype *in vitro*, but it is not yet clear whether this holds *in vivo* (Coleman and Perry, 2002; Araki *et al.*, 2004). Wld^S protein appears to be restricted to the nucleus, so its effect on axons is mediated by other factors (Mack *et al.*, 2001), which may include the NAD-dependent deacetylase SIRT-1 (Araki *et al.*, 2004).

To study the relationship between axonal swelling and Wallerian degeneration, we crossed Wld^S and *gad* mice. Wld^S significantly reduced spheroid numbers without altering the first stages of *gad* pathogenesis, revealing a link between Wallerian degeneration and axonal spheroids in this disease that could extend to other disorders.

Methods

Origin, breeding and genotyping of mice

Homozygous C57BL/Wld^S spontaneous mutants were obtained from Harlan UK (Bicester, UK) and mated with heterozygous *gad* mice, kindly provided by Professor Keiji Wada and Dr Hitoshi Osaka (National Institute of Neuroscience, Tokyo, Japan), following a cross to C57BL/6 to ensure a more homogeneous genetic background. Thus, the genetic background of the experimental mice was 75% C57BL/6, 12.5% CBA/Nga, 12.5% RFM/Nga. Double heterozygotes were identified in the F1 generation by genotyping for *gad* (below) and intercrossed. *gad* homozygotes were identified by genotyping and selected for further study. Wld^S genotype was determined *post mortem* by pulsed-field gel electrophoresis of spleen DNA (Mi *et al.*, 2002). Hemizygous yellow fluorescent protein (YFP) mice of line YFP-H were obtained from Jackson Laboratories (Bar Harbor, MN, USA) and mated with *gad*/Wld^S double heterozygotes. Triple heterozygotes were then mated to *gad*/Wld^S double heterozygotes to produce *gad* homozygotes that were heterozygous for both Wld^S and YFP-H. For *gad* genotyping, tail genomic DNA was extracted at 3 weeks using the Nucleon II kit (Amersham Pharmacia), digested with *Pvu*II, and Southern blotted. It was then hybridized with a ^{32}P-labelled 764-bp probe generated by PCR from *gad* homozygous genomic DNA using primers 5′-ATCCAGGCGGCCCATGACTC-3′ and 5′-AGCTGCTTTGCAGAGAGCCA-3′. Positively hybridizing fragments indicative of the *gad* (0.75 kb) and wild-type (1.6 kb) alleles were then identified by autoradiography. To genotype for inheritance of the YFP-H transgene, the skin of a 1–2 mm ear punch at 21 days was pulled apart and fluorescent axons identified using a Zeiss Axiovert S100 inverted fluorescent microscope through the FITC filter.

Assessment of Wallerian degeneration

gad homozygotes that were heterozygous for *Wld*^{S} and hemizygous for the *YFP-H* transgene were anaesthetized prior to the onset of hindlimb weakness using intraperitoneal Ketanest (100 mg/kg; Parke Davis/Pfizer, Karlsruhe, Germany) and Rompun (5 mg/kg; Bayer, Leverkusen, Germany). The right sciatic nerve (upper thigh) was transected and the wound closed with a single suture. Five days later the mice were killed by cervical dislocation, the swollen first 2 mm of distal sciatic nerve was discarded and the next 2 mm was used for western blotting for heavy neurofilament protein as previously described (Mack *et al.*, 2001). The tibial nerve of the operated leg with a minimum of attached non-nervous tissue was processed for YFP fluorescence as follows. The nerve was stretched by ~10% by pinning onto a Sylgard (Du Pont) dish and fixed with 4% paraformaldehyde (PFA) (BDH Laboratory, UK) in 0.1 M phosphate-buffered saline (PBS) in the dark for 1 h. It was then incubated in 1% Triton X-100 (Sigma, Germany) in 0.1 M PBS for 10 min and washed three times with PBS before mounting in Vectashield (Vector Laboratories, USA). The degree of fragmentation of the representative subset of motor and sensory axons that are YFP-labelled was determined. For more detail, see Beirowski *et al.* (2004).

Preparation of gracile tract sections

Mice aged 126–130 days were anaesthetized using Ketanest and Rompun (100 mg/kg and 5 mg/kg intraperitoneally, respectively) or a higher dose as required for deep terminal anaesthesia. After sternotomy mice were killed by cardiac puncture and instantly intracardially perfused first with a solution containing 10 000 IE/l heparin (Liquemin N 25000; Hoffmann-La Roche) and 1% procainhydrochloride in 0.1 M PBS for 30 s and then with fixative (4% paraformaldehyde in 0.1 M PBS) for 10 min. Brain and spinal cord were carefully removed, further fixed in 4% PFA/0.1 M PBS overnight and extensively washed in 0.1 M PBS. Fixed tissues were extensively rinsed in fresh 0.1 M PBS, dehydrated in an ascending ethanol series and subsequently embedded in paraffin (Paraplast; Sherwood Medical Co., St Louis, MO, USA) applying standard histology techniques. Coronal serial sections (6 μm) were made using a Type HM355 microtome (Microm GmbH) from the entire gracile nucleus in medulla oblongata and cervical gracile fascicle starting at level 535 (Sidman *et al.*, 1971). Serial paraffin sections were mounted on conventional glass slides for use in haematoxylin and eosin (H & E) staining or on poly-L-lysine-coated slides for use in Luxol Fast Blue staining and immunocytochemistry, alternating normally every 2–3 sections. Distinction between gracile nucleus and cervical gracile fascicle was made by applying histomorphological criteria for the typical shapes of coronal sections.

H & E staining and spheroid quantification

Six-micrometre sections were deparaffinized in xylol (Carl-Roth, Germany) for 10 min, rehydrated in a descending ethanol series and rinsed in deionized H_2O for 1 min. Sections were placed in haematoxylin for 5 min, rinsed in tap water for 1 min to allow stain to develop and then placed in eosin for 2 min, dehydrated and mounted in Entellan resin (Merck, Germany). The occurrence of clearly detectable eosinophilic spheroids, indicative of dystrophic axons (Yamazaki *et al.*, 1988; Mukoyama *et al.*, 1989; Kikuchi *et al.*, 1990) was quantified in ~90 sections uniformly dispersed throughout the gracile nucleus of each individual and ~30 sections uniformly dispersed throughout the cervical gracile fascicle. Analysis of lateral columns was performed on these same 30 sections, counting the sum of spheroid numbers on both sides of the spinal cord. In this way, irregular results due to local deviations in spheroid numbers could be ruled out. H & E stained axonal spheroids were generally eosinophilic and appeared glassy or hyaline with a round or oval shape. They varied in diameter (5–50 μm) and sometimes reached a size larger than the nerve cells in gracile nucleus. All specimens were scored blind and agreed by two independent investigators.

Luxol Fast Blue staining and densitometric quantification

Six-micrometre sections from equivalent points in *gad* and *gad/ Wld*^{S} cervical spinal cord and medulla oblongata were processed simultaneously as follows. Sections were deparaffinized in xylol (Carl-Roth, Germany) for 15 min, and processed twice through 100% ethanol for 2 min and 96% ethanol for a few seconds. Slides were transferred to Luxol Fast Blue solution [0.1% Luxol Fast Blue MBS chroma (Merck), 10% acetic acid all made up in 96% ethanol] and incubated at 60°C for 5 h. Sections were then rinsed in 95% ethanol and distilled water for 1 min each, dipped in 0.05% lithium carbonate (Merck) for 1 min, and differentiated in 70% ethanol for a further 1 min. After rinsing in distilled water, sections were examined under light microscope for suitable differentiation between white and grey matter. Nuclear Fast Red staining was carried out for 10 min in 5% aluminium sulphate, 0.1% Nuclear Fast Red followed by rinsing in distilled H_2O, 90% ethanol and 100% ethanol for 1 min each. Slides were incubated in xylol for 5 min and mounted in Entellan resin (Merck). Slides were examined under light microscopy (Nikon Eclipse E200) and evaluated using Bioscan OPTIMAS 6.0 software (Optimas Corp., WA, USA) according to the manufacturer's instructions. For densitometric quantitation, mean grey values were obtained for circumscribed areas of interest using a three-chip monochrome CCD camera, and the background grey value (tissue-free area) was subtracted. Since demyelination occurs selectively in the gracile tract and not in the cuneate tract of *gad* mice by 126–130 days (Mukoyama *et al.*, 1989; our observations), we used cuneate fascicle as a reference area and expressed Luxol Fast Blue staining in gracile tract as a percentage of that in cuneate tract. We applied this procedure to representative Luxol Fast Blue-stained sections of cranial gracile tract: two sections from level C2/C3 representing the cervical gracile fascicle and two sections from level 535 representing the gracile nucleus (Sidman *et al.*, 1971).

Immunocytochemistry of gracile tract

Six-micrometre paraffin sections from equivalent points in *gad* and *gad/Wld*^{S} cervical spinal cord and medulla oblongata were processed simultaneously as follows. Sections were deparaffinized, rehydrated in a descending ethanol series, washed several times in 0.05 M Tris-buffered saline (TBS), and treated with a solution of 6% H_2O_2 in methanol for 20 min to block endogenous peroxidase activity. They were then permeabilized with 0.1% Triton X-100 (Sigma) in 0.05 M TBS additionally containing 0.05 M NH_4Cl, rinsed in fresh TBS three times and subsequently immunoblocked with 5% bovine serum albumin (Sigma) in 0.05 M TBS

for 1 h. First antibody was polyclonal guinea pig anti-glial fibrillary acidic protein (GFAP) (1 : 400 dilution) (Progen, Germany) at 4°C overnight, while negative control sections were incubated without primary antibody. Secondary antibody was goat anti-guinea pig biotin conjugate (1 : 400 dilution) (Sigma) for 1 h at room temperature, and was followed by streptavidin-coupled horseradish peroxidase complex (Vector Laboratories; 1 : 200 dilution) for 1 h. After extensive washing, sections were developed under identical conditions for all specimens with 3,3-diaminobenzidine tetrahydrochloride (Sigma–Aldrich) in 0.1 M phosphate buffer until a clear dark-brown labelling of astrocytes in the gracile tract could be detected. In all cases the control sections without primary antibody incubation showed no labelling of astrocytes. For microscopic examination and TV densitometry, sections were dehydrated and mounted in Entellan resin (Merck). Quantitation was similar to that described for Luxol Fast Blue densitometry. GFAP immunostaining intensities in cranial gracile tract sections were expressed as percentage of GFAP staining intensity in wild-type sections at the same coronal level. We applied GFAP densitometry on representative cranial gracile tract sections from each examined mouse: two sections from level C2/C3 representing the cervical gracile fascicle and two sections from level 535 representing the gracile nucleus (Sidman *et al.*, 1971).

Immunocytochemistry of sciatic nerves

Sciatic nerves from 15-week-old *gad*, *gad*/*Wld*^{S}, or control mice were immersion fixed in 4% PFA/0.1 M PBS for 1 h and washed extensively in 0.1 M PBS before paraffin embedding. Twenty-micrometre sections were immunostained using rabbit polyclonal antibody to ubiquitin (Sigma–Aldrich U5379) and Cy3-conjugated secondary antibody. Confocal images were obtained using a PerkinElmer UltraView LCI confocal microscope coupled to a Nikon Eclipse TE200 microscope, and processed using UltraView software (Perkin-Elmer Life Sciences Ltd, Cambridge, UK).

Statistical analysis of histopathology results

All data (axonal spheroid numbers, TV densitometry intensities) are presented as mean ± SD for the examined genotype groups. Data analysis was performed using PRISM for Macintosh or SPSS for Windows, including Student's *t*-test calculations for paired and unpaired data where appropriate. Significance was considered at $P < 0.05$ and high significance at a $P < 0.01$.

Analysis of neuromuscular pathology

Mice were killed by cervical dislocation and lumbrical muscles immediately dissected under oxygenated Ringer solution. Fixation, immunocytochemistry and signal imaging were then carried out as described previously (Gillingwater *et al.*, 2002). The denervation rate was determined by counting 100–200 endplates in each of two to three lumbrical muscles and the mean value taken for each mouse.

Behavioural tests

The foot splay test (Norreel *et al.*, 2001) was used to estimate the reflex reaction speed of the hind limbs. Mice were gently taken by the neck and tail, the plantar surface of their hind feet painted using a non-toxic children's painting set, and the mouse released from a height of 15 cm to land on white paper. Wild-type mice bring their legs together during descent to land in a controlled manner like a gymnast, whereas *gad* mice fail to do this and land with their feet far apart. The distance between the two hind heels was averaged from 10 successive trials on each testing date (9 and 13 weeks).

In the clasping test, the mouse was suspended by the tail >50 cm from any surface. Clasping time within a 1 min test was scored as flexing or folding of the hind limbs tightly towards the trunk plus any spasmodic stretching. Mice were examined once per week through the period from 6 to 16 weeks. No wild-type mice clasped, regardless of the presence of the *Wld*^{S} mutation.

Results

gad does not weaken the Wld^S *phenotype*

Before assessing the effect of *Wld*^{S} on *gad* pathology we first showed that *Wld*^{S} can protect axons, even in the presence of the *gad* mutation, by inducing Wallerian degeneration in *gad*/*Wld*^{S} mice. Before the lesion, there was no sign of axon degeneration in these nerves, confirming previous reports (Mukoyama *et al.*, 1989). We bred *gad* mice that were heterozygous for *Wld*^{S} and hemizygous for a *YFP-H* transgene (Feng *et al.*, 2000) to allow a rapid and quantitative assessment of Wallerian degeneration (Beirowski *et al.*, 2004) and transected sciatic nerves before the onset of hindlimb weakness. Wallerian degeneration was assessed after 5 days both by western blotting to see degraded heavy neurofilament protein (Fig. 1A) and by fluorescence microscopy to see fragmented YFP-containing axons (Fig. 1B). Nerves unprotected by *Wld*^{S} degenerated as expected (Fig. 1A, middle lane, and Fig. 1B, lower panel) but a single allele of *Wld*^{S} was sufficient to prevent axon degeneration in both readout methods. Thus, *gad* does not significantly weaken the *Wld*^{S} phenotype and it is feasible to test the effect of *Wld*^{S} on *gad* pathology.

Axonal spheroid pathology is reduced by Wld^S

In order to determine the effectiveness of *Wld*^{S} on *gad* axonal spheroid pathology, we counted axonal spheroids in ~90 H & E stained 6-μm paraffin sections from throughout the gracile nucleus and 30 sections from throughout the cervical spinal cord of each 18-week-old *gad* mouse and *gad*/*Wld*^{S} double homozygote. Fifty per cent fewer spheroids were found in gracile nuclei of *gad*/*Wld*^{S} mice than in *gad* mice ($P = 0.0004$) and 63% fewer in cervical gracile fascicle ($P = 0.0011$) (Fig. 2). Intermediate values were observed in *Wld*^{S} heterozygotes, further supporting the result and no spheroids were observed in control animals of this age (data not shown). Spheroids have also been reported in the cervical lateral columns of *gad* mice (Kikuchi *et al.*, 1990). We found far fewer spheroids here than in cervical gracile tract and gracile nucleus, but the number was also significantly reduced by homozygous *Wld*^{S} ($P = 0.046$; $n = 3$) (Fig. 2). We also observed a reduction in axonal spheroids in lumbar spinal cord, from 42 to six in the ventral column and from 13 to four

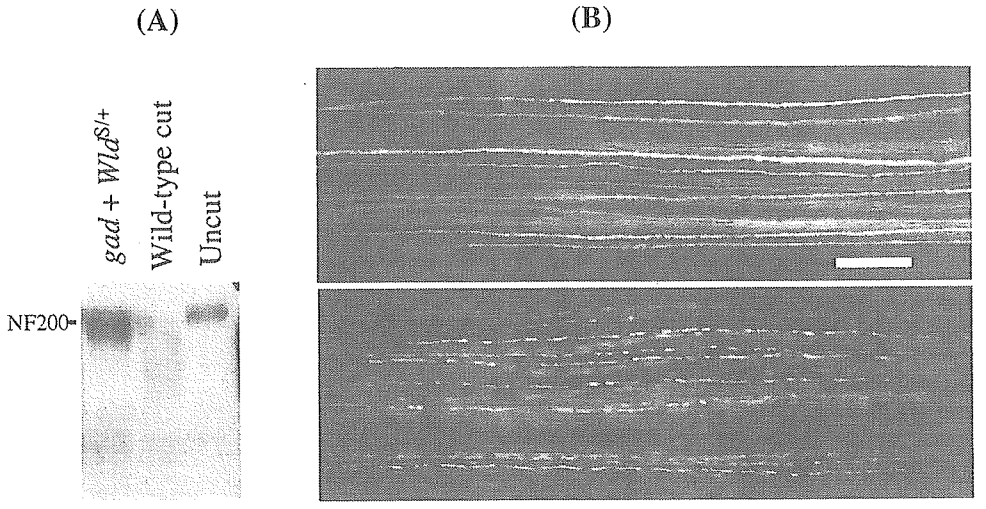

Fig. 1 A single allele of *Wld*^{S} is sufficient to delay Wallerian degeneration even in *gad* mice. (**A**) Western blot showing complete preservation of intact heavy neurofilament protein (NF200) in the distal stump of axotomized *gad* sciatic nerve by heterozygous *Wld*^{S} 5 days after lesion (lane 1). In contrast, no intact NF200 remains after 5 days in axotomized wild-type sciatic nerve (lane 2). Lane 3 is an uncut nerve showing the expected appearance of intact NF200 (gel loading differences probably account for the difference in intensity with lane 1). (**B**) Complete preservation of distal *gad* tibial nerve by heterozygous *Wld*^{S} 5 days after nerve lesion (upper panel), visualized using the *YFP-H* transgene. In contrast, no unfragmented axons remained in a tibial nerve lacking *Wld*^{S} 3 days after a lesion (lower panel). Unlesioned nerves appear exactly as in the upper image (Beirowski *et al.*, 2004). Scale bar = 100 μm.

in the dorsal horn grey matter. Although lumbar regions of only a single *gad* and two *gad/Wld*^{S} mice were studied, these mice were independent of those used for the gracile tract analysis and 3 weeks younger, so these data independently support our conclusion that *Wld*^{S} reduces axonal spheroid pathology in several different regions of *gad* CNS well into late-stage disease.

A reduction in the number of axonal spheroids could result theoretically from either reduced axon pathology or pathology so extensive that the axons are completely destroyed. Kurihara *et al.* (2001) reported that when *gad* pathology was made worse by crossing with *Uch-l3* null mice, extensive axon pathology became detectable at more caudal locations in cervical and thoracic gracile fascicle. We did not observe this in the *Wld*^{S} cross, and *Wld*^{S} homozygotes maintain a rostral–caudal gradient of axonal spheroid pathology (Fig. 2E and F; and thoracic data not shown), indicating that *gad* remains a 'dying-back' pathology in *Wld*^{S} mice but that its progress is delayed.

Secondary measures of axon pathology are also reduced by Wld^S

Further evidence of a reduced loss of axon-myelin units in *gad/Wld*^{S} mice came from a significant reduction ($P = 0.018$) in secondary myelin loss in cervical gracile fascicle in the same animals (Fig. 3A–C). A similar protective trend in the medulla oblongata did not reach statistical significance ($P = 0.059$), probably due to the naturally weaker myelination in this region, but *Wld*^{S} clearly did not cause any deterioration, so the reduction in axonal spheroid numbers (Fig. 2) must reflect reduced pathology and not wholesale axon loss. Furthermore, as the rescued axons remain myelinated, they potentially retain normal conductance properties, at least in these locations. It is unlikely that *Wld*^{S} has any direct effect on myelin, because expression of *Wld*^{S} in glia does not alter Wallerian degeneration (Glass *et al.*, 1993). Thus reduced myelin loss in *gad/Wld*^{S} mice is likely to reflect the maintenance of functional axon-myelin units. *Wld*^{S} also decreased GFAP signal in immunocytochemistry in *gad*, indicating a lower level of astrocyte activation in response to axon damage (Yamazaki *et al.*, 1988) (data not shown). Thus, both direct and indirect measures of spheroidal axon pathology in the gracile tract are reduced by the *Wld*^{S} gene.

Wld^S *operates downstream of axonal ubiquitin depletion in gad*

gad causes axon degeneration through defective ubiquitin metabolism (Osaka *et al.*, 2003), and *Wld*^{S} also interferes with ubiquitin metabolism (Mack *et al.*, 2001; Coleman and Perry, 2002; Zhai *et al.*, 2003). It was important to establish whether *Wld*^{S} blocks the ubiquitin defect in *gad*, an action that would suggest a protective effect restricted to *gad* and other ubiquitin defects, or whether it acts on a downstream step, raising the possibility of delaying axonal spheroid pathology in a wide range of CNS disorders (see above). Interpretation of any change in ubiquitin level in gracile tract would be complicated by the degeneration of those axon branches, so instead we carried out immunocytochemistry for ubiquitin epitopes in the peripheral branch of the same axons in sciatic nerve (Fig. 4). First, we confirmed that axonal ubiquitin was severely depleted in *gad* mice compared with wild-type controls ($P = 0.014$) (Osaka *et al.*, 2003). We then found

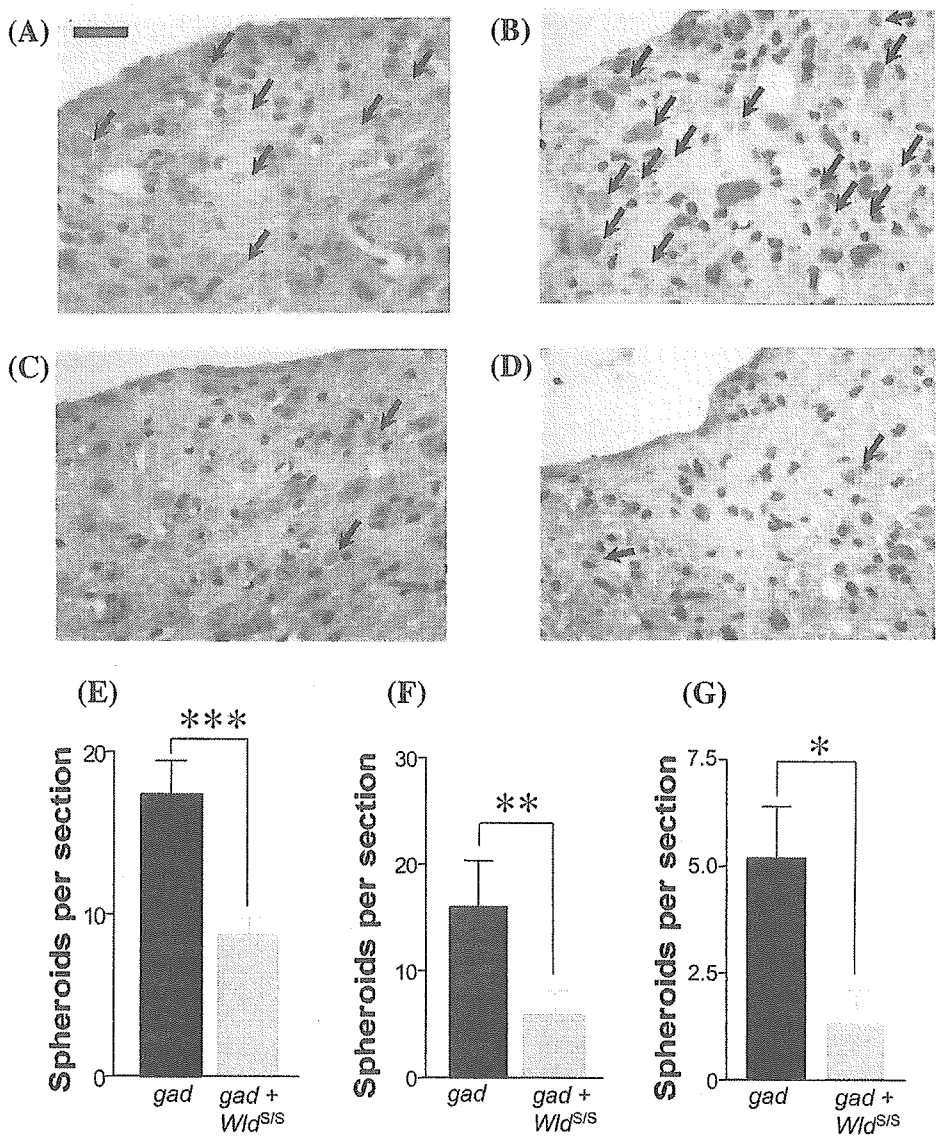

Fig. 2 *Wld*^{S} reduces spheroid body numbers in the gracile tract and lateral columns of *gad* mice. (**A** and **C**) Representative sections from gracile nucleus of (**A**) *gad* and (**C**) *gad/Wld*^{S} mice stained with H & E, showing a large reduction in the number of axonal spheroids (large pink swellings, indicated by arrows) when *Wld*^{S} is present. (**B** and **D**) Representative sections from cervical gracile fascicle of (**B**) *gad* and (**D**) *gad/Wld*^{S} mice. Scale bar (**A–D**) = 25 μm. (**E–G**) Quantitation (mean ± SD) of spheroid counting data in (**E**) gracile nucleus (n = 6), (**F**) cervical gracile fascicle (n = 6) and (**G**) cervical lateral columns (n = 3). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

that a similar defect was present in *gad/Wld*^{S} mice compared with *Wld*^{S} controls (P = 0.0004) and that *Wld*^{S} did not significantly increase the ubiquitin signal either in the presence (P = 0.902) or absence (P = 0.807) of *gad*. Thus, *Wld*^{S} does not correct the depletion of axonal ubiquitin in *gad* and instead operates at a downstream point in spheroid pathology that could be common to other CNS disorders.

Motor pathology

Despite the reduction in axonal spheroids in the gracile tract, there was no apparent reduction in the severity of *gad* symptoms when *Wld*^{S} was present, with no significant difference in hindlimb clasping, (P = 0.82; n = 9) or splay test (P = 0.33; n = 7). Thus, either prevention of swelling in the gracile tract does not preserve the function of those axons, or pathology elsewhere limits any improvement in phenotype of *gad/Wld*^{S} mice. In the absence of any tests to specifically target the function of gracile tract axons, we investigated neuromuscular junction (NMJ) pathology, where dying-back of motor nerve terminals has previously been reported (Miura *et al.*, 1993). At 15 weeks, the degree of denervation was similar between the two strains, with 56.0 ± 6.0% of lumbrical NMJ fully or partially denervated in *gad* mice and 53.5 ± 11.8% in *gad/Wld*^{S} (Fig. 5C and D). This may be because protection of motor nerve terminals at the NMJ by *Wld*^{S} after axotomy is weaker than that of the axon trunk, especially in older mice (Gillingwater *et al.*, 2002). However,

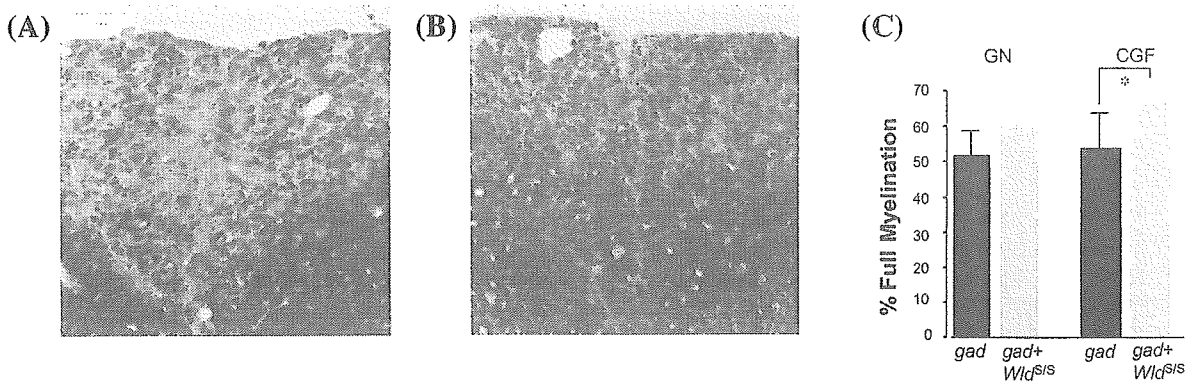

Fig. 3 *Wld*^{S} reduces also secondary demyelination in the gracile tract. (**A** and **B**) Representative cervical gracile fascicles of (**A**) *gad* and (**B**) *gad*/*Wld*^{S} mice stained with Luxol Fast Blue and Nuclear Fast Red, showing the reduction in myelin loss when *Wld*^{S} is present. Scale bar (**A** and **B**) = 25 μm. (**C**) Densitometric quantification (mean ± SD) of Luxol Fast Blue staining (n = 5). *P < 0.05.

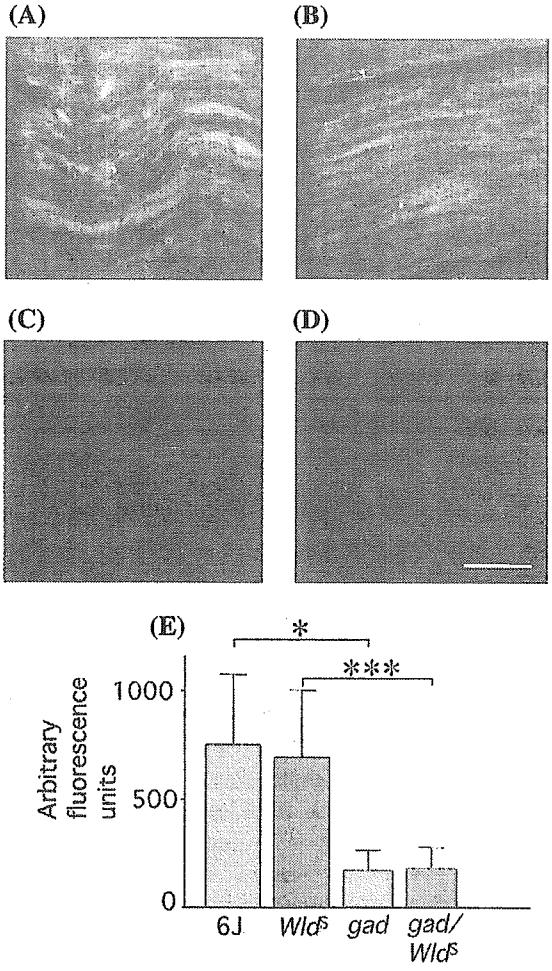

Fig. 4 *Wld*^{S} does not correct the severe depletion of axonal ubiquitin in *gad*. Ubiquitin immunostaining in both (**A**) wild-type and (**B**) *Wld*^{S} mice is greatly reduced in **C** and **D**, respectively, where *gad* is also present. Comparison of **A** with **B** and **C** with **D** also shows that *Wld*^{S} does not alter ubiquitin signal either in the presence of absence of *gad*. (**E**) Quantitation (mean ± SD) of ubiquitin signal. *P < 0.05; ***P < 0.001. 6 J, n = 4; *Wld*^{S}, n = 4; *gad*, n = 4; and *gad*/*Wld*^{S}, n = 10. Scale bar = 50 μm.

at 9 weeks, an age where *Wld*^{S} does protect axotomized motor nerve terminals, neither strain showed any denervation of NMJ in lumbrical muscles (Fig. 5A and B), so there was no time window when both *Wld*^{S} and *gad* exert their opposing effects at the NMJ. Thus, the fact that *Wld*^{S} does not alleviate NMJ pathology in the older mice could explain why *gad* symptoms are not reduced.

Discussion

We report that *Wld*^{S} reduces the occurrence of axonal spheroids in *gad*. This is the first indication that *Wld*^{S} can alleviate axon pathology in chronic CNS disease, thus extending observations made in the PNS that *Wld*^{S} protects axons not only after injury (Lunn *et al.*, 1989) but also in disorders where no physical injury takes place (Wang *et al.*, 2002; Ferri *et al.*, 2003; Samsam *et al.*, 2003). We conclude that axonal spheroid pathology in *gad* and Wallerian degeneration are not independent events and axon degeneration mechanisms are more uniform than morphology would suggest. It follows that Wallerian degeneration, or processes related to it, could contribute to many other CNS disorders where its involvement has not previously been suspected.

The mechanism by which *Wld*^{S} protects axons is still under investigation (Mack *et al.*, 2001; Coleman and Perry, 2002; Zhai *et al.*, 2003; Araki *et al.*, 2004), but appears to involve nuclear Wld^S protein and a factor(s) that communicates its effect to the axon. What is already becoming clear, however, is that *Wld*^{S} directly or indirectly blocks a central step of axon pathology onto which various pathological mechanisms converge (Fig. 6). This is indicated both by the wide range of disorders in which *Wld*^{S} protects axons, as it is inconceivable that *Wld*^{S} blocks different initial events in each case, and by our direct evidence, that early steps of *gad* pathogenesis are unaltered (Fig. 4). Intriguingly, it now seems that a number of different pathological manifestations result from the step delayed by *Wld*^{S}. These are axonal spheroids in *gad*, dying-back axon loss without swelling in peripheral

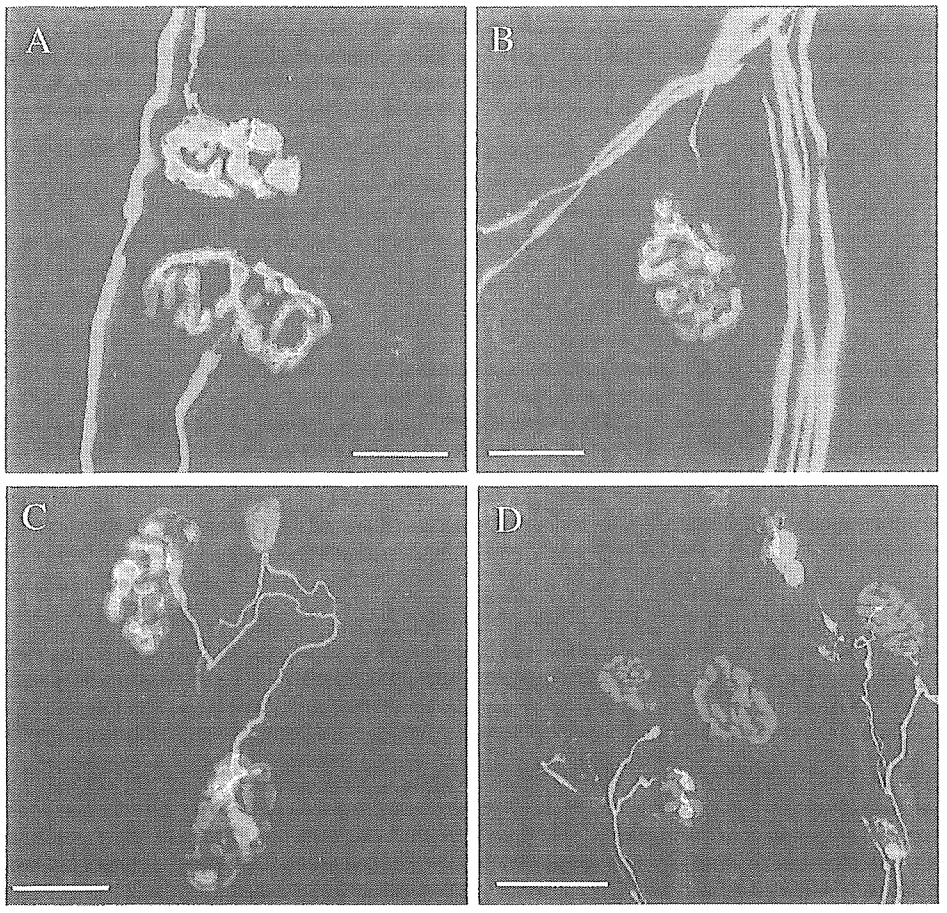

Fig. 5 Denervation at the NMJ. Presynaptic structures labelled with SV2 and neurofilament antibody are shown in green, and postsynaptic structures labelled with TRITC-α-bungarotoxin are in red. At 9 weeks, denervation has hardly begun in (**A**) *gad* or (**B**) *gad/Wld*^{S}. At 15 weeks, both strains show extensive denervation (**C** and **D**, respectively), with partial occupancy of endplates by motor nerve terminals occurring frequently. Scale bar = 25 μm.

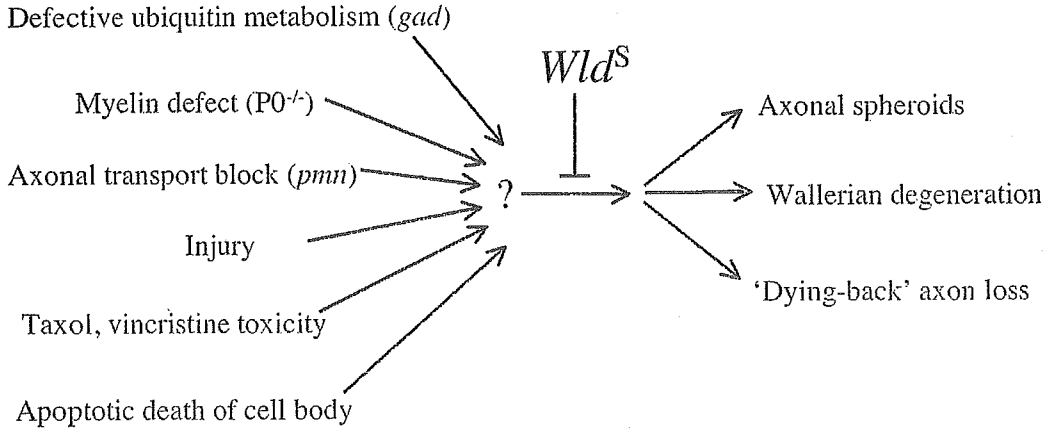

Fig. 6 *Wld*^{S} delays a central step of axonal pathology that lies after the convergence point of multiple degenerative stimuli but upstream of the divergence of several pathological manifestations.

neuropathy and motor neuronopathy, and Wallerian degeneration in CNS and PNS injury. The divergent morphology and topology in these disorders previously suggested independent mechanisms, but the results of directly probing the mechanism using *Wld*^{S} challenge this interpretation.

Many CNS disorders in which there is axonal swelling show accumulation of amyloid precursor protein in the swellings,

indicating impairment of axonal transport in each case and suggesting that their axon degeneration mechanisms are to some extent related. *gad* is one of these disorders, and the others include brain trauma (Gentleman *et al.*, 1993), stroke (Dewar *et al.*, 1999) and other forms of ischaemia (Hughes *et al.*, 2003), multiple sclerosis (Ferguson *et al.*, 1997), and HIV dementia (Medana and Esiri, 2003). This similarity with *gad* suggests that axon degeneration in other disorders may also be related to Wallerian degeneration, a possibility that should now be tested using Wld^S mice or, where appropriate, the newly generated Wld^S rat model (Adalbert *et al.*, in press). However, it is unlikely that Wld^S will stop all forms of axonal swelling, as it appears unable to do so in *Plp* null mice (Edgar *et al.*, 2004). Thus, it should be possible to categorize CNS axonal swelling disorders into those that are altered by Wld^S and those that are not. This will then enable disorders to be grouped together for mechanistic studies rather than focusing on each disorder in isolation.

It is important to consider the spatial and temporal relationship between axonal swelling and axonal breakdown in the light of our data. The lack of good methods for longitudinal imaging of CNS axons has made it difficult to determine whether spheroids first occur as terminal endbulbs of axons whose distal ends have degenerated, or as localized swellings on otherwise morphologically normal axons. Preliminary data from our laboratory using axons of *gad*/YFP-H mice (Adalbert and Coleman., unpublished) suggest that many spheroids in *gad* are not terminal endbulbs, at least in the early stages of the disease. Thus, one model to account for the effect of Wld^S in *gad* is that an 'en passant' spheroid is the first step in pathology, leading to degeneration of the distal axon due to the blockage of axonal transport, a process that fixes the spheroid as a terminal endbulb. In this model, Wld^S might block the Wallerian-like degeneration of the distal end for long enough to allow the spheroid to resolve and the axon to recover. Thus, our data suggest that Wld^S could be used to address the question of whether swollen axons can recover or whether they are destined, inevitably, to degenerate. In a wider context, this is an important issue in several CNS disorders where axonal spheroids occur, including brain trauma and multiple sclerosis (Cheng and Povlishock, 1988; Ferguson *et al.*, 1997).

The above model assumes that Wallerian-like degeneration and axonal swelling in *gad* are separated in space and time, with one causing the other. Alternatively, the mechanism of the axonal swelling itself in *gad* may be related to that of Wallerian degeneration. In support of this model, there are a number of disorders in which CNS axons swell and PNS axons of the same animal degenerate by Wallerian-like degeneration without extensive swelling. In *gad* mice, this occurs even within the same cell, as gracile tract central projections of lumbar primary sensory neurons have spheroids, while peripheral muscle spindles degenerate without swelling (Oda *et al.*, 1992). Similarly, amyotrophic lateral sclerosis (ALS) in humans (Tu *et al.*, 1996; Takahashi *et al.*, 1997), mice (Tu *et al.*, 1996; Oosthuyse *et al.*, 2001) and rats (Howland *et al.*, 2002), together with tauopathy in mice (Lewis *et al.*, 2000; Probst *et al.*, 2000), all show axonal swelling in spinal cord and other CNS areas, but extensive 'Wallerian-like' degeneration without swelling in ventral roots and peripheral nerves. Even injury-induced Wallerian degeneration shows different morphology depending on experimental circumstances. For example, when injured gracile tract axons undergo Wallerian degeneration they swell to up to 10 times their normal diameter, quite unlike Wallerian degeneration in the PNS (George and Griffin, 1994). Thus, a number of observations support a direct mechanistic link between axonal swelling and Wallerian degeneration.

It is not yet clear how related mechanisms might cause swelling in spheroids but axon fragmentation in Wallerian degeneration. Cytoskeletal changes are common to both, so a loosening of cytoskeletal structure could cause disorganized cytoskeleton to accumulate in spheroids but to undergo rapid granular disintegration in Wallerian degeneration. Wallerian degeneration of injured gracile tract axons displays elements of both processes, possibly having an intermediate mechanism: like spheroids, these axons dilate considerably but, typical of Wallerian degeneration, they also rapidly lose their cytoskeletal proteins (George and Griffin, 1994). In traumatic brain injury, observation of Wallerian degeneration and spheroids in the same transverse thin section has been interpreted as degenerating axons having a more proximal spheroid that blocks axonal transport (Cheng and Povlishock, 1988). In view of our findings, an additional explanation needs to be considered, that spheroids and Wallerian degeneration are alternative responses of different axons to the same lesion. Methods for real-time or long-range longitudinal analysis of individual spheroid-containing axons are required to resolve this, similar to new methods already applicable in PNS axons (Pan *et al.*, 2003; Beirowski *et al.*, 2004). What determines whether an axon develops a spheroid or undergoes Wallerian degeneration? Possible explanations include the different glial and haematopoietic cell content of the CNS and the lower rate of axonal transport there (Wujek and Lasek, 1983), but injury type may also be important. Finally, since the discovery of the Wld^S mouse, Wallerian degeneration is no longer considered a passive wasting of distal axons but a regulated self-destruction programme (Buckmaster *et al.*, 1995; Raff *et al.*, 2002). The reduction of axonal spheroid pathology in *gad* by the same gene raises similar questions: rather than being a passive consequence of blocked axonal transport axonal swelling could be, like Wallerian degeneration, a programmed response to axon damage.

Altered ubiquitin metabolism plays important roles in neurodegenerative diseases of the CNS. Genetic mutations in Parkinson's disease include an E3 ligase (Kitada *et al.*, 1998) and possibly *UCH-L1*, the human homologue of the gene mutated in *gad* (Leroy *et al.*, 1998). Ubiquitin-positive inclusions and other evidence indicate abnormal ubiquitylation in Alzheimer's disease (Mori *et al.*, 1987;

van Leeuwen *et al.*, 1998), polyglutamine disorders (DiFiglia *et al.*, 1997; Cummings *et al.*, 1999; Bence *et al.*, 2001) and ALS (Tu *et al.*, 1996; Bruijn *et al.*, 1997). Axons and synapses are particularly vulnerable, as proteasome inhibitors cause specific degeneration of distal neurites (Laser *et al.*, 2003) and ubiquitin-related mutations alter synapse growth (DiAntonio *et al.*, 2001) and stability (Wilson *et al.*, 2002). As Wld^S can counter a downstream effect of defective ubiquitin metabolism, it now becomes important to study its effects on the above disorders.

Wld^S did not alleviate the symptoms of *gad* mice. Unfortunately, methods do not currently exist to assess the function of gracile tract axons, so we cannot rule out the possibility that blocking spheroid formation did not preserve axon function. However, it is likely that continued neuromuscular pathology in *gad*/Wld^S mice also contributes to the symptoms. These mice suffered extensive synapse loss by 15 weeks (Fig. 5), whereas axon pathology was still strongly reduced 3 weeks later (Fig. 2). This supports the hypothesis that different mechanisms underlie synaptic and axonal degeneration, with Wld^S affording only limited protection to synapses, particularly in older mice (Gillingwater and Ribchester, 2001; Gillingwater *et al.*, 2002). Similarly, the synapses of gracile tract axons may have been lost even when those axons are preserved. Our data suggest that synapse pathology is a limiting factor when axons are protected by Wld^S, a finding likely to be important in other models (Ferri *et al.*, 2003; Samsam *et al.*, 2003).

In summary, we conclude that Wld^S alleviates chronic CNS axon pathology in *gad* mice and that formation of distal axonal spheroids in this disease shares features with Wallerian degeneration and 'dying-back' axon loss without spheroids. The effect of Wld^S on other CNS disorders with ubiquitylation deficits and CNS axonal swelling disorders should now be studied. Finally, our data emphasize the importance of finding a way to protect synapses as strongly as Wld^S protects axons.

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