VCP binding influences intracellular distribution of the slow Wallerian degeneration protein, Wld\textsuperscript{S}

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Wallerian degeneration slow (Wld\textsuperscript{S}) mice express a chimeric protein that delays axonal degeneration. The N-terminal domain (N70), which is essential for axonal protection \textit{in vivo}, binds valosin-containing protein (VCP) and targets both Wld\textsuperscript{S} and VCP to discrete nuclear foci. We characterized the formation, composition and localization of these potentially important foci. Missense mutations show that the N-terminal sixteen residues (N16) of Wld\textsuperscript{S} are essential for both VCP binding and targeting Wld\textsuperscript{S} to nuclear foci. Removing N16 abolishes foci, and VCP binding sequences from ataxin-3 or HrdI restore them. \textit{In vitro}, these puncta co-localize with proteasome subunits. \textit{In vivo}, Wld\textsuperscript{S} assumes a range of nuclear distribution patterns, including puncta, and its puncta co-localize with proteasome subunits. \textit{In vivo}, Wld\textsuperscript{S} assumes a range of nuclear distribution patterns, including puncta, and its neuronal expression and intranuclear distribution is region-specific and varies between spontaneous and transgenic Wld\textsuperscript{S} models. We conclude that VCP influences Wld\textsuperscript{S} intracellular distribution, and thus potentially its function, by binding within the N70 domain required for axon protection.

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Introduction

Axonal degeneration contributes to many neurodegenerative disorders including amyotrophic lateral sclerosis (ALS), Alzheimer's disease and multiple sclerosis and often precedes cell death (Ferguson et al., 1997; Ferri et al., 2003; Fischer et al., 2005; Stokin et al., 2005). Hence, understanding its molecular basis could lead to new treatment strategies. An excellent model for studying mechanisms regulating axonal breakdown after toxic or physical injury is the Wld\textsuperscript{S} mouse, which carries a spontaneous mutation causing a tenfold delay in Wallerian degeneration; the process by which the severed distal stump of an injured axon dies (Lunn et al., 1989; Mack et al., 2001). Furthermore, Wld\textsuperscript{S} can delay axon degeneration and attenuate symptoms in mouse models of some ‘dying-back’ disorders (Ferri et al., 2003; Mi et al., 2005; Sajadi et al., 2004; Samsam et al., 2003). Thus, axon degeneration in both injury and disease is a highly regulated process, potentially amenable to therapeutic intervention.

The remarkable delay in axon degeneration in the Wld\textsuperscript{S} mouse results from a tandem triplication, which produces a novel chimeric gene at its internal boundaries (Coleman et al., 1998). The resulting in-frame fusion protein derives its N-terminal 70 amino acids (N70) from the E4 ubiquitin ligase Ube4b, followed by the full coding sequence of Nmnat1, an NAD\textsuperscript+ synthesizing enzyme. The two domains are separated by eighteen amino acids (Wld18) from the Nmnat1 5\textprime; UTR that becomes translated. This cDNA dose-dependently delays Wallerian degeneration when expressed in transgenic mice, rats and flies and also in virally transduced dorsal root ganglion (DRG) explant cultures (Adalbert et al., 2005; Hoopfer et al., 2006; Macdonald et al., 2006; Mack et al., 2001; Wang et al., 2001).

Exactly how Wld\textsuperscript{S} delays Wallerian degeneration remains controversial. Several reports have suggested that increased NAD\textsuperscript+ synthesis is sufficient \textit{in vitro} and strong overexpression of Nmnat1 also confers a degree of axo-protective phenotype in \textit{Drosophila in vivo} (Araki et al., 2004; Macdonald et al., 2006; Wang et al., 2005). However, Nmnat1 alone is not sufficient for any detectable effect in mice, so more N-terminal sequences are also required (Conforti et al., 2007). Nmnat1 and Ube4b portions also appear to act together in modulating potential downstream transcripts or proteins (Gillingwater et al., 2006; Wishart et al., 2007).

Wld\textsuperscript{S} requires its N-terminal domain to bind VCP as well as for phenotype. VCP is an AAA-ATPase with many functions including a critical role in the ubiquitin proteasome system (UPS) (Laser et al., 2006). VCP is partially redistributed by Wld\textsuperscript{S} into discrete nuclear foci (Laser et al., 2006) suggesting a role for the N-terminal sequence in subcellular targeting of Wld\textsuperscript{S}. Neither parent protein alone produce these foci; Nmnat1 has a diffuse nuclear distribution while Ube4b is...
found throughout the cell (Fang et al., 2005; Laser et al., 2006). This novel localization is especially intriguing given that the Wld\(^8\) protein protects axons severed from their cell bodies. Wld\(^8\) protein has been detected in neurites in virally transduced primary cultures, so an intranuclear function remains possible (Wang et al., 2005, 2001) but an indirect nuclear action appears more likely at present, perhaps by pre-loading axons with a Wallerian degeneration inhibitor (Coleman 2005). Thus, understanding how the Wld\(^8\) nuclear foci are formed could shed light on possible functional roles of the N-terminal sequences required for slow Wallerian degeneration phenotype. Furthermore, if Wld\(^8\) were to exert its neuroprotective role outside the nucleus, understanding the behaviour of the more easily detectable intranuclear protein should help predict its behaviour elsewhere in the cell.

We mapped the Wld\(^8\) sequences producing the nuclear foci to the N-terminal sixteen amino acids (N16), which are essential for VCP binding (Morreale et al., submitted for publication). Mutations disrupting VCP binding prevent nuclear foci formation but this localization is restored by substituting alternative VCP binding sequences. VCP downregulation by RNAi also significantly reduces nuclear foci. We show co-localization of Wld\(^8\) puncta and proteasome-containing clastosomes and assess Wld\(^8\) protein distribution throughout the CNS, showing that it varies widely between Wld\(^8\) models, between different CNS regions and within regions. These data regarding the formation, composition and distribution of Wld\(^8\) nuclear foci help to understand how VCP binding may contribute to axon protection.

### Results

#### The N-terminal sixteen amino acids of Wld\(^8\) are essential for nuclear foci formation

To determine which Wld\(^8\) sequences produce nuclear foci, we truncated Wld\(^8\) cDNA constructs fused to C-terminal eGFP, and transfected into PC12 cells. We focused on the N-terminal domain because Nmnat1 has a diffuse nuclear distribution (Laser et al., 2006; Schweiger et al., 2001).

Surprisingly, constructs lacking the first 52, 34 and 16 amino acids of Wld\(^8\) did not produce nuclear foci, suggesting that the N-terminal 16 amino acids (N16) are necessary (Fig. 1, scheme for constructs Supplementary Fig. 8). This contradicts earlier data, where \(\Delta16\)-Wld\(^8\) conjugated to DsRedII did form nuclear foci (Laser et al., 2006). Despite the reduced propensity of DsRedII to aggregate (Yanushevich et al., 2002), it still produces tetramers and...
mislocalizes when conjugated to certain proteins (Zhang et al., 2003). To resolve whether Δ16-WldS forms foci, we produced an untagged form which was detected using the Wld18 antibody against the unique linker region (Samsam et al., 2003). This showed exclusively diffuse nuclear staining in PC12 cells (Fig. 1). Hence, the Δ16-WldS puncta observed previously (Laser et al., 2006) are an artefact of DsRedII tetramerization or aggregation. We also observed a similar effect when conjugating Nmnat1 to DsRedII (Supplementary Fig. 1). Western blotting of transfected cultures confirmed that these differences were not due to any difference in protein expression level (Fig. 1G).

**VCP binding is essential for nuclear foci formation in vitro**

N16 also contains a motif that binds valosin-containing protein (VCP) (Laser et al., 2006). Thus, if VCP binding helps direct WldS distribution, replacing N16 with other VCP binding sequences should restore this pattern. We swapped N16 for equivalent-length regions from HrdI and ataxin 3 which contained the VCP binding sequences (Boeddrich et al., 2006; Morreale et al., submitted for publication), and transfected into PC12 cells. Immunostaining showed that both of these chimeric proteins produce nuclear foci, suggesting a role for VCP binding sequences in producing nuclear puncta (Fig. 2).

To eliminate the possibility that deleting N16 disrupts nuclear foci formation through a more general disturbance of WldS secondary or tertiary structure we next prevented VCP binding using point mutations that do not alter predicted secondary structure. We find R10 and R13 to be essential for VCP binding (Morreale et al., submitted for publication), so we transfected PC12 cells with constructs in which these arginines had been mutated separately to alanines, and assessed WldS distribution. These mutations did indeed disrupt nuclear foci formation in PC12 cells (Fig. 2). Conversely, mutation of arginine 11, which is not required for VCP binding, had no effect (Fig. 2). Again, Western blotting confirmed that expression level was little changed between these cultures (Fig. 2H). Thus, the correlation between VCP binding and a punctate distribution of WldS holds down to specific single amino acid changes within N16.

**Competition for VCP binding**

The ability of sequences from HrdI and ataxin 3 to substitute for N16 suggests that overexpression of such proteins might suppress

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**Fig. 2. VCP binding is important for WldS to form nuclear foci.** Red staining (α-Wld18 immunostaining, alexa 568 secondary): conjugation of VCP binding sequences from ataxin-3 (panel A) and HrdI (panel B) to Δ16-WldS can restore foci formation in PC12 cell culture (some foci marked by arrows). Table G shows the first sixteen amino acids of each construct, containing the VCP binding motifs, with the arginine-rich region aligned in bold. Green staining (α-Wld18 immunostaining, alexa 488 secondary): Panel C shows nuclear foci produced by WldS in some cells. D and F: point mutations in arginines 10 and 13 (R → A) of WldS, which are required for VCP binding, disrupt nuclear foci formation. This is unaffected by mutation of arginine 11 (panel E), which is not necessary for the interaction with VCP. Hence, VCP binding appears necessary for nuclear foci formation in vitro. Scale bars represent 10 μm. Western blots (H) show protein expression levels of WldS and mutant constructs using the Wld18 antibody to demonstrate that localization is not due to protein levels. β-actin loading control also shown.
nuclear foci by competing for VCP binding. To test this, we co-transfected HrdI-FLAG with WldS–eGFP. There was no effect on WldS foci, but this appears to be due to HrdI being a cytoplasmic protein (Schuberth and Buchberger 2005), whose nuclear levels are not high enough to compete (Supplementary Fig. 2). Ube4b, in contrast, does enter nuclei albeit at relatively low levels (Fang et al., 2005; Hatakeyama et al., 2001), so we also co-transfected Ube4b-FLAG with WldS–eGFP. Its nuclear levels were also too low to disrupt WldS foci, but interestingly Ube4b itself was partially redistributed into foci, possibly by binding to unoccupied sites on hexameric VCP (Supplementary Fig. 2). Similar results were obtained with a WldS–mCherry fusion (data not shown). Thus, WldS redistributes not only VCP (Laser et al., 2006), but also some of its binding partners.

**VCP RNAi disrupts nuclear foci**

To confirm that VCP binding, rather than any other function of N16, is critical for WldS localization to foci, we then down-regulated VCP by RNAi in HeLa cells co-transfected with WldS–mCherry (or WldS–eGFP, data not shown) using siRNAs VCP6 and VCP7 (Qiagen) and a negative control siRNA. After 2 days, half of the dishes were fixed and imaged to assess nuclear foci and the remainder were lysed for VCP Western blotting.

VCP RNAi decreased VCP protein levels compared to untreated controls or negative control siRNA (Fig. 3). This was not due to cell impairment or death in the siRNA-treated cultures as total protein levels are similar (β-actin staining). WldS nuclear foci were dramatically reduced 48 h after transfection, from an average of 70% of transfected cells having foci to 6–7% (ANOVA Games–Howell p<0.0001 for VCP6 and VCP7, Fig. 3). Therefore, VCP levels strongly influence the localization of WldS nuclear foci consistent with the proposed role of VCP in foci formation.

**WldS is often punctate, but differentially distributed in vivo**

We next characterized intranuclear WldS distribution in vivo in the spontaneous mutant mouse, transgenic 4836 mice and line 23 transgenic rats (Adalbert et al., 2005; Mack et al., 2001). Previous data show that WldS has a diffuse nuclear pattern in motor neurons (Fang et al., 2005; Gillingwater et al., 2006), but that foci are present in over 90% of cerebellar granule cells (Gillingwater et al., 2006). Here, we expand on this, showing that 82% of molecular layer cells also express small numbers of large inclusions, while Purkinje cells lack WldS staining (Fig. 4, Table 1). In granule cells, the mean diameter of the inclusions was 0.63 ±0.03 μm (n=46), with the mean nuclear diameter measuring 6.35 ±0.15 μm (n=46). The mean volumes were: nucleus 145.0±10.3 μm³, inclusion 0.16±0.02 μm³. Hence in cerebellar granule cells the inclusion constitutes about 0.1% of the total nuclear volume.

These data also show great variation in WldS expression pattern both between and within other regions of the CNS in WldS mice (Table 1 and Fig. 4). We divided the expression patterns into four categories: 1) large nuclear inclusions; 2) numerous small nuclear inclusions; 3) strong diffuse nuclear expression; 4) very weak diffuse/no staining in the nucleus. Where cells fell into more than one category we counted them twice. The number of nuclei demonstrating each staining pattern is expressed as a percentage of the total topro-3 positive nuclei, but because these figures also include topro-3 positive non-neuronal cells, they may underestimate neuronal WldS expression.

![Fig. 3](image-url)
We observed variation in the expression pattern of WldS protein, both regionally and intercellularly (Fig. 4 and Table 1). In the hippocampus 84% of cells in CA3 but only 10% in CA1 show small nuclear inclusions, and dentate gyrus granule cells lack visible inclusions. Cerebral cortex showed similar variations in intensity and staining pattern. Surprisingly, there was little or no discernable staining in superior cervical ganglion (SCG) neurons (Supplementary Fig. 3), despite these having a clear phenotype when cultured in vitro (Buckmaster et al., 1995). WldS expression may be altered in vitro due to growth factors and other components in the culture medium or diffusely distributed WldS may confer protection below the detection limit of immunocytochemistry here. In neurons of the dorsal root ganglion (DRG) staining varied from a diffuse nuclear pattern in the large neurons, to sizeable and occasionally unusually-shaped inclusions in the small neurons. These included doughnut shapes, rings, loops and swirls which we have classified here as a fifth category; ‘tubes’ (Supplementary Fig. 3, Table 1).

The localization of WldS in transgenic 4836 mice (Mack et al., 2001) and line 23 rats (Adalbert et al., 2005), where expression of the chimeric protein is driven by the β-actin promoter, is markedly different (Fig. 5, Table 2). No inclusions were observed in cerebellar granule cells, where nuclear foci are particularly prominent in spontaneous WldS mice (Fig. 4). However, foci were visible in cells other than granule cells or Purkinje cells in transgenic rat cerebellum and inclusions resembling tubes were observed in these other cell types in transgenic mouse cerebellum. Expression in the hippocampus also differed from the spontaneous mutant mouse, being stronger in CA1 in transgenics but far weaker in CA3. In dentate gyrus there was discernible expression in transgenic mice but not rats. Hence, in vivo formation of nuclear foci is clearly a complex process which varies depending not only on the brain region but the specific conditions in each cell.

VCP binding is necessary but not sufficient for nuclear foci formation in vivo

In order to test whether VCP binding is necessary for nuclear foci in vivo, we studied the distribution of A16-WldS expressed in a transgenic mouse recently generated by our laboratory (Conforti et al., in preparation). Many nuclei showed strong diffuse staining across several brain regions but large foci similar to those observed in WldS mice were not present (Fig. 6). In some nuclei very small speckles could occasionally be seen but these were much smaller and less frequent than the foci seen in spontaneous mutant WldS mice, and in WldS transgenics (see Figs. 3 and 4). Hence, these data support the in vitro data showing that N16 is required for nuclear foci.

We also studied Atx-WldS transgenic mice, where N16 is replaced with the ataxin-3 VCP binding region (Conforti et al., in preparation). We were surprised to find no nuclear foci in any of the
brain regions analysed (Fig. 6). Small speckles were occasionally observed, but smaller and less frequent than in WldS mice (Figs 4 and 5). Thus, the in vivo data suggest that VCP binding is necessary but not sufficient to target WldS to nuclear foci. This is consistent with the observation that nuclear foci are not present in all PC12 cells expressing WldS and that some neuronal subtypes lack foci (Figs. 4 and 5, Supplementary Fig. 4).

WldS nuclear foci co-localize with proteasome-containing clastosomes

Understanding the precise intranuclear localization and interactions of WldS may prove relevant to the mechanism of axon protection, even if the foci themselves are not directly involved, as the N-terminal domain required for their formation is essential for this mechanism (Conforti et al., 2007). Therefore, characterization of these foci could shed light on the function of the N-terminal sequences.

We thus obtained fluorescently-conjugated (GFP or YFP) markers for PML bodies, Cajal bodies, gems, speckles and para-speckles (kind thanks to Professor A. Lamond and Dr. J. Sleeman), and assessed whether WldS co-localized in 2-day differentiated PC12 cells or HeLa cells. For coilin (marking Cajal bodies) and ASF (marking splicing speckles) co-localization could only be assessed in HeLa cells as these proteins rarely produce foci in PC12 cells. For all other constructs, the co-localization results were the same in both PC12 (Supplementary Fig. 5) and HeLa cells (Fig. 7). We rarely observed complete co-localization between WldS and any of these proteins (Fig. 7). However PML foci, and to a lesser extent coilin foci, were often observed adjacent to or slightly overlapping WldS, which suggests weak or transient association (Fig. 7 and Supplementary Fig. 6).

As WldS derives partly from the ubiquitin ligase Ube4b and interacts with UPS protein VCP, we also assessed co-localization with proteasome-containing clastosome foci (Lafarga et al., 2002). WldS–FLAG puncta co-localized with proteasome foci using proteasome 20S subunit C2 polyclonal antibody (Abcam) (Fig. 7A) and partial colocalization was also observed using a second antibody (BIOMOL, Supplementary Fig. 6). Co-localization was also observed using WldS–eGFP instead of WldS–FLAG (data not shown). These data are consistent with previous suggestions that WldS may be linked to proteasomal function or inhibition (MacInnis and Campenot 2005; Zhai et al., 2003).

Foci-positive Schaffer collaterals, but not weak-expressing mossy fibres, are protected from Wallerian degeneration in adult hippocampal slices

Finally, we tested whether differences in WldS protein pattern affect its axon-protection phenotype. WldS expression was higher in CA3, which also expressed more nuclear foci than in the dentate gyrus and this was the basis for a novel electrophysiological test. First, we made field potential recordings from acute hippocampal slices using conventional methods. Next, we compared recordings from slices 1–2 days after cutting both the mossy fibres (originating

<table>
<thead>
<tr>
<th>% Tubes</th>
<th>% large Inclusions</th>
<th>% Speckles</th>
<th>% strong Diffuse</th>
<th>% weak Diffuse/none</th>
</tr>
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<tbody>
<tr>
<td>Caudate</td>
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<td>55</td>
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<td>0</td>
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<tr>
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<td>75</td>
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<td>85</td>
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</table>

The percentage of cells in each region displaying a particular WldS protein nuclear distribution pattern was assessed from low power confocal images. The distribution pattern was divided into tubes (linear-type structures), large inclusions, speckles (multiple small inclusions), strong diffuse and weak diffuse/no discernible staining. Some cells were placed into more than one category (e.g. where the cell showed a large inclusion with strong diffuse staining as well) and would therefore be counted twice. An extended version of this table is provided with the supplementary information to this paper.
from low-expressing dentate gyrus neurons) and Schaffer-collateral pathways (originating from higher-expressing CA3 neurons).

The fibre volley elicited by stimulated mossy fibre distal stumps was lost in Wld\textsuperscript{S} slices within 48 h (11 V stimulus response: 0 days: $-1.34\pm0.45$ mV, $n=4$; 2 days: $-0.07\pm0.07$ mV, $n=4$; $p<0.0001$, 2-way ANOVA of full input-output curves). By contrast, fibre volleys could still be stimulated in the corresponding Schaffer-collateral pathways (11 V stimulus response: 0 days: $-1.34\pm0.16$ mV, $n=5$; 1 day: $-1.85\pm0.30$ mV, $n=6$; 2 days: $-0.98\pm0.28$ mV, $n=4$; $p>0.05$ compared to 0 day), but were absent in wild-type slices (11 V stimulus response: 0 days: $-2.23\pm0.40$ mV, $n=4$; 1 day: $-1.53\pm0.33$ mV, $n=8$; 2 days: $-0.30\pm0.15$ mV, $n=8$, $p<0.0001$ 2-way ANOVA, compared to 0 day; Fig. 8). These persistent fibre volleys were blocked by adding tetrodotoxin (1 μM) to the ACSF bathing the slices. In all slices, the synaptic EPSPs were unchanged 24 h following axotomy, but were completely lost by 48 h (data not shown).

Thus, functional axon preservation only occurred in the CA3/Schaffer-collateral pathway of Wld\textsuperscript{S} mice, where about 80% of the cell bodies of the visible nuclear foci in addition to weak diffuse staining. The severed distal axons of dentate gyrus granule cells however, which contain lower levels of nuclear Wld\textsuperscript{S} expression, did not retain their function.

Discussion

The N-terminal Wld\textsuperscript{S} protein domain is essential for delaying Wallerian degeneration in vivo, but how it fulfils this role is not understood (Conforti et al., 2007). We provide evidence that the VCP binding sequence within this domain is essential for it to form nuclear foci. These puncta can be disrupted by removing N16 both in vitro and in vivo, by introducing point mutations into arginines required for VCP binding and are restored in vitro by substituting N16 for the VCP binding sequences from ataxin 3 or HrdI. Foci formation is also influenced by VCP levels as demonstrated by siRNA, and the VCP binding protein Ube4b appears to be partially redistributed into these foci. We also demonstrate that Wld\textsuperscript{S} foci co-localize with 20S proteasome subunits in PC12 cell cultures. In vivo, we show that Wld\textsuperscript{S} adopts an array of different intranuclear localizations including producing puncta.

Understanding the formation and composition of the Wld\textsuperscript{S} nuclear foci is important as it may shed light on possible roles of the N-terminal domain of this protein. There does not appear to be either a positive or negative correlation between foci and delayed axonal degeneration, as both motor neurons, which lack foci (Mack
et al., 2001), and CA3, which does express foci, have the phenotype. However, VCP binding within this N-terminal domain is important for both foci formation as shown here and probably for the phenotype (Conforti et al., 2007). Hence, investigation of the WldS nuclear foci could provide information relevant to the protective mechanism. Here, we expand on observations that VCP binds to WldS and is partially redistributed by it to nuclear foci (Laser et al., 2006), showing that binding of VCP and its expression levels also influence the intranuclear location of WldS in vitro. As VCP is abundant in cytoplasm, our data also imply that the location of any extranuclear WldS could also be influenced by VCP binding.

In addition to partially redistributing VCP (Laser et al., 2006) WldS also competes for VCP binding with other proteins such as Hrd1 (Morreale et al., submitted for publication). This could inhibit the normal function of VCP, though this is unlikely as it is very abundant and only a small amount is redistributed by WldS (Laser et al., 2006). WldS may also be able to indirectly influence the localization of VCP binding proteins, as it does partially with Ub4b. These may then interact physically or functionally with other components of the spots, or with the Nmnat1 portion of WldS.

The Nmnat1 domain will affect the localization of the nuclear NAD⁺ synthesis machinery, perhaps causing very high local NAD⁺ concentrations in nuclear puncta or at sites where WldS may accumulate due to VCP binding. As WldS mouse brain lacks a detectable increase in NAD⁺ despite the four-fold increase in Nmnat activity (Mack et al., 2001), NAD⁺ synthesized by WldS may be rapidly and locally used within foci. This could tie together evidence suggesting that Nmnat1 activity is important for slow Wallerian degeneration in vitro, where it is likely very highly overexpressed (Araki et al., 2004; Jia et al., 2007; Wang et al., 2004), and data showing that Nmnat1 overexpression alone is not sufficient in vivo, where puncta are not formed and VCP binding does not occur (Conforti et al., 2007).

It is interesting that conjugation of tetrameric DsRedII to Δ16-WldS also induces it to produce nuclear puncta morphologically similar to those of full-length WldS, Atx-WldS and HrdI-WldS (Laser et al., 2006). Our preliminary data indicate WldS forms oligomers, particularly tetramers and hexamers (not shown), consistent with the tetrameric or hexameric nature of Nmnat1 (Zhou et al., 2002; Emanuelli et al., 1992). If WldS is indeed oligomeric there may be multivalent interactions between WldS and hexameric VCP (or DsRedII oligomers), producing a lattice structure which grows to form the visible ‘spots’. More than one WldS oligomer would need to bind each VCP hexamer, although it remains unclear whether all six sites are available simultaneously. However, this does not rule out the possibility of a lattice structure being formed, particularly given that nuclear puncta are produced when either Δ16-WldS or hexameric Nmnat1 are conjugated to tetrameric DsRedII.

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Table 2

| Distribution profile of WldS protein in the transgenic mouse and rat |
|------------------------|-----------------|-----------------|-----------------|-----------------|
|                        | % Tubes | % large Inclusions | % Speckles | % strong Diffuse | % weak Diffuse/none |
| **Transgenic mouse**   |         |                   |          |                 |                   |
| Caudate                | 0       | 55                | 0        | 0               | 100               |
| Cerebellum: granule cells | 0     | 2                 | 0        | 0               | 100               |
| Cerebellum: Purkinje cells | 0   | 0                 | 0        | 0               | 100               |
| Cerebellum: other cell types | 6   | 2                 | 0        | 0               | 100               |
| Cortex: layer 4        | 0       | 8                 | 0        | 73              | 27                |
| Cortex: layer 6        | 0       | 8                 | 0        | 72              | 28                |
| Dentate                | 0       | 7                 | 15       | 0               | 100               |
| Hippocampus: CA1       | 0       | 1                 | 65       | 20              | 80                |
| Hippocampus: CA3       | 0       | 0                 | 3        | 0               | 100               |
| Motor neurone          | 0       | 0                 | 100      | 0               |                   |

| **Transgenic rat**     |         |                   |          |                 |                   |
| Caudate                | 0       | 3                 | 15       | 0               | 100               |
| Cerebellum: granule cells | 0   | 2                 | 0        | 0               | 100               |
| Cerebellum: Purkinje cells | 0   | 0                 | 0        | 0               | 100               |
| Cerebellum: other cell types | 0  | 17                | 0        | 17              | 83                |
| Cortex: layer 4        | 0       | 2                 | 0        | 17              | 83                |
| Cortex: layer 6        | 0       | 0                 | 2        | 15              | 85                |
| Dentate                | 0       | 5                 | 0        | 27              | 73                |
| Hippocampus: CA1       | 0       | 4                 | 6        | 0               | 100               |
| Hippocampus: CA3       | 0       | 7                 | 4        | 4               | 47                |
| Motor neurone          | 0       | 0                 | 100      | 0               |                   |
| DRG                    | 0       | 0                 | 19       | 19              | 81                |

The percentage of cells in each region displaying a particular WldS protein nuclear distribution pattern was assessed from low power confocal images. The distribution pattern was divided into large inclusions, multiple small inclusions (speckles), strong diffuse and weak diffuse/no discernible staining. Some cells were placed into more than one category, as in the analysis for native WldS mice (see Table 1).
A further intriguing observation made here is that the WldS foci co-localize with subunits of the 20S proteasome in culture. As no ubiquitinated WldS was detected in a previous study (Laser et al., 2006), it probably does not accumulate at the proteasome simply because it is degraded there. The distribution of the proteasome has been reported to be similar in vivo between WldS and wild-type mice (Fang et al., 2005), so its localization does not appear to be different between the two strains.

Our data add to existing links between WldS and the UPS. The VCP binding sequences within WldS are derived from the E4 ubiquitin ligase Ube4b, which polyubiquitinates its substrates, targeting them for degradation by the proteasome. As WldS lacks the catalytic U-box of Ube4b it has been suggested that it may exert a dominant-negative effect on endogenous Ube4b function. Though no general inhibition of the UPS has been observed, it is plausible that specific Ube4b targets could be upregulated because they are not degraded efficiently, as supported by a recent publication showing reduced ubiquitination and increased expression of CD200 in the spinal cord of WldS mice (Chitnis et al., 2007). However, no increase in CD200 levels has been detected in WldS transgenic rats, suggesting that this effect may not mediate the WldS phenotype (Adalbert et al., 2005). Proteasome inhibitors can also delay Wallerian degeneration (MacInnis and Campenot 2005; Zhai et al., 2003). Hence, the observed co-localization between WldS and the proteasome further implies that the UPS may be involved in slow Wallerian degeneration.

VCP and UPS components (or ubiquitinated proteins) have been identified in inclusions found in several neurodegenerative conditions (Diaz-Hernandez et al., 2004; DiFiglia et al., 1997), and VCP alone has been observed in protein accumulations in amyotrophic lateral sclerosis, Parkinson's disease, Alzheimer's disease and motor neuron disease with dementia (Ishigaki et al., 2004; Mizuno et al., 2003). One common question in many of these conditions is whether protein inclusions themselves are toxic. Several reports suggest that this is not always the case (Slow et al., 2006). Here, we show that the spontaneous WldS mutant mouse expresses large inclusions in the nuclei of over 90% of its cerebellar granule cells, occupying 0.1% of the total nuclear volume. However, despite these accumulations these mice are viable with no apparent health defects. While we cannot comment on specific neurodegenerative conditions, this observation suggests that nuclear protein aggregates per se are not toxic.

It is also interesting to note that VCP, required to produce the WldS puncta, is a binding partner of ataxin 3 (Boeddrich et al., 2006; Zhong and Pittman 2006). It has also been suggested to be a binding partner of expanded polyglutamine (Hirabayashi et al., 2001), and though a recent report suggests that this interaction is not direct, a polyglutamine expansion does appear to enhance binding between VCP and ataxin-3 (Boeddrich et al., 2006). VCP has been reported to modulate ataxin-3 aggregation in vitro, at low levels increasing aggregation while at high levels suppressing it, and to reduce pathogenicity in a Drosophila model (Boeddrich et al., 2006). Homologs of VCP in Caenorhabditis elegans have been shown to suppress polyglutamine aggregation (Yamanaka et al., 2004). Hence, while the specific components of these aggregates will be different from the WldS foci, it is intriguing to speculate that their mechanisms of formation could potentially be related, and hence data gathered on the construction of the WldS puncta could have wider-ranging implications for other VCP-containing protein aggregates that occur in disease.

In addition to assessing co-localization between the proteasome and WldS foci, we also looked for an association with PML (PML bodies), SMN (gems), coilin (Cajal bodies), ASF (speckles) and PSP1 (paraspeckles). Though direct co-localization was rarely
observed between WldS and these bodies, we cannot rule out the possibility of partial or transitory associations occurring. It is interesting that PML foci, and to a lesser extent coilin, did occasionally appear directly adjacent to the WldS foci (Fig. 7 and Supplementary Fig. 6), particularly given that PML bodies have been observed to associate with both the proteasome and coilin in
amplitude of the fibre volley has been normalized to the mean amplitude recorded with the 11 V stimulus for the appropriate pathway in acute slices. These values were as follows: wild-type Schaffer-collateral = \(\text{4 h}\); following slice preparation and pathway axotomy (\(\text{4 h}\)). Middle panel; in \(\text{Wld}^6\) mice input–output relationships for fibre volleys recorded within 4 h or 48 h following slice preparation (and pathway axotomy) are not significantly different (\(n=4\) (4 h); \(b=4\) (48 h)). Right-hand panel; in contrast fibre volleys from mossy fibres in \(\text{Wld}^6\) mice are absent 48 h following slice preparation and pathway axotomy (\(p<0.0001\) compared to acute slices, 2-way ANOVA comparing full input–output curves). For comparison the input–output relationship for this pathway and recorded from acute slices at 4 h (\(o\)) is shown (\(n=4\) (4 h); \(n=8\) (48 h)). Middle panel; in \(\text{Wld}^6\) mice input–output relationships for fibre volleys recorded within 4 h or 48 h following slice preparation (and pathway axotomy) are not significantly different (\(r=4\) (4 h); \(n=4\) (48 h)).

Fig. 8. Schaffer collaterals, but not mossy fibres, show protection from degeneration in \(\text{Wld}^6\) mouse hippocampal slices. A) Extracellular field potential waveforms from wild-type and \(\text{Wld}^6\) spontaneous mutant mice from the pathways, as indicated, and in response to a 9 V stimulus (each waveform is the average of 10 traces). Field potentials were recorded within 4 h (upper traces) or 48–52 h following slice preparation (48 h; lower traces). Where fibre volleys are present, these are indicated by arrows. Scale bars: 5 mV and 2.5 ms. B) Left-hand panel, in wild-type mice the fibre volleys in recordings from the CA1 region were not present 48 h (●) following slice preparation and Schaffer-collateral pathway axotomy (\(p<0.0001\) compared to acute slices, 2-way ANOVA comparing full input–output curves). For comparison the input–output relationship for Schaffer-collateral fibre volleys recorded from acute slices at 4 h (●) is shown (\(n=4\) (4 h); \(b=8\) (48 h)). Middle panel; in \(\text{Wld}^6\) mice input–output relationships for fibre volleys recorded within 4 h or 48 h following slice preparation (and pathway axotomy) are not significantly different (\(r=4\) (4 h); \(n=4\) (48 h)). Right-hand panel; in contrast fibre volleys from mossy fibres in \(\text{Wld}^6\) mice are absent 48 h following slice preparation and pathway axotomy (\(p<0.0001\) compared to acute slices, 2-way ANOVA comparing full input–output curves). For comparison the input–output relationship for this pathway and recorded from acute slices (4 h) is shown (\(n=5\) (4 h); \(n=4\) (48 h)). For each of the input–output relationships the amplitude of the fibre volley has been normalized to the mean amplitude recorded with the 11 V stimulus for the appropriate pathway in acute slices. These values were as follows: wild-type Schaffer-collateral = −2.2 mV; \(\text{Wld}^6\) Schaffer collateral = −1.3 mV and \(\text{Wld}^6\) mossy fibre = −1.3 mV.

One probable reason for the variation in distribution between the spontaneous mutant and the transgenics is that the spontaneous mutant expresses \(\text{Wld}^6\) under the native Ube4b promoter, whereas in the transgenic mice and rats it is driven by the \(\beta\)-actin promoter. However, this alone does not explain the variation for two reasons: firstly, in the cerebellum Ube4b itself is predominately expressed in Purkinje cells (Kaneko et al., 2003), which exhibit very weak \(\text{Wld}^6\) immunostaining; secondly, the \(\beta\)-actin promoter would be expected to produce ubiquitous expression across neurons, but this is not the case. It is also possible that the variation in the distribution of \(\text{Wld}^6\) in the transgenic mice could be due to suppression of the \(\beta\)-actin promoter in some cell types. With regard to the expression of nuclear foci, as VCP binding is critical for their formation and VCP levels affect foci formation in culture, we speculate that VCP expression or levels of other yet unidentified components of the \(\text{Wld}^6\) foci could affect their propensity to form in different cell types.

We also assessed the distribution of \(\text{Wld}^6\) in our transgenic mouse strains \(\Delta{16-\text{Wld}^6}\) and \(\text{Atx-Wld}^6\), showing that removal of \(\text{N16}\) and...
consequently the VCP binding sequence disrupts foci formation in brain, but that addition of the ataxin 3 VCP binding sequence is unable to restore the foci in vivo (though it can do so in vitro). Hence, there are differences between in vitro and in vivo situations possibly due to Wld\(^6\) or VCP levels or other factors as mentioned above.

Finally, we demonstrated here that there is a relationship between visible immunoreactivity for Wld\(^6\) and slow Wallerian degeneration in the hippocampus of the spontaneous mutant mouse. We have previously shown that the amount of axonal and synaptic protection correlates with Wld\(^6\) protein expression level (Mack et al., 2001; Gillingwater et al., 2002, 2006). Thus, one possibility is that the relatively low level of expression of Wld\(^6\) protein in CA3 neurons (compared with, say, cerebellar neurons, cortical neurons or motor neurons) is not sufficient to protect synapses in either the mossy fibre or Schaffer-collateral pathways in the hippocampus from axotomy-induced degeneration. While we cannot yet definitively resolve the question of why the Wld\(^6\) protein is so differently distributed across the CNS, these data are important to consider when breeding Wld\(^6\) mice with disease models to determine if there is an effect on symptoms and progression. The axons of Schaffer collaterals, which are derived from the Wld\(^6\)-positive CA3 region of the hippocampus, in which 80% of cell bodies have visible nuclei, continue to elicit fibre volleys upon stimulation after 2 days in culture. By contrast DG-derived mossy fibres with weaker Wld\(^6\) expression from the same slice lose their functional activity within this time period. Higher expression is however visualised in the DG of transgenic mice, and hence these transgensics may be useful where Wld\(^6\) expression in the spontaneous mutant mouse appears to be incompatible with certain experiments.

In summary we show that VCP binding within N16 is required for formation of the Wld\(^6\) nuclear foci, which also contain proteasome subunits in vitro. In vivo, the distribution of this protein is diverse and we observe a correlation between its visible expression levels and axonal protection in the hippocampus which may be considered in future studies involving crosses with Wld\(^6\) mice. Our studies also have wider implications for how VCP binding could influence Wld\(^6\) function, wherever in the cell its neuroprotective action actually occurs. Though there appears to be no functional correlation between the Wld\(^6\) foci and axonal protection, it is intriguing that foci formation requires VCP binding within a domain essential for the protective phenotype. Hence study of these foci, their formation and interactions could generate further clues into the underlying mechanisms of slow Wallerian degeneration.

Experimental methods

Constructs

N-terminally truncated Wld\(^6\) cDNAs were PCR-amplified from Wld\(^6\) in pH\(\beta\)Apr-1 (Mack et al., 2001) using primers as below. PCR products were cloned into the pEGFP-N1 vector (Clontech), producing a C-terminal eGFP tag.

Reverse primer for all constructs (mutated Wld\(^6\) stop codon underlined and 5′ BamHI site in bold):

- 5′-GGCGGATCCCTCCGACAGTGGAAATGGTTGTG-3′
- 5′-TAGCCCAGCTTTAGAGGAAGCAGGTGCTTGGTGGACAGACCT
- 3′-AAGCTTTTGTCGAC-3′

For untagged Δ16-Wld\(^6\), the transgenic construct in pH\(\beta\)Apr-1 was used. Briefly, truncated Wld\(^6\) was PCR-amplified from full-length Wld\(^6\) in pH\(\beta\)Apr-1 (Mack et al., 2001) using high-fidelity Pfu polymerase (Stratagene). Linearised Δ16Wld\(^6\)-β\(\beta\)Apr-1 above) was used as a template together with two long complementary primers containing the ataxin-3 VCP binding motif conjugated to the start of Δ16-Wld\(^6\). The forward primer is shown below (HindIII site bold, artificial start codon underlined, Δ16-VCP binding motif italicized). The long reverse primer is the exact reverse complement of this sequence:

- 5′-AGCTTTAGAGGAAGCAGGTGCTTGGTGACAGACCT
- 3′-AAGCTTTTGTCGAC-3′

In addition, a short F primer binding within the ataxin-3 VCP binding motif (5′-TAGCCAAGCTTTAGAGGAAGCAGTGGCTTGGTGACAGACCT-3′) and the FT4-Bam reverse primer (above), which binds at the end of Wld\(^6\), were added to the reaction mix to amplify the full-length Atx-Wld product. The PCR product was precipitated, HindIII/BamHI digested and cloned into pH\(\beta\)Apr-1.

HrdI-Wld was produced using the same strategy as for Atx-Wld. The product was PCR-amplified using linearised Δ16-Wld\(^6\) as a template combined with two long complementary primers encoding the sixteen amino acid HrdI VCP binding sequence conjugated to the start of Δ16-Wld\(^6\). The long forward primer sequence was (HindIII site bold, artificial start codon underlined, HrdI VCP binding motif italicized):

- 5′-AGCTTTAGAGGAAGCAGGTGCTTGGTGACAGACCT
- 3′-AAGCTTTTGTCGAC-3′

Into the reaction mix was added a short forward primer previously within the HrdI region (5′-TAGCCAAGCTTTAGAGGAAGCAGTGGCTTGGTGACAGACCT) and the reverse primer FT4-Bam. The resulting HrdI-Wld product was then cloned into the pH\(\beta\)Apr-1 vector with HindIII/BamHI.

Point-mutated Wld\(^6\) constructs to disrupt VCP binding were PCR-amplified as described by Morreale et al. (submitted for publication) by mixing primers as below with Wld\(^6\) in pH\(\beta\)Apr-1 (template), and the Wld\(^6\)-ΔN16 F and FT4Bam primers:

- Wld\(^6\)-ΔN16 F: TATAGTCCCAAGCTTTAGAGGAAGCAGTGGCACGACCT
- R10A F: AGCGCTGACGAGATTGGCGGCGCTGTCGCA
- R10A R: TGGACCGGCGCTGTCGCAATCTGCTACGCT
- R11A F: GCTGACGAGATTGGCGGCGCTGTCGCA
- R11A R: TGGACCGGCGCTGTCGCAATCTGCTACGCT
- R13A F: GATTGCGACGAGGGCGCTGTCGCA
- R13A R: CCAGCAAGCTTGACGACGCGCTGTCGCA

Wld\(^6\)-FLAG was amplified from Wld\(^6\) in pH\(\beta\)Apr-1 using the primers below, and cloned into pcDNA3 using HindIII/BamHI (Invitrogen). All constructs described were confirmed by sequencing (Cogenics Lark UK).
Wild<sup>5</sup>-Forward (HindIII site bold, start codon underlined):

\[ 5'\text{-TAGCCCAAGCTTTAGAGGAAGCGTGGAGGAGCT-} \text{GAGCCTGCT-3'} \]

Wild<sup>5</sup>-FLAG reverse (BamHI site bold, mutated stop codon underlined, FLAG tag italicized):

\[ 5'\text{-CGCGGAGTCCACTTTGTCATGCCTTCTTGGATACCCCA-} \text{GAGTGGGAGTGTGGCTGGC-3'} \]

To produce Wild<sup>5</sup>–mCherry, we BamHII/EcoRI digested mCherry from the pRSETB vector (courtesy of the Tsien Lab) and ligated it into pcDNA3 (Invitrogen). Wild<sup>5</sup> with a mutated stop codon was then digested from the Wild<sup>5</sup>–eGFP vector with HindIII/BamHI and ligated N-terminal to mCherry such that the coding sequences were in frame.

HdRI-FLAG (synonym: synoviolin) and Ube4b-FLAG were generated as in Morreale et al. (submitted for publication) by PCR using the primers below. These were digested with HindIII/BamHI and ligated into pH<sup>5</sup>-dr4 (GIBCO) and 100 ng/ml NGF (Invitrogen). HeLa cells were cultured as described above and seeded into 6-well dishes (for Western blot) or IBIDI coverslip-bottom dishes (for immunocytochemistry). These were transfected the following day using HiPerfect transfection reagent (Qiagen) following a modified protocol. Briefly, 1.2 μl of siRNA stock and/or 100 ng Wild<sup>5</sup>–mCherry (or e-GFP) plasmid were diluted in 50 μl HBS buffer (20 mM HEPES, 150 mM NaCl, pH 7.4). Separately 6 μl HiPerfect was diluted in 50 μl HBS per dish, incubated 5 min at room temperature and mixed with the siRNA/siRNA solution. After a further 10 min the 100 μl mixture was added to cells in 450 μl OptiMEM medium and cultures returned to the incubator. Four hours after transfection medium was supplemented with 650 μl HeLa growth medium. Cells were fixed with 4% PFA or lysed and scraped in 300 μl RIPA buffer as appropriate 4 h after transfection. Imaging data from 3 experiments was analysed using ANOVA, Games–Howell post-hoc test.

### Western blotting

Cell lysates were mixed in a ratio of 2:1 with laemmli (BioRad) containing 5% β-mercaptoethanol, boiled and run on 10% SDS-PAGE gels which were transferred semi-dry to nitrocellulose membrane. Membranes were blocked in 5% milk in 0.2% tween/PBS for 1 h and incubated at 4 °C overnight in an appropriate primary antibody [anti-Wld18 at 1:2000 (Samsam et al., 2003), anti-VCP 1:500 (BD Transduction Laboratories), anti-β-actin 1:5000 (Abcam)]. After washing in 0.2% PBS/tween blots were incubated for 1 h in secondary antibody (anti-mouse or anti-rabbit HRP; both 1:3000 GE Healthcare), washed again, treated with ECL (GE Healthcare) and exposed to film.

### Mice

Spontaneous mutant C57BL/6Olalisd-Wld<sup>(ild)</sup> mice and C57BL/6 (wild-type) mice were obtained from Harlan Olac Laboratories (Bicester, UK) and housed within the animal care facilities in Edinburgh. Transgenic mouse line 4836 and transgenic rat line 23 housed in the Babraham Institute were engineered to express the Wld<sup>2</sup> gene under a β-actin promoter, as described previously (Adalbert et al., 2005; Mack et al., 2001). All animal work was performed under the UK Animals (Scientific Procedures) Act 1986 under Project Licence numbers 80/1778 and 60/3277.

### Immunohistochemistry

Immunofluorescence staining of 2–3 month old spontaneous mutant Wild<sup>5</sup>, line 79 transgenic mice and line 23 transgenic rat brains was performed on 20 μm cryostat-cut sections from 4% PFA perfuse-fixed mouse brains or freshly cut 100 μm slices fixed in 4% PFA (Fisher). There was no difference in the staining pattern obtained with either method. Slices were blocked overnight in 4% bovine serum albumin (BSA, Sigma) with 0.5% Triton X-100 (Sigma) in PBS. Wild<sup>18</sup> antibody (Samsam et al., 2003); 1:500 dilution in blocking solution) was applied overnight and, after washing with PBS, a TRITC-conjugated anti-rabbit secondary antibody (DAKO; 1:20 dilution in PBS) was applied overnight. Slices were then washed in PBS and incubated in To-pro 3 (Molecular Probes) for 10 min before mounting in Mowiol. Staining was visualised on a laser scanning confocal microscope (BioRad Radiance 2000,
Hemel Hempsted, UK) and Z-series were merged using Lasersharp (BioRad) software.

To quantify nuclear WldS staining, several images were taken from each region using the same laser intensities, magnification and Z-series stacks. Nuclei containing different WldS protein expression patterns were counted and expressed as a percentage of the total To-pro 3 stained nuclei per image. These percentages were averaged over all the images within a particular brain region.

High magnification confocal images from 4 separate WldS mice and staining runs were used to measure the diameter of the cerebellar granule cell inclusions. Z-series were generated using a 60× objective (N.A. 1.4) with 8× zoom and imported into Image Pro (Media Cybernetics). Since the nuclei were close together, their diameters were measured manually using a line measuring tool. WldS inclusions were measured using an automatic calculation function (results expressed in μm). These data were used to calculate the volumes of the nuclei and inclusions.

Images were imported into Adobe Photoshop and where necessary the brightness and contrast were adjusted to distinguish the WldS and To-pro 3 staining. This was done with reference to the original image. Tissues from 3–5 month old Δ16-WldS, Azts-WldS and appropriate WldS and C57BL/6J controls were processed as follows. Observations were made from three line 1 homozygote and two line 2 hemizygote Δ16-WldS mice and three homozygote mice from each of lines 1, 6 and 7 of Azts-WldS. Twenty micrometre cryostat sections from 4% PFA-perfused brains were cut, washed with 0.05 M TBS and incubated for 10 min in cell penetrator solution (6 g NH4Cl and 50 μl Triton X-100 in 20 ml 0.05 M TBS). These were blocked in 5% albumin fraction V (Merck) in TBS for 1 h followed by incubation with 1:500 α-To-pro 3 in 0.8% albumin fraction V/TBS overnight at 4°C. Samples were then washed with TBS and 1:200 alpha-fluor 568 goat anti-rabbit (Molecular Probes, cat # A11036) in 0.8% albumin fraction V/TBS applied for 1 h (room temperature). After a final wash, slides were mounted with Vectashield containing DAPI, and visualised at 40× magnification on a Zeiss LSM 510 META confocal microscope.

Electrophysiology

Mice (2 months old) were killed by cervical dislocation and their brains removed to oxygenated, ice-cold, high magnesium artificial cerebrospinal fluid (ACSF, 125 mM NaCl, 26 mM NaHCO3, 25 mM glucose, 2.5 mM KCl, 1.25 mM NaH2PO4, 1 mM CaCl2, 4 mM MgCl2). For studies of hippocampal function in acute preparations, 400 μm slices were cut on a Vibratome 1000+ tissue slicer (Campden Instruments, Loughborough, UK). In half the slices, a sterile scalp knife was used to cut through the mossy fibre pathway at the point where they exit the dentate gyrus. In the remaining slices the Schaffer-collateral pathway was axotomised at the CA3/CA1 boundary. All the slices were then transferred to a holding chamber where they were suspended, on a fine net, in high magnesium ACSF, oxygenated with 95% O2/5% CO2, at 32°C – 1°C and incubated for 10 min in cell penetrator solution (6 g NH4Cl and 50 μl Triton X-100 in 20 ml 0.05 M TBS). These were blocked in 5% albumin fraction V (Merck) in TBS for 1 h followed by incubation with 1:500 α-To-pro 3 in 0.8% albumin fraction V/TBS overnight at 4°C. Samples were then washed with TBS and 1:200 alpha-fluor 568 goat anti-rabbit (Molecular Probes, cat # A11036) in 0.8% albumin fraction V/TBS applied for 1 h (room temperature). After a final wash, slides were mounted with Vectashield containing DAPI, and visualised at 40× magnification on a Zeiss LSM 510 META confocal microscope.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mcn.2008.03.004.

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