Differential Proteomics Analysis of Synaptic Proteins Identifies Potential Cellular Targets and Protein Mediators of Synaptic Neuroprotection Conferred by the Slow Wallerian Degeneration (WldS) Gene

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Non-somatic synaptic and axonal compartments of neurons are primary pathological targets in many neurodegenerative conditions, ranging from Alzheimer disease through to motor neuron disease. Axons and synapses are protected from degeneration by the slow Wallerian degeneration (WldS) gene. Significantly the molecular mechanisms through which this spontaneous genetic mutation delays degeneration remain controversial, and the downstream protein targets of WldS resident in non-somatic compartments remain unknown. In this study we used differential proteomics analysis to identify proteins whose expression levels were significantly altered in isolated synaptic preparations from the striatum of WldS mice. Eight of the 16 proteins we identified as having modified expression levels in WldS synapses are known regulators of mitochondrial stability and degeneration (including VDAC1, Aralar1, and mitofilin). Subsequent analyses demonstrated that other key mitochondrial proteins, not identified in our initial screen, are also modified in WldS synapses. Of the non-mitochondrial proteins identified, several have been implicated in neurodegenerative diseases where synapses and axons are primary pathological targets (including DRP-2 and Rab GDP dissociation inhibitor β). In addition, we show that downstream protein changes can be identified in pathways corresponding to both Ube4b (including UBE1) and Nmnat1 (including VDAC1 and Aralar1) components of the chimeric WldS gene, suggesting that full-length WldS protein is required to elicit maximal changes in synaptic proteins. We conclude that altered mitochondrial responses to degenerative stimuli are likely to play an important role in the neuroprotective WldS phenotype and that targeting proteins identified in the current study may lead to novel therapies for the treatment of neurodegenerative diseases in humans. Molecular & Cellular Proteomics 6: 1318–1330, 2007.

Despite a traditional focus on the neuronal cell body for uncovering mechanisms underlying neurodegenerative disease, many recent studies have highlighted the important role that non-somatic neuronal compartments play in the instigation and progression of neurodegeneration (1, 2). As a result, distal neuronal compartments such as synapses and axons are now regarded as primary pathological targets in a broad spectrum of human and animal neurodegenerative conditions, ranging from Alzheimer disease through prion disease to motor neuron disease (2). And yet despite our increasing awareness of the vulnerability of synapses and axons, our understanding of the independent mechanisms regulating non-somatic neurodegeneration remains inadequate, and few therapeutic strategies exist that are capable of targeting these distinct cellular compartments.

Several recent breakthroughs in our understanding of mechanisms underlying non-somatic degeneration have taken advantage of the slow Wallerian degeneration (WldS)1 mutation that selectively protects synapses and axons (3).

1 The abbreviations used are: WldS, slow Wallerian degeneration; Aralar1, calcium-binding mitochondrial carrier protein 1; DRP-2, dihydropyrimidinase-related protein 2; NAD, nicotinamide adenine dinucleotide; Nmnat1, nicotinamide mononucleotide adenylyltransferase 1; STI1, stress-induced phosphoprotein 1; UBE1, ubiquitin-activating enzyme E1; VCP, valosin-containing protein; VDAC, voltage-dependent anion-selective channel protein; ANOVA, analysis of variance; SNAP, soluble N-ethylmaleimide-sensitive factor attachment protein; GDI, GDP dissociation inhibitor; 2D, two-dimensional; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; IPA, Ingenuity Pathways Analysis; S, soluble; M, membrane; CAPZ α2, F-actin capping protein α-2 subunit.
The Wilds gene encodes a fusion protein comprising the full length of nicotinamide mononucleotide adenyllytransferase 1 (Nmnat1; a NAD⁺-synthesizing enzyme) coupled by a unique 18-amino acid sequence to the N-terminal 70 amino acids of the ubiquitination enzyme Ube4b (10, 13). Transgenic expression of this fusion gene and its corresponding chimeric protein is sufficient to confer the full Wilds neuroprotective phenotype (10–12). Wilds protein product is localized exclusively to neuronal nuclei (7, 10, 14). This is intriguing as the protein provides substantial protection for axons and synapses, but cell bodies degenerate normally (15–17). The localization of Wilds protein in cellular compartments other than those that are protected by its action indicates that it confers its neuroprotective effects indirectly through its effects on protein expression or function in distal, non-somatic cellular compartments.

There is currently a considerable amount of controversy over whether individual fragments of the Wilds gene or the chimeric gene in its entirety is responsible for conferring the neuroprotective phenotype. Some studies have identified an important role for the Ube4b fragment, thereby implicating changes in the function of the ubiquitin-proteasome system in the Wilds phenotype (18–20). Other studies have suggested that the Nmnat1 fragment alone may be required to confer neuroprotection, thereby implicating changes in the function of NAD-dependent pathways in the Wilds phenotype (21–23). However, these findings are inconsistent with recent in vivo and in vitro studies suggesting that Nmnat1 alone is not capable of replicating the Wilds phenotype (24, 25). Moreover other experiments have suggested that the chimeric gene in its entirety may be required to elicit maximal modification of neurodegenerative pathways (26). One major step toward resolving this controversy will be to identify the downstream proteomic changes that occur in distal, non-somatic neuronal compartments as a result of Wilds expression.

In the present study we carried out a differential proteomics analysis of synaptic proteins in Wilds and wild-type mice, focusing on synaptosomes extracted from the striatum that have a robust neuroprotective phenotype after traumatic nerve injury (27). Our data revealed modifications in the synaptic proteome relating to both ubiquitination and NAD-dependent pathways, supporting the hypothesis that changes in both may be required to elicit the full Wilds neuroprotective phenotype. Significantly we also showed that several protein changes identified in Wilds synapses are likely to result in altered mitochondrial responses to neurodegenerative stimuli.

**EXPERIMENTAL PROCEDURES**

**Synaptosome Preparation**—Two-month-old, female C57Bl/6 (wild type) and Wilds mice were obtained from Harlan Olac Laboratories (Bicester, UK) and housed within the animal care facilities in Edin-burgh. Care was taken to ensure that the wild-type mice did not contain the α-synuclein gene deletion that was present in a substrain of Harlan Olac Bl6 mice. We confirmed the α-synuclein-positive status of our wild-type mice by Western blotting for α-synuclein in our synaptosome protein extract (see below; data not shown). Mice were killed by cervical dislocation, and brains were rapidly removed before the striatum was dissected.

Eighteen striata were removed in total from both wild-type and Wilds mice and then pooled into groups of three striata (i.e. n = 9 mice, n = 3 samples per genotype, each sample composed of three pooled striata). Striata were then homogenized in an ice-cold isotonic sucrose solution (0.32 m sucrose, 1 mM EDTA, 5 mM Tris-HCl, pH 7.4). Homogenate was centrifuged in a fixed angle rotor at 900 × g for 10 min, and the supernatant (S1) was collected. The pellet (P1) was resuspended in sucrose solution and centrifuged again at 900 × g for 10 min. The resulting supernatant (S1') was combined with S1 and centrifuged in a fixed angle rotor at 20,000 × g for 15 min. The supernatant (S2) was discarded, and the pellet (P2) containing synaptosomes was washed in a Krebs-like buffer (118.5 mM NaCl, 4.7 mM KCl, 1.18 mM MgCl₂, 0.1 mM K₂HPO₄, 20 mM HEPES, 1.3 mM CaCl₂, 10 mM glucose, pH 7.4) and then centrifuged at 14,000 × g for 10 min.

**Sample Homogenization and Protein Solubilization**—Sample homogenization and protein solubilization were carried out as described previously (28). For both wild-type and Wilds mice, synaptosome preparations from each of the three replicates were homogenized separately on ice with 300 μl of hypotonic ice-cold sucrose buffer (0.25 m sucrose, 10 mM HEPES, 1 mM EDTA, 1% (v/v) Sigma P8340 protease inhibitor mixture, pH 7). The lysate was centrifuged using a TL100 ultracentrifuge (Beckman) at 35,000 × g for 10 min at 4 °C, and the supernatant (soluble (S) fraction) was retained. The pellet was then homogenized on ice in 300 μl of ice-cold detergent solution (2% n-octyl glucoside, 0.5 mM aminohexanoic acid), the lysate was centrifuged as before, and the second supernatant (membrane (M) fraction) was retained. Total soluble protein content in each supernatant was determined using the Pierce BCA protein assay kit (Perbio Science, Cheshire, UK). Protein extracts were frozen on dry ice and stored at −70 °C if not immediately required.

**2D Gel Electrophoresis and Visualization**—2D gel electrophoresis and visualization was carried out as described previously (28). Briefly first dimension IEF was carried out using IPG gel strips run on an IEFphor IEF unit (Amersham Biosciences) according to the manufacturer’s instructions. To each 13-cm IPG strip (pH 3–10), 100 μg of protein was applied in 250 μl of rehydration buffer (7 mM urea, 2 mM thiourea, 65 mM DTT, 0.5% (v/v) Triton X-100, 0.5% pH 3–10 IPG buffer, 0.1% (w/v) bromphenol blue). Strips were rehydrated for 14 h followed by IEF for a total of 17,500 V-h at 20 °C. Following IEF, strips were incubated with agitation for 15 min in equilibration solution (50 mM Tris-HCl, 6 mM urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.1% (w/v) bromphenol blue) containing 1% (w/v) DTT and then for 15 min in equilibration solution containing 2.5% (w/v) iodoacetamide. Focused IPG strips were placed on top of self-cast 16.5 × 19-cm linear 10% acrylamide gels. Second dimension SDS-PAGE was carried out at 20 °C and run at 20 mA/gel (29). For visualization, gels were incubated with agitation in fixing solution (10% (v/v) ethanol, 7% (v/v) acetic acid) for 30 min and then in SYPRO Ruby protein stain (Bio-Rad) for at least 3 h. Gels were destained in fixing solution for at least 2 h and washed in distilled water for at least 30 min.

**Analysis of 2D Gels and Protein Spot Identification**—Analysis of 2D gels and protein spot identification were carried out as described previously (28). Analysis of 2D gels was carried out using ImageMaster 2D Platinum software (Amersham Biosciences) according to the manufacturer’s instructions. Protein spots were excised from the gels and in-gel digested with trypsin using a Model 4700 Proteomic System (Applied Biosystems). Peptide mass fingerprints and their corresponding MASCOT scores were used to identify and quantify proteins.
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previously (28). Briefly 2D gels were run in duplicate for the soluble and membrane fractions of the tissue obtained from each of the three replicate groups of animals in the two different experimental conditions (wild type and Wld\(^{e}\)), giving a total of 24 gels included in the analysis. After SYPRO Ruby staining the gels were imaged at 302 nm UV light on an Alpha Innotech FluorChem 8900 Imaging System (Alpha Innotech Ltd., Cannock, UK). Image analysis was carried out using standard procedures in the Phoretix 2D Evolution 2005 software (Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK). Gel images were morphed and aligned to correct for differences in protein separation and migration between gels. Protein spots were detected automatically and verified manually using the montage view of the Phoretix software. After background subtraction the measured volume of each protein spot was normalized as a percentage of the total integrated spot volume on each gel using standard Phoretix protocols. Protein spots with normalized volumes $<$0.03% were not included in further analysis. Matched spots across the two experimental conditions were systematically compared for differences in mean normalized spot volume using a two-way ANOVA with significance accepted at the $p < 0.05$ level.

**MALDI-TOF Mass Spectrometry**—Protein spots showing significant changes in mean normalized volume were selected for spot picking, trypsin digestion, and MALDI-TOF mass spectrometry to identify their protein content. Selected protein spots were manually excised from gels and placed into 96-deep well plates (Abgene), covered with distilled H$_2$O, and stored at $-20^\circ$ C until digestion. Protein in-gel trypsin digestion and MALDI target spotting were performed manually. Gel plugs were washed three times for 30 min each with 200 $\mu$L of 50 mM ammonium bicarbonate in 50% ACN, dehydrated with 100% ACN, dried, and then rehydrated with 200 $\mu$L of sequencing grade modified trypsin (Promega, Madison, WI) in 20 mM ammonium bicarbonate. The samples were then covered and incubated overnight at 32 $^\circ$C. The digested protein mixture was spotted onto a MALDI target with an equal volume (0.7 $\mu$L) of a saturated solution of 4-hydroxy-$\alpha$-cyanoacinnamic acid in 50% ACN with 0.1% trifluoroacetic acid.

Mass spectra were acquired on a Voyager DE-STR mass spectrometer (Applied Biosystems, Foster City, CA) recording peptide masses between 700 and 3500 Da. The acquired data were postprocessed to generate peak lists using Data Explorer version 4.0 (Applied Biosystems). Briefly MALDI spectra were base line-corrected with some noise reduction before they were desalted avoiding only the monoisotopic masses. They were then calibrated using trypsin autolysis peaks (generated at 842.5100, 1045.5642, and 2211.1046) after which the masses were extracted and submitted for database interrogation. The PS1 system (as supplied with the Voyager DE-STR mass spectrometer, Applied Biosystems) interrogates the database with an error of 100 ppm. Each selected hit is then used to re-calibrate the spectrum and then match to that hit using a more stringent error of 15 ppm (manual searches and checking of spectra were accomplished using the more widely accepted variance of 50 ppm). All peptide masses collected from mass spectra were submitted for matching at the Swiss-Prot database (February 21, 2006; 243,975 protein entries; us.expasy.org/) using Protein Prospector (version 4.07, MS-Fit; Ref. 28) and the PS1 Mass Spec searching program (as supplied with the Voyager DE-STR mass spectrometer, Applied Biosystems). Search parameters were as follows: N terminus, "His"; C terminus, "acid"; $M_s$ searched from 1000 to 100,000 and pI searched from 3 to 10; minimum number of peptides to match, 4; only five reported hits; one missed cleavage allowed. Possible modifications included peptide N-terminal Gln to pyro-Glu, oxidation of Met, protein N terminus acetylated, and Cys modified by carbamidomethylation. Additional considerations included the species data available in the protein database and the gel coordinates ($M_s$/pI) of the gel spot to be matched. To eliminate redundancy caused by proteins appearing in multiple databases under different names and accession numbers, the Swiss-Prot nomenclature and accession numbers (which have minimal redundancy and a high level of integration with other databases) were adopted for all protein identifications. Wherever two proteins from the same family were identified as a match, they could be distinguished by first analyzing the experimentally collected spectra for matched peptides in terms of calibration and signal intensity; second, the pl and $M_s$ of the identified proteins (pre/proprotein and mature cleaved/modified forms) could be compared with the gel location to confirm identity; and third, a comparison of the peptides identified could show one matched protein to contain a greater number of peptides or an extra peptide sequence not contained within the other. Although such instances were not identified in the current study, should none of these methods distinguish between any two matched proteins, they would be recorded as co-migrating proteins, and the gel feature is likely to contain both.

**MALDI-TOF Mass Spectrometry Using ZipTips**—In-gel digested protein samples of low peptide yield were concentrated using reverse-phase C$_{18}$ ZipTips (Millipore). After overnight digestion samples were acidified by dilution to a final concentration of 0.1% TFA or until the pH was about 3. The ZipTip was placed on a 10-$\mu$L pipette, set to maximal volume, wet by aspirating three times in 100% acetonitrile, and then equilibrated in the same way prior to peptide binding in 0.1% TFA. Solvent was discarded after each aspiration. Peptide binding was achieved by repeated aspiration (7–10 times) in the sample well. The bound peptides were then washed twice with 10 $\mu$L of 0.1% TFA, discarding the eluant, before direct elution onto the MALDI target with about 1 $\mu$L of a saturated solution of $\alpha$-cyano-4-hydroxycinnamic acid matrix in 50% acetonitrile, 0.1% TFA.

**Quantitative Western Blotting**—40 $\mu$L of whole brain or synaptosomal protein was separated by SDS-polyacrylamide gel electrophoresis on 4–20% precast NuPage 4–12% BisTris gradient gels (Invitrogen) and then transferred to PVDF membrane overnight. The membranes were then blocked using Odyssey blocking buffer (LI-COR Biosciences) and incubated with primary antibodies according to the manufacturers’ instructions (neurofilament, Calbiochem; synapsin, Calbiochem; synaptophysin, Synaptic Systems; voltage-dependent anion-selective channel protein 1 (VDAC1), GeneTex; ubiquitin-activating enzyme E1 (UBE1), thiorodoxin, cytochrome c, and VDAC2, Abcam; GDP dissociation inhibitor (GDI) 2, Protein Tech Group; soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP), BIOMOL; stress-induced phosphoprotein 1 (St1), BD Biosciences; dihydropyrimidinase-related protein 2 (DRP-2) was generated by Dr. Calum Sutherland, University of Dundee). Odyssey secondary antibodies were added according to the manufacturer’s instructions (goat anti-rabbit IRDye 680 and goat anti-mouse IRDye 800). Blots were imaged using an Odyssey Infrared Imaging System (LI-COR Biosciences). Scan resolution of the instrument ranges from 21 to 339 $\mu$m, and in this study blots were imaged at 169 $\mu$m. Quantification was performed on single channels with the analysis software provided.

**Transmission Electron Microscopy**—Anesthetized C57Bl6 and Wld\(^{e}\) mice (intraperitoneal injection of Ketanest (100 mg/kg) and Rompun (5 mg/kg)) were killed by perfusion fixation with 0.1 $\mu$L phosphate buffer containing 4% paraformaldehyde and 2.5% glutaraldehyde before removing the brain and immersing it in fixative for a further 12 h. Brains were then washed in 0.1 $\mu$L phosphate buffer before cutting free floating 70-$\mu$m-thick coronal sections on a Vibratome. Sections containing the striatum were postfixed in 1% osmium tetroxide in 0.1 $\mu$L phosphate buffer for 45 min. Following dehydration through an ascending series of ethanol solutions and propylene oxide, all sections were embedded on glass slides in Durcupan resin. Regions of striatum were cut out from a randomly
selected section using a scalpel and glued onto a resin block for sectioning. Ultrathin sections (60–70 nm) were cut and collected on Formvar-coated grids (Agar Scientific), stained with uranyl acetate and lead citrate in an LKB “Ultrstainer,” and then quantitatively assessed in a Philips CM12 transmission electron microscope. Negatives taken in the microscope were scanned into an Apple Macintosh G5 computer using an Epson 4870 Photo flat bed scanner at 600dpi and subsequently processed using Adobe Photoshop.

In Silico Protein Network Analysis—To obtain further insight into potential cellular pathways that may be modified as a result of protein changes identified in our experiments, the Ingenuity Pathways Analysis (IPA) application (Ingenuity Systems) was used. IPA dynamically generates networks of gene, protein, small molecule, drug, and disease associations on the basis of “hand-curated” data held in a proprietary database. More than 90% of the information in this database is “expert-curated” and is drawn from the full text of peer-reviewed journals. Less than 10% of interactions have been identified by techniques such as natural language processing. Networks generated by IPA have a maximum of 35 members and are enriched for the input proteins or genes. To enhance the explorative interpretation of data, networks are ranked according to a score calculated via a right tailed Fisher’s exact test. This test outputs a value that takes into account the original input gene or proteins of interest and the size of the network generated. The value enables the application to approximate how relevant the network is to the analysis. It should be noted that this score does not indicate the biological relevance or quality of the network but simply provides a useful indicator with which to start an explorative analysis. Further information on the computational methods implemented in IPA can be obtained from Ingenuity Systems.

In this study, the Swiss-Prot protein accession numbers for the 16 proteins identified in the proteomics analysis described above were uploaded to the IPA application. The software was then used to generate networks of association between the input proteins and genes/proteins present in the IPA database.

RESULTS

We chose to investigate protein changes in isolated nerve terminal (synaptosome) preparations from Wild® mice to identify the eventual non-somatic protein targets of upstream cellular pathways modified by Wild® expression. Importantly we undertook this analysis in synapses from an identified anatomical region, the striatum, where a strong synaptotrophic phenotype has been demonstrated previously after cortical lesion (27). We investigated protein levels in isolated synaptic preparations to identify synapse-specific targets of Wild® and to avoid the possibility that low abundance proteins of interest may be masked by potentially more abundant somatic and/or myelin proteins in whole tissue preparations containing cell bodies and axons.

Age- (2 months) and gender (female)-matched wild-type (C57Bl6) and Wild® (C57Bl6/Wild®) mice were used to make striatal synaptosomes. The striatum was dissected out from nine mice of each strain and pooled into three groups of three striata for the preparation of synaptosomes (see “Experimental Procedures”). The synaptosomes were homogenized, and a two-step protein extraction protocol was used to obtain an S fraction and a pelletted M fraction from each sample. To confirm that our synaptosome preparations were synaptically enriched, we first quantified expression levels of known presynaptic proteins using Western blots on total protein extracted from whole brain versus synaptosome preparations (Fig. 1). These experiments showed that levels of the synaptic protein synaptophysin were significantly elevated in our synaptosome preparations compared with whole brain preparations (increase of 211 ± 43%, mean ± S.E.; p < 0.01, two-tailed unpaired t test). Quantification of expression levels from these blots (see “Experimental Procedures”) also revealed no differences in synaptophysin or neurofilament protein levels between wild-type and Wild® mice (Fig. 1 and also see Fig. 5).

Representative 2D electrophoresis gels of proteins present in striatal synaptosome preparations are shown in Fig. 2 (S fraction) and Fig. 3 (M fraction). Protein spots were detected automatically using the Phoretix 2005 gel analysis software and verified manually. Poorly defined spots and spots with normalized volumes of <0.03% were excluded from further analysis. In total, 679 spots were matched between the wild-type and Wild® samples and analyzed for significant differences in normalized volume between the Wild® and wild-type tissue. Although the total number of proteins in the synaptic proteome is unknown, it is likely to consist of up to 3000 proteins (30). It is likely, therefore (see Fig. 6 below and “Discussion”), that our current analysis has missed other proteins that are changed in Wild® synapses either through incomplete separation on the gels or masking by more abundant proteins of a similar size and charge.

Differential analysis and presence/absence analysis of the gels from both soluble and membrane fractions of synaptosome preparations identified 20 spots with significant differences in their expression levels (ANOVA, p < 0.05; Table I). Fig. 4 shows examples of three-dimensional peaks derived from the optical density profile of wild-type and Wild® gel images for four of the 16 identified proteins (DRP-2, UBE1, cytosolic acyl-coenzyme A thioester hydrolase, and stress-induced phosphoprotein 1). The reproducibility of such peaks is shown for UBE1 and STI1 in Supplemental Fig. 1.

All 20 spots of interest were picked and digested with trypsin, and the subsequent fragments were analyzed by
MALDI-TOF. Good spectra were obtained for 17 of these, and three could not be positively identified. The 17 spots contained 16 different proteins (Table II). Of the 16 proteins identified, 12 showed modified expression levels (Table I). The other four were present on the wild-type gels but not visible in the Wlds gels (Table I). None of the proteins identified in Wlds synapses were found to have altered localization within the synaptic preparation (i.e., down-regulated in the soluble fraction but correspondingly up-regulated in the membrane fraction). It is therefore likely that most of the protein changes detected were due to differences in the level of expression and/or post-translational modifications.

To validate altered expression levels for our candidate proteins, we performed quantitative fluorescent Western blots for all candidate proteins (where antibodies were available) on freshly prepared synaptosomal protein extract (Fig. 5). Without exception, these experiments confirmed our original 2D gel screen findings. For example, UBE1 expression was up-regulated by 35.5 ± 2.9% (mean ± S.E.) in Wld^s synaptosomes (compare Fig. 5 with Table I). Similarly, VDAC1 expression was down-regulated by 29.3 ± 1.7% (compare Fig. 5 with Table I). Expression levels of other synaptic proteins not altered in our 2D gel experiments (neurofilament and synapsin proteins; see Fig. 1) did not vary by more than 1.5% between wild-type and Wld^s preparations.

Surprisingly, eight of 16 proteins identified by our screen are known to be localized to mitochondria (Table I). As some mitochondrial proteins showed elevated expression levels and others showed reduced levels, it is unlikely that our results were simply due to differences in the number of mitochondria present in Wld^s synapses. Rather, our data suggest that proteins involved in regulating the sensitivity of mitochondria to degenerative stimuli are altered in Wld^s synapses. To ask whether mitochondrial modifications were restricted to those proteins identified above, we used quantitative Western blotting to examine expression levels for other mitochondrial proteins not picked up by our original proteomics screen. These analyses showed that expression levels of cytochrome c, VDAC2, and thioredoxin were also modified in Wld^s synapses (Fig. 6).

As numerous studies examining synaptic form and function in Wld^s mice have yet to find any phenotype other than modified responses to degenerative stimuli (5, 10, 11, 27) it is unlikely that basal function of healthy synaptic mitochondria is altered in Wld^s mice as a result of the protein changes identi-
Mitochondria were contained within the plane of section of synapses (Fig. 7). Thus, protein modifications present in Wld(e) synapses are only found in an altered phenotype when placed under stress following a neurodegenerative stimulus. To investigate this, we used electron microscopy to examine mitochondria in pre- and postsynaptic compartments from the striatum of wild-type and Wld(e) mice (the same region used to prepare synaptosome preparations; a minimum of 40 synaptic profiles per strain). As expected, there were no observable differences in the gross morphology, location, or number of mitochondria in Wld(e) synapses (Fig. 7). Mitochondria were identified as actin (spot numbers 144 and 157), and actin filament assembly and organization (35), it is possible that loss of this protein at the synapse leads to modified actin function and/or deposition. In addition, it is notable that actin occupies a central position within this protein network. Two of the 17 spots of interest were identified as actin (spot numbers 144 and 157), which was also found to be absent in Wld(e) synapses (noting that the majority of other forms of actin are apparently not changed in these forms were absent in Wld(e) synapses; see Fig. 3). These modified forms of actin may be due to the difference in levels of the F-actin capping protein α-2 subunit (CAPZ α2), which was also found to be absent in Wld(e) gels (Table I). As CAPZ α2 is a known regulator of actin filament assembly and organization (35), it is possible that loss of this protein at the synapse leads to modified actin function and/or deposition.

### Table I

| Spot | Acc. no. | Protein | Fr. | Average change in Wld(e) | ANOVA p | Mito? |
|------|----------|---------|-----|--------------------------|---------|------|
| 637  | P51174   | Acyl-CoA dehydrogenase, long chain-specific, mitochondrial precursor | S   | 808 | 0.0030 | Y    |
| 780  | Q9112    | Cytosolic acyl-coenzyme A thioester hydrolase | S   | 473 | 0.0358 | Y    |
| 446  | Q08553   | DRP-2 | S   | 291 | 0.0014 | N    |
| 476  | Q8R339   | Dihydrolipoamide-residue acetyltransferase | S   | 244 | 0.0377 | Y    |
| 802  | P28663   | β-SNAP | S   | 143 | 0.0080 | N    |
| 657  | Q8BFR5   | Elongation factor Tu, mitochondrial precursor | S   | 86  | 0.0433 | Y    |
| 157  | Q02053   | UBE1 | S   | 60  | 0.0057 | N    |
| 116  | Q92DG2   | Dihydrolipoamide-residue succinyltransferase | M   | –93 | 0.0067 | Y    |
| 326  | Q8CAQ8   | Mitochondrial inner membrane protein (mitofilin) | S   | –369 | 0.0089 | Y    |
| 861  | Q69332   | VDAC1 | S   | –408 | 0.0015 | Y    |
| 460  | Q60865   | STI1 | S   | –413 | 0.0271 | N    |
| 650  | Q61598   | Rab GDIβ | S   | –840 | 0.0030 | N    |
| 58   | Q8BH59   | Aralar1 | M   | Abs | N/A | Y    |
| 187  | P51863   | Vacuolar ATP synthase subunit B | M   | Abs | N/A | N    |
| 202  | P77542   | CAPZ α2 | M   | Abs | N/A | N    |
| 144  | P60710   | Actin | M   | Abs | N/A | N    |
| 157  | P60710   | Actin | M   | Abs | N/A | N    |

Notable potential interactions identified by this analysis included those between VDAC1 and Bax (e.g. Bax binds to VDAC1, accelerating channel opening and outflow of cytochrome c from mitochondria (31)) and between UBE1 and Jun (e.g. UBE1 is necessary for the degradation of Jun in G0/S phase transition fibroblasts (32)). Both of these potential interactions serve to raise the hypothesis that protein modifications in Wld(e) mice may be able to directly modulate apoptotic or apoptosis-related cascades known to be active in synaptic compartments (33, 34) with or without mitochondrial involvement. In addition, it is notable that actin occupies a central position within this protein network. Two of the 17 spots of interest were identified as actin (spot numbers 144 and 157), both of which were present in wild-type gels but absent from Wld(e) gels. This finding indicates that different post-translationally modified (e.g. ubiquitinated or phosphorylated) forms of the protein may exist in striatal synapses and that two of these forms were absent in Wld(e) synapses (noting that the majority of other forms of actin are apparently not changed in Wld(e) synapses; see Fig. 3). These modified forms of actin may be due to the difference in levels of the F-actin capping protein α-2 subunit (CAPZ α2), which was also found to be absent in Wld(e) gels (Table I). As CAPZ α2 is a known regulator of actin filament assembly and organization (35), it is possible that loss of this protein at the synapse leads to modified actin function and/or deposition.
In this study we used differential proteomics techniques combined with MALDI-TOF peptide mass fingerprinting and quantitative Western blotting to identify proteins that are modified at a basal level in an identified population of central nervous system synapses known to be protected from neurodegeneration in \textit{Wlds} mutant mice. Sixteen proteins were identified whose expression levels were significantly altered in \textit{Wlds} synapses compared with those in age- and sex-matched wild-type controls. Prominent among these changes were eight proteins known to be localized to mitochondria (including VDAC1, calcium-binding mitochondrial carrier protein 1 (Aralar1), and mitofilin), suggesting that altered mitochondrial responses to neurodegenerative stimuli may play a critical role in the \textit{Wlds} neuroprotective phenotype. Other non-mitochondrial proteins identified that may be important for the \textit{Wlds} phenotype included the ubiquitin-activating enzyme UBE1 and DRP-2. These findings provide additional experimental support for the hypothesis that changes attributable to both the ubiquitin-proteasome pathway and NAD-associated pathways may be required to elicit the full \textit{Wlds} phenotype. The emergence of such specific protein changes from our essentially open ended analysis of global synaptosomal proteins, correlating with demonstrable \textit{in vivo} neuroprotection (27), argues in favor of these changes being an important feature of the \textit{Wlds} phenotype.

\textit{Changes in Mitochondrial Proteins in \textit{Wlds} Synapses—} Our finding that at least 11 known mitochondrial proteins (combining our proteomics screen and subsequent validation) had modified expression levels in \textit{Wlds} synapses suggests that mitochondria may be primed to have a more robust response to degeneration-inducing stimuli in \textit{Wlds} mice and therefore

![Image](https://www.mcponline.org/download/Molecular%20%26%20Cellular%20Proteomics%206.8.png)
Synaptic Protein Modifications in Wld⁻ Mice

TABLE II

Mass spectrometry and peptide fingerprinting data for identified proteins that show significant changes in Wld⁻ synaptosomes

For each protein the molecular weight score (MOWSE), the number of peptides matched from the total number of peptides submitted to Protein Prospector, the relative molecular weight (M) and pI, and the overall intensity of the monoisotopic peptide peaks as a percentage of the total intensity are given. A minimum of four peptides were required for each protein match; errors between the predicted and measured peptide masses are expressed as average (Avg) ± S.D. (in ppm). It should be noted that proteins represented by spots 116 and 476 were not found at the predicted position on the 2D gel reflecting their molecular weight and pI. This is likely to represent post-translational modifications removing residues 1–68 of spot 116 and residues 1–81 of spot 476. These modifications resulted in molecular weight/pI of 41.5/6.0 for spot 116 and 59.1/5.7 for spot 476.

| Spot | Protein                                                                 | Mᵦ  | pI   | MOWSE score | Hit/submitted | Intensity | Avg ± S.D. |
|------|-------------------------------------------------------------------------|------|------|-------------|---------------|-----------|------------|
| 446  | DRP-2                                                                   | 62.2 | 5.9  | 2.52E+05    | 9/75          | 9         | 6 ± 7      |
| 637  | Acyl-CoA dehydrogenase, long chain specific, mitochondrial precursor    | 47.9 | 8.5  | 1.83E+04    | 10/54         | 31        | 6 ± 7      |
| 802  | β-SNAP                                                                  | 33.6 | 5.3  | 3.62E+07    | 15/65         | 44        | 6 ± 8      |
| 476  | Dihydrolipoyllysine-residue acetyltransferase                           | 67.9 | 8.8  | 5.8E+04     | 16/31         | 72        | 4 ± 5      |
| 780  | Cytosolic acyl-coenzyme A thioester hydrolase                           | 42.5 | 8.9  | 9.11E+01    | 6/37          | 29        | 5 ± 5      |
| 157  | UBE1                                                                    | 117.8| 5.4  | 9.23E+10    | 16/75         | 27        | 7 ± 9      |
| 650  | Rab GDIβ                                                                | 50.5 | 5.9  | 6.1E+03     | 5/28          | 16        | 5 ± 5      |
| 861  | Voltage-dependent anion-selective channel protein 1                     | 32.4 | 8.6  | 1.5E+08     | 10/35         | 46        | 7 ± 8      |
| 326  | Mitochondrial inner membrane protein (mitofilin)                        | 83.9 | 6.2  | 2.59E+03    | 6/61          | 18        | 4 ± 5      |
| 460  | ST1                                                                     | 62.5 | 6.4  | 1.50E+07    | 18/75         | 33        | 8 ± 9      |
| 657  | Elongation factor Tu, mitochondrial precursor                            | 49.5 | 7.2  | 2.40E+06    | 11/75         | 14        | 4 ± 6      |
| 116  | Dihydrolipoyllysine-residue succinyltransferase                         | 48.9 | 9.1  | 5.00E+04    | 9/60          | 17        | 8 ± 9      |
| 59   | Aralar1                                                                 | 74.6 | 8.4  | 8.30E+04    | 9/75          | 16        | 5 ± 6      |
| 187  | Vacular ATP synthase subunit B                                          | 40.3 | 4.9  | 1.20E+04    | 9/20          | 44        | 5 ± 7      |
| 202  | CAPZ α2                                                                 | 32.9 | 5.6  | 3.51E+04    | 5/20          | 30        | 5 ± 6      |
| 144  | Actin                                                                   | 41.8 | 5.3  | 7.50E+05    | 8/35          | 29        | 5 ± 6      |
| 157  | Actin                                                                   | 41.8 | 5.3  | 7.50E+05    | 9/29          | 41        | 5 ± 7      |

Fig. 5. Bar chart showing validation of protein expression changes in Wld⁻ mouse synaptosomes compared with wild type (WT) using quantitative Western blotting where antibodies were available. There was no significant change in the levels of two control synaptic proteins (left side of graph; also see Fig. 1): neurofilament (NF) and synapsin. Every candidate protein examined showed significant changes (p < 0.05; comparison against neurofilament levels; ANOVA with Tukey’s posthoc test) consistent with those identified by our 2D gel screen. Bars, mean ± S.E. (n = minimum of 6 per protein).

Fig. 6. Bar chart showing significant changes in expression levels for other mitochondrial proteins in Wld⁻ mouse synaptosomes (p < 0.001; comparison against neurofilament levels; ANOVA with Tukey’s posthoc test), not identified in initial 2D gel screens, measured using quantitative Western blotting. Bars, mean ± S.E. (n = minimum of 3 per protein). NF, neurofilament.

play a key role in the neuroprotective phenotype. Precisely how such changes in the mitochondrial proteome could bring about modified responses to degenerative stimuli remains to be determined. However, it is known that VDAC1 (also known as outer mitochondrial membrane protein porin 1) is a pore-forming protein that constitutes the major pathway for movement of adenine nucleotides through the outer mitochondrial membrane (36). It can sensitize cells to various extracellular apoptosis-inducing stimuli (37). Similarly down-regulation of mitofilin has been implicated in regulating apoptosis at least in HeLa cells (38). Thus, VDAC1 and/or mitofilin may be key proteins in regulating modified responses of mitochondria, and hence neurodegeneration, in Wld⁻ synapses. It also re-
mains to be determined whether the non-mitochondrial protein changes identified in the present study are occurring as a result of, are causing, or are occurring in parallel with changes in the mitochondrial proteome.

The finding that mitochondria are likely to play an important role in synaptic protection conferred by the \( Wld^s \) gene is supported by several previous studies. For example, Ikegami and Koike (39) showed that neurite degeneration is controlled through non-apoptotic processes resulting from mitochondrial dysfunction, and Ferreirinha et al. (40) showed that ab-

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**Fig. 7.** A and B, electron microscopic analysis of striatal synapses showed no difference in the gross morphology, localization, or number of synaptic mitochondria between wild-type (A; arrow indicates a single presynaptic mitochondrion) and \( Wld^s \) mice (B; black arrows indicate presynaptic mitochondria in two different nerve terminals). C, note the presence of well defined cristae (white arrows) and mitochondrial membranes (black arrow) in nerve terminal mitochondria from \( Wld^s \) mice. D, quantification of the numbers of mitochondria per nerve terminal profile showed no difference between wild-type (WT) and \( Wld^s \) mice (mean ± S.E.; \( p = 0.9127 \), two-tailed t test; \( n = 115 \) wild-type synapses; \( n = 64 \) \( Wld^s \) synapses). Scale bars, 0.2 \( \mu m \) (A and B) and 0.1 \( \mu m \) (C).

**Fig. 8.** Principal Ingenuity Pathways Analysis network of interactions involving nine of the 16 proteins with modified expression in \( Wld^s \) synapses. Proteins within this network identified as being modified in \( Wld^s \) synapses are indicated using adjacent circles (red, up-regulated in \( Wld^s \); green, down-regulated in \( Wld^s \); blue, absent in \( Wld^s \)). Solid connecting lines indicate the presence of a direct interaction, and dashed connecting lines indicate an indirect interaction. All suggested indirect interactions were confirmed manually using Ingenuity Pathways Analysis software to identify published evidence indicating an interaction between the two proteins. \( CAPZA2 \), F-actin capping protein \( \alpha-2 \) subunit; \( NAPB \), \( \beta \)-SNAP; \( TUFM \), elongation factor Tu; \( ACTB \), actin; \( GDI2 \), Rab GDP dissociation inhibitor \( \beta \); \( SLC25A12 \), Aralar1; \( DPYSL2 \), DRP-2; \( EGFR \), epidermal growth factor receptor; \( BDNF \), brain-derived neurotrophic factor; \( ALPP \), alkaline phosphatase, placental; \( APP \), amyloid beta (A4) precursor protein (peptidase nexin-2, Alzheimer disease); \( KITLG \), Kit ligand; \( MYCN \), v-myc myelocytomatosis viral related oncogene, neuroblastoma derived.
normal synaptic and axonal mitochondrial morphology can be detected in advance of clinical symptoms in a mouse model of hereditary spastic paraplegia. Similarly it has been shown that a loss of mitochondrial membrane potential and segregation of energy-competent mitochondria are early events in neurite degeneration (41, 42). As degeneration of non-somatic neuronal compartments plays a key role in many neurodegenerative diseases (1, 2), it is not surprising to find that non-somatic mitochondria are also well established as critical mediators in neurodegenerative diseases (43, 44). For example, mitochondria have been implicated in the pathogenesis of Parkinson disease, motor neuron disease, and following demyelination (45, 46), all conditions where the Wld<sup>e</sup> gene offers significant levels of neuroprotection (6, 8, 47). It is also possible that the selective protection of synapses and axons, but not neuronal soma (15), by the Wld<sup>e</sup> gene is caused by differences in responses to degeneration found in synaptic, axonal, and somatic mitochondria. This hypothesis is consistent with previous studies showing differences in the functional status of somatic versus non-somatic neuronal mitochondria in normal (i.e. non-Wld<sup>e</sup>-expressing) neurons (39, 48).

Changes in Proteins Linked to Both Ube4b and Nmnat1 Components of the Wld<sup>e</sup> Gene—Our finding that the ubiquitinating enzyme UBE1 is up-regulated in Wld<sup>e</sup> synapses provides support for the hypothesis that altered ubiquitination plays an important role in conferring the Wld<sup>e</sup> phenotype at the level of the synapse (10, 18–20, 49) as it demonstrates that key components of the ubiquitination pathway other than Ube4b are modified in protected non-somatic neuronal compartments. This hypothesis is consistent with previous studies demonstrating an important role for the ubiquitin-proteasome system in regulating synaptic form, function, and composition (50–53).

The most parsimonious explanation for modulation of a ubiquitination factor in Wld<sup>e</sup> synapses must be based around the fact that the N-terminal 70 amino acids of the mult ubiquitination factor Ube4b are present in the Wld<sup>e</sup> chimeric gene (13). However, this fragment does not contain the U-box motif responsible for conferring its normal activity (20), making it unlikely that this fragment of Ube4b directly affects ubiquitination activity in the distal extremities of the neuron. The recent demonstration that valosin-containing protein (VCP; p97/Cdc48) binds to the N-terminal 70-amino acid Ube4b fragment of Wld<sup>e</sup> suggests an alternative mechanism as VCP influences the cellular localization, and presumably therefore the activity, of the ubiquitin-proteasome machinery (20). It is possible that VCP function or localization, modified as a result of its interaction with Wld<sup>e</sup> protein, may be responsible for ultimately mediating non-somatic levels of UBE1.

Alongside these changes in the ubiquitination pathway, several of the other proteins identified as having different expression patterns in Wld<sup>e</sup> synapses are known to play an important role in NAD metabolism and hence can be linked to the Nmnat1 component of the Wld<sup>e</sup> gene. For example, Aralar1 is important for the transfer of NAD(P)H into mitochondria (54). Similarly VDAC1 is a known component of the NAD pathway, affecting cellular equilibrium via maintenance of cellular redox homeostasis (55). NAD levels, which can be manipulated by changing expression levels of Nmnat1, have also been shown previously to at least partially replicate the Wld<sup>e</sup> phenotype in vitro (Refs. 21–23, but see Refs. 24 and 25). It is possible, therefore, that NAD-mediated neuroprotection of non-somatic compartments may be acting through modulating Aralar1 and/or VDAC1 levels.

The identification of protein changes affecting both ubiquitin- and Nmnat1/NAD-dependent pathways as a result of Wld<sup>e</sup> expression suggests that the presence of both major components of the nuclearly localized Wld<sup>e</sup> gene product (Ube4b and Nmnat1) are translated into downstream protein modifications within non-somatic compartments protected by Wld<sup>e</sup> similar to previous reports of the role of Wld<sup>e</sup> in regulating gene transcription (26). Future studies should therefore address the requirement for changes in both pathways to be present to confer full levels of Wld<sup>e</sup>-mediated neuroprotection.

Changes in Levels of Non-mitochondrial Proteins in Wld<sup>e</sup> Synapses—In addition to the identification of changes in proteins directly linked to mitochondria, several non-mitochondrial proteins were identified that may be important for modulating synaptic vulnerability. DRP-2 levels, which were increased in neuroprotected Wld<sup>e</sup> synapses, show a progressive decrease in Alzheimer disease (56), a condition where synaptic degeneration plays a key role in disease initiation and progression (Ref. 57; also see Ref. 2). Similarly DRP-2 plays a role in motor neuron disease (58) and schizophrenia (59). Both elongation factor Tu (which was up-regulated in Wld<sup>e</sup>) and vacuolar ATP synthase (down-regulated in Wld<sup>e</sup>) have also been implicated in the pathogenesis of Alzheimer disease (60, 61). Rab GDIβ (down-regulated in Wld<sup>e</sup>) has been implicated in gracile axonal dystrophy (62). This finding is of particular interest as the Wld<sup>e</sup> gene has been shown to inhibit axonal spheroid pathology in the gracile axonal dystrophy (gad) mouse (9). The mutation in gad mice is a loss of ubiquitin C-terminal hydrolase 11 (Uch-11). Thus, Rab GDIβ may be another protein altered as a result of modifications in the ubiquitin-proteasome system in Wld<sup>e</sup> mice.

Of the proteins identified in protected Wld<sup>e</sup> synaptic terminals in this study, only one has been shown previously to have direct neuroprotective effects: ST11 (also known as p60 and Hsc70/Hsp90-organizing protein). ST11 mediates the heat-shock response by mediating the association of the molecular chaperones Hsc70 and Hsp90 (63), leading to a rescue of cells from apoptosis (64, 65). Our findings suggest that ST11 may also play a role in regulating synaptic vulnerability. This may have significant implications for our understanding of, and development of therapeutics for, prion disease (another neurodegenerative condition with significant synaptic degeneration; for a review, see Ref. 2) as ST11 is known to interact with the cellular prion protein (PrPc) thereby mediating neuroprotection and neuritogenesis (65, 66).
**Protein Expression Levels Versus mRNA Expression Levels in Wld<sup>e</sup>**—Previous experiments have identified conserved mRNA expression changes for 11 genes as a result of Wld<sup>e</sup> expression (26). Somewhat surprisingly, none of these identified mRNA changes were shown to have corresponding in vivo protein level modifications in the current experiments. There are three possible explanations for this result. First, it is conceivable that none of the identified proteins are localized to nerve terminals, instead being confined to somatic cellular compartments. This is the most likely explanation for those genes identified with a well documented role in either the nucleus and/or cell soma (e.g. Pttg1 (67) and Ssr1 (68)). Second, it is possible that mRNA changes do not necessarily equate to changes at the protein level. And third, protein expression differences may have been present in our synaptosome preparations but were not detected because either they were masked by other proteins on the gel, or the proteins of interest were only present in very low abundance. Indeed our finding that mitochondrial proteins not identified by our initial screen are also modified in Wld<sup>e</sup> synapses supports this hypothesis. The latter is a distinct possibility because in the current experiments we excluded very faint protein spots (with normalized volumes <0.03%) from our analysis. 2D gel electrophoresis is not optimal for the detection of such lower abundance proteins whose levels may be more reliably assessed using other methods such as Western blotting. Unfortunately reliable antibodies are not currently available for most of the protein products of genes whose mRNA levels are modified as a result of Wld<sup>e</sup> expression (26). Future experiments will be required to determine whether any of the mRNA changes reported previously are capable of modulating expression levels for any of the proteins identified in the current study or whether the mRNA changes are occurring in parallel to, rather than in sequence with, such protein modifications.

**Conclusions**—The protein changes identified by the current study may represent critical regulators of neurodegeneration in synaptic compartments. Therapeutic targeting of mitochondrial responses to neurodegenerative stimuli is likely to be beneficial for patients with conditions where synapses, and most likely axons, are primary pathological targets.

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