Inhibition of Pin1 reduces glutamate-induced perikaryal accumulation of phosphorylated neurofilament-H in neurons

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Running Head: Inhibition of Pin1 reduces neurotoxicity

Abbreviations: Cdk5: Cyclin dependent kinase 5; p-NF-H: phosphorylated Neurofilament-H; Pin1: Prolyl Isomerase 1

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Abstract

Under normal conditions, the proline-directed serine/threonine residues of neurofilament tail-domain repeats are exclusively phosphorylated in axons. In pathological conditions such as amyotrophic lateral sclerosis (ALS), motor neurons contain abnormal perikaryal accumulations of phosphorylated neurofilament proteins. The precise mechanisms for this compartment-specific phosphorylation of neurofilaments are not completely understood. Although localization of kinases and phosphatases is certainly implicated, another possibility involves Pin1 modulation of phosphorylation of the proline-directed serine/threonine residues. Pin1, a prolyl isomerase, selectively binds to phosphorylated proline-directed serine/threonine residues in target proteins and isomerizes cis isomers to more stable trans configurations. In this study we show that Pin1 associates with phosphorylated neurofilament-H (p-NFH) in neurons and is colocalized in ALS-affected spinal cord neuronal inclusions. To mimic the pathology of neurodegeneration we studied glutamate-stressed neurons which displayed increased p-NF-H in perikaryal accumulations that colocalized with Pin 1 and led to cell death. Both effects were reduced upon inhibition of Pin1 activity by the use of an inhibitor juglone and down-regulating Pin1 levels through the use of Pin1 siRNA. Thus, isomerization of lys-ser-pro repeat residues that are abundant in NF-H tail domains by Pin1 can regulate NF-H phosphorylation, which suggests that Pin1 inhibition may be an attractive therapeutic target to reduce pathological accumulations of pNF–H.

Keywords: AD/ALS/neurotoxicity/NF-H/phosphorylation/Pin1
**Introduction**

The functions of most proteins are regulated by post-translational modifications, of which phosphorylation is probably the most common. In neurons, phosphorylation of cytoskeletal proteins is tightly regulated and compartmentalized. Although proline directed kinases are found in both cell bodies and axons, the multiple repeat lysine-serine-proline (KSP) sites in the neurofilament (NF) tail domains are known to be almost exclusively phosphorylated in the axonal compartment of mammalian and squid neurons (Julien and Mushynski, 1982; Lee et al., 1987; Nixon et al., 1994; Grant and Pant, 2004). Defects in the relative kinase and phosphatase activities and/or deregulation of compartment-specific phosphorylation result in neurodegenerative disorders: Aggregated forms of hyperphosphorylated tau and phosphorylated NF (p-NF) are found in pathological cell body accumulations in the central nervous system of patients suffering from Alzheimer’s disease (AD), Parkinson’s disease (PD) and ALS. Antibodies against phosphorylated neurofilament heavy/middle chain NF-H/M recognize epitopes found in perikaryal inclusions, neurofibrillary tangles and paired helical filaments of AD (Cork et al., 1986; Wang et al., 2001), in Lewy bodies (Forno et al., 1986; Pollanen et al., 1993) and in inclusions in spinal cord motor neurons (Sobue et al., 1990; Itoh et al., 1992; Al-Chalabi and Miller, 2003).

Within the last decade, a novel level of modulation of protein phosphorylation has emerged, namely, the factors that regulate the structural conformation and stability of proteins, particularly those phosphorylated at Ser/Thr-Pro (S/T-P) sites by proline directed kinases. Most proline-directed kinases (Weiwad et al., 2004) and phosphatases (Wulf et al., 2005) are highly selective for trans S/T-P bonds. Peptidyl-prolyl cis/trans isomerases, such as Pin1, specifically target
phosphorylated S/T-P sites and by virtue of the proline residue, can “toggle” an inactive cis isomer to the more stable trans form, often with altered function. Pin 1 plays a key role in diverse cellular functions, including the cell cycle, differentiation, cancer, neurodegeneration, DNA damage response and apoptosis (Lu et al., 1996; Liou et al., 2003; Lu, 2004; Thorpe et al., 2004; Wulf et al., 2005). Pin 1 is localized in nuclei of most cells, where it modulates the functions of several mitotic proteins such as cyclin D1, cdc2 and transcription factors such as p53, c-Jun N-terminal kinase (c-JNK), β-catenin, and tau, a regulator of microtubule dynamics in mitosis (Lu et al., 1996). Pin1 also regulates structure and function of RNA polymerase II during transcription (Xu et al., 2003). It is overexpressed in transformed cells and is thus implicated in oncogenesis (Bao et al., 2004). In neurons, however, Pin1 is distributed in both nucleus and cytoplasm, increases during neuronal differentiation and its expression correlates with the phosphorylation of tau, although all potential phosphorylation sites are not equally phosphorylated (Hamdane et al., 2006). Phosphorylation at one Pin 1 binding site, Thr 231, that activates tau dephosphorylation in vitro (Lu et al., 1999; Zhou et al., 2000), is down-regulated during differentiation, suggesting that Pin 1 regulates tau dephosphorylation by inducing conformational changes, at least at this site. In vitro, Pin1 specifically binds to tau proteins that are phosphorylated on their Thr 231 residue (Wintjens et al., 2001; Lu et al., 2002; Hamdane et al., 2006). Significantly, the Thr 231 phosphoepitope of tau is exclusively found in mitotic cells, is a marker of abnormal tau hyperphosphorylation (Hamdane et al., 2003) and is specifically detected in a conformation-dependent manner in neurofibrillary tangles (NFT) in AD brains, thus linking Pin1 to neurodegeneration (Lu et al., 1999; Augustinack et al., 2002a). This link was dramatically confirmed by Pin1-null mice which display age dependent motor and behavioral defects, tau pathologies, and neuronal loss (Liou et al., 2003), results which suggest that Pin 1
protects against neurodegeneration, but how? One suggestion is based on a model of neurodegeneration stating that stressed post-mitotic neurons (e.g., exposed to β-amyloid peptides) are abnormally induced to enter the cell cycle, leading to the appearance of mitotic proteins (including p-T231-tau) resulting in neuronal apoptosis (Neve and McPhie, 2006). The protective role of Pin1 is to alter the phosphorylated conformation of mitotic proteins to enable dephosphorylation of tau at Thr 231 by exposing the site to PP2A phosphatase activity. The absence of Pin1 in null mice or the low soluble Pin1 levels seen in AD brains, prevents phosphatase action at the hyperphosphorylated tau sites [reviewed in (Lu, 2004)].

Like tau, neurofilaments contain many S/T-P phosphate acceptor sites that are targeted by several different kinases and phosphatases. In contrast to tau, however, NFs, particularly NF-H, are enriched with numerous KSP repeat motifs in the tail domain (43-100 depending on species), sites for proline-directed kinase phosphorylation. Phosphorylation of these tail domain sites is normally restricted to the axonal compartment as NF polymers are assembled and transported along the axon and results in extension of sidearms that interact with other NFs, NF-associated proteins and microtubules to form a stable, structural lattice. We suggest that Pin1, distributed throughout the neuron, is directly involved in modulating NF phosphorylation to affect its distribution in the cell body and axon. By deregulating the system via induction of neurodegeneration (glutamate excitotoxicity), to evoke NF pathologies resembling those seen in ALS, we have been able to identify a role for Pin1 in the regulation of NF phosphorylation.

Materials and Methods

All experiments were replicated a minimum of 4 times and results shown are representative Western blots or images. All quantitative data were obtained though densitometric scanning and
Antibodies

Pin1 antibodies were obtained from Cell Signaling Technologies (Beverly, MA) and Oncogene Research Products and used for Western blotting (1:1000) and immunohistochemistry (1:100) respectively. RT-97 antibody was provided by Drs Ralph Nixon and Veeranna (Nathan Kline Institute, Orangeburg, NY) and used at 1:500-1000 dilutions for immunofluorescence and 1:5000 dilution for Western blotting. SMI31 was obtained from Covance (Princeton, NJ) and used at 1:500 for immunofluorescence and 1:2500 for Western blotting. Anti-tubulin antibody (clone DM1A), total NF (clone N52) and DAPI (Sigma-Aldrich, St. Louis, MO) were used at 1:10000 for Western blotting and 1:1000 for nuclear counter-staining respectively.

Plasmids and Pin1-siRNA

Dominant-negative Pin1 was produced by making a point mutation to produce an alanine at serine 16- using the Quikchange Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. Transfection of wild-type and dominant-negative Pin1 constructs were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Wild-type Pin1 was cloned into pGEX-5X-1 obtained from Amersham Biosciences (Piscataway, NJ) for GST expression studies. Control non-silencing and Pin1-siRNAs (silencing) were designed as follows. Control siRNA (non-silencing) sense and antisense sequences were: 5’-r(UUUUCCGAACGUGUCACGU)d(TT)-3’  5’-r(ACGUGACACGUUCGGAGAA)d(TT)-3’ respectively.

Pin1 siRNA (silencing) sense and antisense sequences were: 5’-
r(GCUCAGGCGAGUGUACUA)dTdT-3’ 5’-r(UAGUACACUCGCCUGAGC)dTdT-3’, respectively. The sense and antisense strands were annealed to create the double-stranded siRNA at a 20µM concentration. Control siRNA and Pin1 siRNA were dissolved in suspension buffer to obtain a 20µM solution and heated at 90°C for 1 min, then incubated at 37°C for 60 mins before transfection. Final concentrations (40nM) of siRNAs were transfected into E18 primary cortical neurons (5DIC) using Lipofectamine 2000 reagent according to the manufacturer’s instructions. After 48 hours the cells were either fixed for immunohistochemical analyses or lysed with lysis buffer for Western blot analyses.

**Reagents**

5-Hydroxy-1, 4-naphthoquinone (juglone) and L-glutamic acid (glutamate) were purchased from Sigma-Aldrich. Juglone was prepared as described (Chao *et al.*, 2001) and used at 30 µM in DRG while glutamate was used at 0.1 mM and 10 µM for 6 hours in cortical neurons and dorsal root ganglion neurons respectively.

**GST-Pin1 protein expression and pull down assays**

GST and GST-Pin1 were expressed in E.Coli and purified according to the manufacturer’s instructions (Amersham Biosciences). Purified GST and GST-Pin (20mg of each) were then used in GST-pull down assays from rat brain lysates. 10% Rat brain lysates were prepared in IP lysis buffer containing 50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1% Triton-x-100, 1 mM EDTA, 1 mM EGTA, 5 mg/ml leupeptin, 2 mg/ml aprotinin, 5 mg/ml pepstatin and 1mM PMSF described previously (Kesavapany *et al.*, 2004). GST and GST-Pin1 were incubated with the lysates overnight at 4°C, washed three times and then separated by SDS-PAGE on 4-20% acrylamide
gels. Gels were stained with Coomassie, destained and differential bands excised and sent for mass spectrometric identification. For Western blotting analyses, samples were transferred onto nitrocellulose and p-NF-H was detected using RT-97 antibody.

**Mass Spectroscopic Identification**

Excised Coomassie stained and destained gel slices were subjected to in-gel tryptic digestion and then dried digests were processed as previously described (Jaffe et al., 2004).

**Co-immunoprecipitation**

Rat brain was homogenized in ice-cold IP lysis buffer (see above) using 40 strokes of a Dounce homogenizer on ice. Homogenates were centrifuged for 30 minutes at 4°C at 14,000 rpm. Supernatants were precleared using Protein A sepharose beads (Sigma) and 500 μg of total protein was used in immunoprecipitation experiments using the polyclonal Pin1 antibody and immunoglobulin was captured using Protein A sepharose beads overnight at 4°C. After three washes with IP lysis buffer, immunoprecipitates were heated in 2x SDS sample buffer, separated by SDS-PAGE and transferred onto nitrocellulose membranes for Western blotting analyses. Phosphorylated NF-H was immunodetected using RT-97 antibody. Co-immunoprecipitations of similarly prepared rat brain lysates using RT-97 antibody specific for phosphorylated NF-H were also carried out to detect Pin1 with the Pin1 antibody.

**SDS-PAGE and sample preparation**

10% total homogenates of spinal cord were made as described previously (Kesavapany et al., 2004). Samples were heated for 10 minutes at 95°C and then aliquoted and frozen at -80°C.
Western blots were prepared from equal volumes of total lysates. Soluble lysates were produced by using 40 strokes of a Dounce homogenizer in ice cold IP lysis buffer containing 50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1% Triton-x-100, 1 mM EDTA, 1 mM EGTA, 5 mg/ml leupeptin, 2 mg/ml aprotinin, 5 mg/ml pepstatin and 1 mM PMSF, incubated for 20 minutes on ice and then centrifuged at 14,000 rpm for 20 minutes to obtain the soluble supernatant. Protein concentrations for soluble lysates were determined with the bichinchinoic acid (BCA) assay system (Pierce, Rockford Il). Western blots were obtained from equal amounts of soluble lysates. All neuronal cell lysates were made by scraping the neurons directly into 2x SDS sample buffer and heating at 95°C for 10 minutes. Protein balancing for total homogenates and confirmation of protein loading was achieved through immunodetection of tubulin. Once cooled, samples were separated by SDS-PAGE and processed for Western blotting analyses. Signals were scanned and densitometrically measured using a GS800 scanner and its accompanying Quantity One program (Biorad, Hercules, CA).

ALS and AD tissue
Closely matched, age and post-mortem time, control and ALS affected spinal cord sections and spinal cord tissues were obtained from the Robarts Research Institute Brain Bank, London Ontario, Canada. Matched control and AD tissue were obtained from the Harvard Brain Tissue Resource Center. Samples that were matched in terms of region (Brodmann layer), age and postmortem time were used to produce total and soluble lysates (described earlier).

Immunohistochemical staining of spinal cord sections
Paraffin embedded control and ALS affected spinal cord sections were prepared for
immunostaining through xylene treatment and gradual rehydration with 95% - 75% ethanol. Sections were blocked and then incubated with primary antibodies overnight at 4°C in 0.6% Triton-X-100/3% bovine serum albumin (Sigma). Secondary antibodies were incubated for 1 hour at room temperature, in the dark and slides were coverslipped using GelMount (Biomeda). Images were captured with a 20X objective on a Zeiss LSM510 using LSM Image Software managed with Adobe Photoshop.

**Primary rat DRG cultures and treatments**

Pregnant female rats or mice were sacrificed and embryos were removed from the uterus at embryonic day 15, as previously described (Olah *et al.*, 2001). DRG neurons were plated onto poly-lysine coated glass coverslips and grown for 4 days before treatment +/- juglone/glutamate. For sequential treatment, neurons were first pre-treated with 30 µM juglone for 3 hours and then with 10 µM glutamate for 6 hours. Two homogenate preparations were prepared, a total homogenate where neurons were harvested directly into SDS sample buffer and heated before processing and a soluble homogenate (described above) to determine soluble levels of Pin1.

**Primary rat cortical neuron cultures and treatments**

E16-18 rat cortical neuron cultures were produced as described previously (Kesavapany *et al.*, 2004). Typically, neurons were transfected after 3 days in culture (DIC), incubated for 5 hours and then treated with glutamate. Neurons were grown for seven days before treatment +/- juglone/glutamate. For sequential treatment, neurons were first pre-treated with 30 µM juglone for 3 hours and then with 0.1 mM glutamate for 6 hours. Two homogenate preparations were prepared, a total homogenate where neurons were harvested directly into SDS sample buffer and heated before processing and a soluble lysate to determine soluble levels of Pin1 prepared with
IP lysis buffer described earlier.

**Immunocytochemical staining of neuronal cultures**

Primary rat cortical and DRG neurons were plated on poly-lysine coated glass coverslips, and processed for immunocytochemistry as previously described (Kesavapany *et al.*, 2004). Coverslips were mounted using GelMount (Biomedica, Foster City, CA). In situ cytotoxicity kits were obtained from Roche (Indianapolis, IN) and TUNEL staining was performed according to the manufacturer’s instructions prior to immunocytochemistry. Images were captured with an oil immersion 63X objective on a Zeiss LSM510 using LSM Image Software managed with Adobe Photoshop.

**Preparation of molecular models**

Molecular models were prepared with the aid of DeepView (Guex & Peitsch, 1997). The NFH tail domain has an amino acid composition characteristic of ‘intrinsically unstructured’ protein (Fink, 2005). This was confirmed for the C-terminal 600 residues of human NF-H by applying the IUPred algorithm via the website, http://iupred.enzim.hu/ (Dosztanyi *et al.*, 2005). Because nearly all the proline-associated serines in this NF-H domain become phosphorylated in mature axons (Jaffe *et al.*, 1998a; Jaffe *et al.*, 1998b), these serines evidently become accessible to kinases during this processing. Thus it seems reasonable to model each unphosphorylated repeat unit as a loop centered on the SP residues. Accordingly, in constructing Figure 8, the DeepView loop-building tool was applied to each repeat unit as an extended peptide, and then these were ligated and minimized. The cis transition of SP2 was modeled by rotating the S-P w torsion by 180° while leaving the N-terminal residues stationary.
Results

Pin1 associates with the phosphorylated neurofilament-heavy chain (p-NF-H).

We used GST pull down assays to investigate whether Pin1 binds to p-NF-H. GST and GST-Pin1 were expressed and purified by immobilizing the protein on glutathione sepharose beads. The bound GST fusion proteins were then incubated with rat brain lysates overnight. The samples, after washing, were separated by SDS-PAGE and gels were stained and destained. Bands present in GST-Pin1 pull down but not in the GST lanes were excised, subjected to in gel tryptic digestion and identified using MS-MS mass spectroscopy. Peptides from 2 different proteins were conclusively identified including tau and the band at 200 kDa (arrowed in Fig. 1A) that was NF-H. Two peptides that belonged to NF-H were identified as TLDVKSPEAK and SPADKFPEK where the serine in the first peptide was phosphorylated. Other bands on the gels were MAP1b (X1) and NF-M (X2). X3 was found to contain keratin which is a common contaminating band found in these types of studies and X4 was a degradation product of GST-Pin1. One band was identified as tau but we chose to focus our efforts on NF-H since tau has been well characterized by other groups. NF-M studies are part of our future plans. To confirm this result, Pin1 was immunoprecipitated from rat brain lysates and p-NF-H was immunodetected using RT-97 antibody. Pin1 readily pulled down phosphorylated NF-H (Figure 1B). Pin1 co-immunoprecipitated with p-NF-H when p-NF-H was pulled down using RT-97 antibody (Fig. 1C).

NF-H phosphorylation is increased in Alzheimer’s disease tissue

Although the majority of reports have concentrated on hyperphosphorylated tau as the major cytoskeletal pathology in Alzheimer’s disease [reviewed in (Stoothoff and Johnson, 2005)], p-NF-H is also a constituent of aggregates in AD brain (Sternberger et al., 1985; Cork et al., 1986;
Wang et al., 2001). We wanted to compare the levels of NF-H phosphorylation in control and AD tissue. Closely matched control and AD homogenates were subjected to SDS-PAGE before immunodetection of p-NFH. We found a three-fold increase in p-NF-H in AD samples compared to controls, but found no change in soluble Pin1 levels. Total levels of NF-H did not change (Fig. 2).

**NF-H phosphorylation is increased in ALS without any change in Pin1 levels**

Because cytoskeletal proteins, especially NF-H, are reported to be aberrantly phosphorylated in ALS spinal cord and affected motor neurons (Straube-West et al., 1996; Strong et al., 2001; Rao and Nixon, 2003), we compared p-NF-H expression in ALS affected spinal cord tissue with that in normal controls. Western blots of total homogenates from ALS spinal cords together with controls (closely matched as to age and post-mortem time) were immunoassayed for p-NF-H using RT-97 antibody. Although soluble Pin1 levels can change during AD (Lu et al., 1999), we found that soluble Pin1 levels were unchanged in these ALS samples compared to controls. Nevertheless, p-NF-H was elevated in all ALS samples compared to controls (Fig. 3A) without any change in total NF levels.

**p-NF-H and Pin1 co-localize in aggregates found in ALS spinal cord.**

To determine whether Pin1 colocalizes with p-NF-H aggregates in ALS spinal cord, spinal cord sections were immunostained for Pin1 and for p-NF-H. p-NF-H and Pin1 staining were evident in all control neurons and both were uniformly distributed throughout the sections (Fig. 3B; a-c). By contrast, distinct differences in the staining pattern were observed in the ALS affected sections (Fig. 3B; d-f). Aggregates of p-NF-H were observed in ALS affected sections and the
uniform nature of Pin1 and p-NF-H staining was disrupted, with Pin1 co-localizing in the NF-H aggregates. Specificity for aggregate staining was confirmed by sections without primary antibody (Fig. 3B – bottom panels).

**Glutamate toxicity in dorsal root ganglion neurons (DRG) produces accumulations of p-NF-H and Pin1**

DRG neuronal cultures have been used as models for the neurotoxicity seen in ALS (Durham et al, 1997). DRG neurons subjected to excitotoxic or oxidative stress exhibit increased kinase activity and accumulate p-NF-H in their cell bodies (Shea et al., 2004). Upon glutamate treatment, we observed an approximate two-fold increase in p-NF-H levels relative to untreated neurons. When DRG neurons were treated with juglone, a specific Pin1 inhibitor (Lee et al., 2001), prior to glutamate exposure, NF-H phosphorylation remained at untreated levels. Pin1 levels were unchanged by glutamate treatment (Fig. 4A). DRG neurons, identically treated, were also fixed and immunostained (Fig. 4B; a-i). Though Pin1 was expressed in cell body and neurites in untreated DRG neurons, p-NF-H was confined to their processes (Fig. 4B; a-c). Glutamate treatment caused a marked increase in cell body staining of p-NF-H, where it co-localized with Pin1 with a concomitant loss of Pin1 expression in neurites (Fig. 4B; d-f). Juglone treatment prior to glutamate treatment, abolished the cell body accumulation of p-NF-H (Fig. 4B; g-i). We used different glutamate treatment concentrations in the cortical and DRG neuron experiments because cortical neurons have a higher tolerance to glutamate exposure; we treated cortical neurons with 0.1 mM glutamate and DRG neurons, which are more sensitive to glutamate, with 10 µM glutamate (Crawford et al., 2000; Lee et al., 2001).
Glutamate toxicity increases p-NF-H accumulation in cortical neuron perikarya.

Seven-day old cortical neuronal cultures were treated with or without glutamate for 6 hours or pretreated for 3 hours with 30 µM juglone prior to glutamate treatment. The neurons were harvested for SDS-PAGE and Western blotting analyses (Fig. 5A); identically-treated neurons on coverslips were fixed and immunostained for p-NF-H and Pin1 (Fig. 5B). There was a two-fold increase in p-NF-H in cortical neurons treated with glutamate. This level was reduced nearly to that of controls when neurons were incubated with juglone prior to glutamate exposure. Pin1 levels were not significantly changed (Fig.5A; bar graph). There was a marked increase in p-NF-H staining in the neuronal cell body without any change in Pin1 staining; the two proteins colocalized in this compartment (Fig. 5B; e-h). The cell body accumulation of p-NF-H was reduced when neurons were incubated with juglone prior to glutamate exposure (Fig. 5B; panels i and l), co-localization of Pin1 and p-NF-H was also reduced. Untreated neurons exhibited little or no staining of p-NF-H in the cell body although Pin1 was predominantly localized in this area (Fig. 5B; a-d). We also obtained identical results using another well characterized p-NF-H antibody, SMI31 (data not shown).

Glutamate mediated increase in phosphorylated NF-H is reduced by dominant negative Pin1 and Pin1-siRNA transfection.

To confirm that Pin1 activity was responsible for this effect, we used transfections of dominant negative Pin1 (DN-Pin1) as well as transfections of Pin1-siRNA. Dominant-negative Pin1 was constructed in the laboratory of Silvio Gutkind and has been well characterized in the laboratory (unpublished data). 5 day old neurons were transfected with dominant negative Pin1 and after 5 hours they were treated with glutamate for 6 hours. Dominant-negative Pin1 transfected neurons were identified through its GFP tag which showed that the transfected neuron exhibited
decreased p-NF-H staining. Additionally, in transfected neurons, cell body accumulation of p-NF-H was reduced (Fig. 6A). Identically transfected and treated neurons were harvested for SDS-PAGE analyses. Neurons treated with glutamate exhibited a two-fold increase in p-NF-H levels compared to non-treated neurons and this was reduced back to ‘normal’ levels when dominant-negative Pin1 was over-expressed. Transfected dominant-negative Pin1 migrated as a GFP fusion protein at approximately 50kDA while endogenous Pin1 migrated at approximately 18kDA. Statistical analyses showed that this mediation of p-NF-H was significant (Fig. 6B). Additionally, reduced phosphorylated NF-H levels were evident in Pin1-siRNA transfected neurons according to Western analyses. Using immunocytochemistry, it was found that the extensive accumulations of phosphorylated NF-H in the neuronal perikarya were reduced in Pin1-siRNA transfected neurons (Fig. 6C). Identically transfected and treated neurons were harvested and p-NF-H and Pin1 were immunodetected. Glutamate treatment caused a robust increase in p-NF-H levels in control siRNA transfected neurons which was reduced when Pin1-siRNA was transfected, confirming the inhibitor as well as the DN-Pin1 data (Fig. 6D). Note also that the Pin1 siRNA resulted in a 50% knockdown in the expression of Pin1 compared to the control siRNA.

Inhibition of Pin1 reduces glutamate-mediated apoptosis.

Glutamate can be neurotoxic and prolonged treatment can induce apoptosis (Lee et al, 2000). The phosphorylation of NF-H and its accumulation in the cell body has also been described as leading to neurotoxicity and apoptosis (Rao and Nixon, 2003). To determine whether phosphorylated NF-H accumulations in cell bodies correlates with neuronal apoptosis in our system and whether inhibition of endogenous Pin1 affects this, 7-day old cortical neurons were
untreated, treated with glutamate or pretreated with 30 µM juglone prior to glutamate exposure. Approximately 10% of untreated cortical neurons were TUNEL positive; this increased to 40% upon glutamate treatment. Pretreatment with juglone prior to glutamate treatment reduced TUNEL positive neurons to approximately 15%, approaching numbers comparable to non-treated neurons (Fig. 7A). Representative TUNEL images are shown while quantitation in the bar graph represents TUNEL positive counts from four separate experiments where 10 independent fields counted (Fig. 7B). In glutamate-treated cortical and DRG neurons p-NF-H was also elevated with no changes in soluble or total Pin1 levels. We chose to employ a 6 hr treatment of glutamate, as this produced extensive accumulation of p-NF-H accompanied by neuronal death. When treatment times were extended, the percentage of TUNEL positive neurons increased but neurons also began to detach from the coverslips (data not shown).

**Discussion**

Recent studies implicating Pin1 in tau hyperphosphorylation and paired helical filament (PHF) accumulation in neurodegeneration (Augustinack et al., 2002a; Augustinack et al., 2002b; Liou et al., 2003) lend support to the hypothesis that Pin1 may also modulate NF phosphorylation in normal and pathological situations. NF-H/M contain multiple proline-directed Ser/Thr repeat residues in the C-terminal tail domain. Normally these only become phosphorylated in the axonal compartment. Pin1 specifically targets phosphorylated Ser/Thr-P sites and switches a kinase inaccessible cis conformation to an accessible trans configuration. This can affect structure and function of the target protein (Wulf et al., 2004). Our data from excitotoxic-stressed neurons suggest that Pin1 regulates at least some of the phosphorylated KSP repeats in the NF-H carboxy-terminal tail domains.
Neuronal stress (e.g., oxidative, excitotoxic) disrupts the relative activities of kinases and phosphatases that tightly regulate topographic phosphorylation. This disruption involves abnormal activation of proline directed kinases such as Cdk5, Erk1/2, SAPK, and p38, and results in p-NF-H (and/or hyperphosphorylated tau) accumulations within perikarya, leading to cell death (Shea et al., 1988; Davis et al., 1995; Brownlees et al., 2000; Shea et al., 2004). By examining the glutamate-induced neurodegeneration model, which evokes NF pathologies resembling those seen in ALS, we have identified a role for Pin1 in the regulation of NF phosphorylation. We show that phosphorylated NF-H (p-NF-H) is overexpressed in ALS spinal cord tissues and colocalizes with Pin1 in aggregates in ventral horn cells, similar to the Pin1 co-purification with PHF tangles in AD (Lu et al., 1999). We find that p-NF-H is also overexpressed in AD brains and, as in ALS, soluble Pin 1 expression is unchanged, in contrast to the decline of Pin1 reported in AD brain (Lu et al., 1999) and hippocampus (Butterfield et al., 2006; Sultana et al., 2006). The reasons for these differences are not understood. Pin1 GST pull down assays of rat brain lysates show that Pin 1 associates with p-NF-H, and that both proteins colocalize in axons of untreated DRG and cortical neurons, but not in cell bodies. However, glutamate-induced neurotoxicity produces overexpression of p-NF-H accompanied by increased p-NF-H in perikarya where it colocalizes with Pin1, a result rescued by Pin1 activity inhibition, either with juglone pretreatment, and/or with co-transfection with dominant negative Pin1 or Pin1 SiRNA. Moreover, these cells become apoptotic, possibly because of obstructed p-NF-H transport into axons (Shea et al., 2004). All this occurs in the absence of any change in soluble Pin1 expression and suggests that Pin 1 catalytic or binding activity is responsible. In contrast to Pin1 rescue of tau hyperphosphorylation by dephosphorylating a key site, Thr 231, thus preventing neurodegeneration (Davis et al., 1995; Lu et al., 2003; Galas et al., 2006), Pin 1 stimulates
perikaryal phosphorylation of NF-H in stressed neurons. How can this difference be reconciled?

A model of its role in regulating tau phosphorylation in stressed neurons has been suggested. Glutamate stress, surprisingly, induces tau dephosphorylation in cortical neurons containing phosphorylated fetal tau, while p-tau abnormally accumulates in cell bodies (Davis et al., 1995). Oxidative stress also induces tau dephosphorylation with specific phosphorylated epitopes exhibiting different levels of dephosphorylation (Galas et al., 2006). Significantly, there is no change in Pin 1 levels. Juglone, the Pin1 inhibitor, partially prevents dephosphorylation of the Thr 231 epitope, while in a Pin 1 knockout, stress-induced dephosphorylation at Thr 231 is prevented without affecting dephosphorylation at other sites (Galas et al., 2006). This suggests that Pin1 regulates tau phosphorylation by targeting dephosphorylation of a key binding site responding to unbalanced kinase: phosphatase activities. This may result from cis-trans isomerization and induced conformational changes that expose other phosphorylated tau sites to phosphatases. It is important to acknowledge that in addition to groups implicating Pin1 reductions with increased prevalence to AD and other dementias, Pin1 presence has also been found to be involved in disease and apoptotic processes. A recent report showed that Pin1 activity enhanced the formation of Lewy Bodies, a pathological hallmark found in Parkinson’s disease and overexpression of dominant-negative Pin1 inhibited formation of these inclusions (Ryo et al., 2006). Thus, Pin1 activity impacts target proteins with distinct results in different neurodegenerative diseases. Another recent report showed that Pin1 is involved in mitochondrial mediated apoptosis. The activation of c-Jun N-terminal kinase (JNK) signaling induced the disassociation of Pin1 from the neuron specific JNK scaffold protein JIP3, which promotes the binding of Pin1 with phosphorylated BH3-only protein BIMEL. This stabilized BIMEL and
activated the mitochondrial apoptotic machinery (Becker and Bonni, 2006). Whether this process is involved in neurodegeneration remains to be seen.

Because NF-H possesses numerous KSP repeat phosphate acceptor sites in the tail domain, which are prime targets of several proline dependent kinases, Pin1 regulation of these sites may differ fundamentally from its regulation of the one important site in tau, Thr 231. Interpretation of our data suggests a novel model in which Pin1 is a key player in the topographic regulation of NF phosphorylation (Pant and Veeranna, 1995; Grant et al., 2006). According to the model, NF monomers and oligomers within the cell body are transiently prevented from polymerizing into filaments by phosphorylation of specific NF head domain Ser residues by PKA and PKC (Sihag et al., 1988; Hisanaga et al., 1994). Phosphorylation of head domain sites in a head-to-tail tetramer (and higher) NF-subunit oligomers, may interact with exposed tail domain KSP sites to block initiation of their phosphorylation. This is supported, in part, by the observation that PKA phosphorylation of NF-M head domain sites in rat cortical neurons inhibits phosphorylation of tail domain KSP sites (Zheng et al., 2003). Hence, though Pin1 is present, and presumably active in neuronal cell bodies (Hamdane et al., 2006), phosphorylated S/T-P target sites are unavailable for Pin1 binding. Within the axon initial segment, however, as NF oligomers bind to microtubules (MTs) for transport into the axon, S/T phosphatases are activated, the head domain sites are dephosphorylated and NF polymerization begins. During axonal transport tail domain sites thus become accessible to proline directed kinases and as phosphorylation proceeds, the tails extend, cross linking adjacent NFs and interacting with microtubules. Pin1 now plays a major role within the axon, by inducing cis-trans isomerizations of critical p-S/T-P sites, driving the tail domains to extend and become stabilized. The details of Pin1 interactions with axonal
pNF are described below; meanwhile, we shall focus on the destabilizing effect of stress on the processing of NFs within the cell body.

Glutamate excitotoxicity evokes an influx of Ca2+ that activates several proline directed kinases. Cdk5, for example, is hyperactivated when calpain, a calcium dependent protease, cleaves p35 to produce a more active and stable p25 (Lee et al., 2000). Additionally, Erk1/2 and other stress activated kinases such as SAP/JNK and p38 are also elevated in response to ischemic and excitotoxic stimuli (Zheng et al., 2003). The kinase:phosphatase activity ratio in the cell body is altered to favor proline directed kinases. Moreover, the influx of Ca2+ activates calcineurin, a phosphatase which may dephosphorylate NF head domain p-Ser sites and induce abnormal NF polymerization within the cell body. Phosphorylated tail domain multiple KSP repeats are exposed evoking Pin 1 binding and promoting sustained, aberrant, perikaryal accumulation of p-NF-H. This, in turn, inhibits axonal transport that leads to cell death. Inhibition of Pin 1 activity with juglone, a dominant-negative form of Pin1 or siRNA mediated reduction of the levels of Pin1, prevent a sustained extension and phosphorylation of C-terminal tail domain KSP repeats and stops p-NF-H accumulation in the cell body.

An alternative mechanism may involve Pin1 directly. Since Pin1 activity can be switched off by PKA phosphorylation at Ser16 (Lu et al., 2002), this regulation may control NF phosphorylation within neuronal perikarya during neurofilament protein synthesis. Some forms of AKAP, which can bind both PKA and phosphatases such as calcineurin, function to regulate their relative activities in relation to presynaptic AMPA receptor insertion (Houslay, 2006), and perhaps function similarly with regard to neurofilament phosphorylation. Thus ‘stress conditions’, such
as glutamate treatment, may turn off PKA in response to increased Ca2+ influx and aberrantly activate Pin1.

If there is a physiological role for Pin1 in the axonal compartment, it may incorporate these facts: (1) Pin1 only acts after phosphorylation at (S/T)-P repeat sites (2) proline-directed kinases will not phosphorylate serines or threonines that are cis-bonded to proline residues, and (3) NF-H tail domains, which contain approximately 43/44 S/T-P repeat units, are not detectably phosphorylated in normal neuronal perikarya, and yet become extensively phosphorylated during axonal transport and assembly into neurofilaments. Figure 8 illustrates one way that Pin1 may participate in the normal process of NFH tail domain phosphorylation in axons. Although there is little information about the tertiary structure of this domain, it appears to be unstructured, (i.e. does not contain neither sheets nor helices) (Fink, 2005). We assume, for purposes of discussion, that the unphosphorylated tail domain is globular with only the most C-terminal repeats on its surface accessible to kinases, while more N-terminal repeat units are sterically sequestered within the core. However, the negative charges introduced by multiple proline-directed S/T-phosphorylations will cause the repeat units to unfold and allow kinases access to adjacent repeat units except at certain sites where instead, phosphorylation induces cis isomerization of the p-S/T-P bond. We propose that in such cases and in the absence of active Pin1, the cis bond not only blocks kinase action, but also produces a local conformation that blocks access to new phosphorylation sites (Fig. 8B-3). When active Pin1 is available, however, it’s binding to the pS/T-P sites will regenerate the trans isomers, induce a conformational change and expose adjacent S/T-P sites to initiate further kinase action. Because Pin1 can only act on
phosphorylated S/T-P sites, one or more phosphorylated cis S/T-P bond must already be present so that Pin1 can isomerize it and cause further unraveling of the tail domain.

As shown here, Pin1 action on NF-H during neurotoxic conditions can result in aberrant phosphorylation and aggregation of neurofilaments in cultured neurons. This suggests that regulation of Pin1 binding to NF-H is necessary for normal post-translational control of topographic NF-H tail domain phosphorylation within neuronal perikarya and the axonal compartment. Thus, inhibiting Pin1 activity or reducing its levels during neurotoxicity, is an attractive target for therapeutic intervention to reduce the formation of these aggregates in neuronal cell bodies. Pin1 antagonists, currently being sought in the treatment of cancer, may potentially be beneficial to suppress the neurotoxic accumulation of phosphorylated NF-H found in neurodegenerative disorders such as AD, PD and ALS.

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References


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Figure Legends

Figure 1. **Pin1 binds to phosphorylated neurofilament-heavy chain (p-NF-H).** (A) GST pull-down assays in rat brain lysates. Samples were separated by 10% SDSPAGE, gels were Coomassie stained. Protein bands corresponding to approximately 200kDa were excised and identified by MS-MS mass spectrometry. This protein was identified as NF-H (arrowed). Other bands present in the gel are as follows: X1 – MAP1b, X2 – NF-M, X3 – keratin and X4 – GST-Pin1 degradation product. Tau, GST-Pin1 and GST are also indicated. A representative gel from 5 experiments with identical results is shown. (B) A co-immunoprecipitation using anti-Pin1 antibody (Oncogene). Samples were separated by SDS-PAGE and subjected to Western blotting using RT-97 antibody which specifically detects p-NF-H. Arrowed band shows the species co-immunoprecipitation with Pin1. + and – refer to presence and absence of immunoprecipitating antibody, respectively. (C) Co-immunoprecipitation was done using RT-97 antibody to immunoprecipitate p-NF-H. Pin1 was detected in the immunoprecipitate. Arrowed bands shows p-NF-H and Pin1 species. + and – refer to presence and absence of immunoprecipitating antibody, respectively.

Figure 2 **NF-H phosphorylation is increased in Alzheimer’s disease (AD) tissue**

Total protein lysates from age/region matched controls (CON) and AD brain were made and
separated by SDS-PAGE. The presence of p-NF-H (top panel) and Pin1 (middle panel) were immunodetected and equal loading was confirmed by tubulin. The increase in p-NF-H was evident in AD samples without any change in soluble Pin1 levels. Quantitation is shown in the bar graph below expressed as ±SEM of densitometric measurements of p-NF-H and Pin1 signals normalized to tubulin measurements. Total NF levels were unchanged.

**Figure 3 NF-H phosphorylation and aggregation are increased in ALS spinal cord tissues.**

(A) Total lysates of spinal cord tissues from control and ALS were separated by SDS-PAGE. The Western blot shows an increase in phosphorylated NF-H in ALS tissue compared to the controls (CON) without any significant change in Pin1 levels. Quantitation is shown in the bar graph below with tubulin showing equal loading. Phosphorylated NF-H and Pin1 densities normalized to tubulin densities (obtained through densitometric scanning) expressed as ±SEM. Total NF levels were unchanged. (B) Spinal cord sections from ALS patients and closely matched controls were immunostained for Pin1 (red) and p-NF-H using RT-97 (green). In the ventral region of gray matter the distribution of both Pin1 and phosphorylated NF-H were uniform in the area. (a-c). However, in the ventral horn of the white matter of ALS spinal cord, phosphorylated NF-H and Pin1 co-localized in aggregates (d-f). Controls without primary antibody did not show any staining. Scale bars represent 20µm.

**Figure 4 Glutamate excitotoxicity induces phosphorylated NF-H accumulations in DRG neuronal cell bodies.** (A) Five day old DRG neurons were non-treated, treated with 10mM glutamate for 4 hours or pre-treated with 30µM juglone then treated with 10µM glutamate for 4 hours. Total lysates from the DRG samples were subjected to Western blotting to detect p-NF-H and Pin1. p-NF-H increased upon treatment with glutamate which was reduced to non-treated
levels when DRG neurons were pretreated with juglone prior to glutamate exposure. Tubulin expression served as loading control. Quantitation shown in bar graph at right as ±SEM of 4 separate experiments. (B) Five day old DRG neurons were non-treated (panels a-c), treated with 10µM glutamate for 6 hours (panels d-f) or pretreated with 30mM juglone then treated with 10µM glutamate for 6 hours (panels g-i). In non-treated DRG neurons, Pin1 was localized to the cell body while p-NF-H was found mainly in the processes. Upon glutamate treatment there was an increase in p-NF-H and co-localization with Pin1 in the cell bodies. This was reduced when neurons were pretreated with juglone prior to glutamate exposure. Scale bar represents 20µm.

**Figure 5 Glutamate-mediated increases in p-NF-H levels in cortical neurons is inhibited by juglone** (A) Seven day old primary cortical neurons were non-treated, treated with 0.1mM glutamate for 6 hours or pre-treated with 30µM of the Pin1 inhibitor juglone for 3 hours then treated with 0.1mM glutamate for 6 hours. Total lysates were subjected to Western blotting. p-NF-H increased in the glutamate (Glut) treated samples and were reduced to non-treated (NT) levels when neurons were treated with juglone prior to glutamate exposure (Jug+Glut). Soluble Pin1 levels did not change. Bar graph on the right shows the results of 4 experiments expressed as ±SEM. (B) Neurons were non-treated (NT: a-d), glutamate treated (Glut: e-h) and pretreated with juglone prior to glutamate exposure (Jug+Glut: i-l), fixed and stained for p-NF-H using RT-97 antibody (red), Pin1 (green) and DAPI (blue). Non-treated neurons exhibited p-NF-H staining in the processes with little or no staining in the cell body which increased upon glutamate treatment. Cell body p-NF-H staining was reduced when neurons were pre-treated with the Pin1 inhibitor prior to glutamate treatment. Scale bar represents 20µm.
Figure 6 Glutamate-mediated increase in phosphorylated NF-H is reduced by overexpression of dominant-negative Pin1 and Pin1-siRNA.

(A) Five day old cortical neurons were transfected with dominant-negative Pin1 and after 5 hours were treated with 0.1 mM glutamate (Glut). Neurons were immunostained and p-NF-H was detected using RT-97 (red) and DN Pin1 detected through expression of GFP. Only neurons transfected with DN Pin1 exhibited reduced phosphorylated NF-H in the cell body. Scale bar represents 20 μm. (B) Identical samples from (A) were harvested for lysates. Total cell lysates were made and separated by SDS-PAGE and then subjected to Western blotting. Phosphorylated NF-H (p-NF-H) was detected using RT-97. Pin1 was immunodetected using anti-Pin1 antibody. Phosphorylated NF-H was reduced in the DNPin1 transfected sample. Transfected Pin1 (tPin1) migrated at approximately 50 kDa as it was a GFP fusion protein compared to 18 kDa of endogenous Pin1 (ePin1). Equal loading was confirmed by detection of tubulin. The Western blot panels are representative of 5 independent experiments and quantitation of p-NF-H is shown in the bar graph on the right where densitometric measurements of RT-97 were normalized to tubulin measurements and expressed as ±SEM. (C) Five day old neurons were transfected with either control siRNA (a-d; e-h) or Pin1-siRNA (i-l) and then treated with 0.1 mM glutamate (Glut). Neurons were immunostained and p-NF-H was detected using RT-97 (red) and Pin1 detected by using Pin1 antibody. Only neurons transfected with Pin1-siRNA exhibited reduced phosphorylated NFH in the cell body. Scale bar represents 20 μm. (D) Identical samples from (C) were harvested and separated by Western blotting for immunodetection of p-NF-H and Pin1. Signals were normalized to tubulin levels. Pin1-siRNA reduced glutamate-induced increases in p-NF-H.
Figure 7 Inhibition of Pin1 reduces glutamate mediated neuronal cell death

(A) Seven day old cortical neurons were non-treated (a), treated with 0.1mM glutamate for 6 hours (b) or pretreated with juglone then glutamate (c) and presence of apoptotic neurons examined by TUNEL-FITC staining. Nuclei were counter-stained using DAPI. TUNEL positive cells increased upon glutamate treatment and declined after neurons were treated with juglone prior to glutamate treatment. Scale bar represents 20 µm. (B) Quantitation is shown in the bar graph where 5 independent areas containing a minimum of 50 neurons were counted. Neuronal death increased nearly 4 times upon exposure to glutamate and this was reduced nearly two-fold when neurons were treated with juglone prior to glutamate treatment.

Figure 8 Hypothetical mechanism for Pin1 regulation of NF-H phosphorylation.

The three adjacent KSP repeat units used to illustrate this model are the human NF-H sequence 742-761. We arbitrarily diagram the kinase phosphorylation of NF tail domain repeats as normally starting at the most C-terminal repeat unit and proceeding toward their N-terminus. The more N-terminal repeat units are assumed to be sterically shielded from kinase access by burial within the tail domain until the adjacent C-terminal units are phosphorylated: (1) Tail domain phosphorylation occurs at kinase-accessible C-terminal repeats (yellow spheres); phosphorylation ‘unwinds’ the outer repeat units and permits kinase access to additional repeat units. (2) In some cases, two or more phosphorylations will initiate a trans to cis isomerization of the last phosphorylated S-P bond. This causes a local conformation that does not expose further tail domain phosphorylation sites. However, if active Pin1 is available it will rapidly return the cis p-S/T-P, to trans and allow additional phosphorylation to proceed normally in axons. (3) However, if this occurs in perikarya, premature extension of sidearms may prevent neurofilament subunit transport out of perikarya and cause aggregation of p-NF-H subunits.
Figure 6

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