

Neurofilament tail phosphorylation: identity of the RT-97 phosphoepitope and regulation in neurons by cross-talk among proline-directed kinases

Veeranna,*'† Ju-Hyun Lee,*'† Tej K. Pareek,‡ Howard Jaffee,§ Barry Boland,*'¶ K. Yaragudri Vinod,** Niranjana Amin,†† Ashok B. Kulkarni,‡ Harish C. Pant†† and Ralph A. Nixon*'†'‡‡

*Center for Dementia Research, Nathan Kline Institute for Psychiatric Research, Orangeburg, New York, USA †Department of Psychiatry, New York University School of Medicine, New York, New York, USA

Laboratory of Cell and Developmental Biology, NIDCR, NIH, Bethesda, Maryland, USA

§Mass Spectrometry Facility, NINDS, Functional Genomics Section, NIH, Bethesda, Maryland, USA

¶Laboratory for Neurodegenerative Research, UCD School of Biomolecular and Biomedical Science, Conway Institute,

University College Dublin, Dublin, Ireland

**Analytical Psychopharmacology, Nathan Kline Institute for Psychiatric Research, Orangeburg, New York, USA

††Laboratory of Neurochemistry, NINDS, NIH, Bethesda, Maryland, USA

‡‡Department of Cell Biology, New York University School of Medicine, New York, New York, USA

Abstract

As axons myelinate, establish a stable neurofilament network, and expand in caliber, neurofilament proteins are extensively phosphorylated along their C-terminal tails, which is recognized by the monoclonal antibody, RT-97. Here, we demonstrate *in vivo* that RT-97 immunoreactivity (IR) is generated by phosphorylation at KSPXK or KSPXXXK motifs and requires flanking lysines at specific positions. extracellular signal regulated kinase 1,2 (ERK1,2) and pERK1,2 levels increase in parallel with phosphorylation at the RT-97 epitope during early postnatal brain development. Purified ERK1,2 generated RT-97 on both KSP motifs on recombinant NF-H tail domain proteins, while cdk5 phosphorylated only KSPXK motifs. RT-97 epitope generation in primary hippocampal neurons was regulated by extensive cross-talk among ERK1,2, c-Jun Nterminal kinase 1,2 (JNK1,2) and cdk5. Inhibition of both ERK1,2 and JNK1,2 completely blocked RT-97 generation. Cdk5 influenced RT-97 generation indirectly by modulating JNK activation. In mice, cdk5 gene deletion did not significantly alter RT-97 IR or ERK1,2 and JNK activation. In mice lacking the cdk5 activator P35, the partial suppression of cdk5 activity increased RT-97 IR by activating ERK1,2. Thus, cdk5 influences RT-97 epitope generation partly by modulating ERKs and JNKs, which are the two principal kinases regulating neurofilament phosphorylation. The regulation of a single target by multiple protein kinases underscores the importance of monitoring other relevant kinases when the activity of a particular one is blocked.

Keywords: cytoskeleton, neurodegenerative disease, cdk5, JNK, ERK, GSK3- β .

J. Neurochem. (2008) 107, 35-49.

Neurofilaments (NF), the major cytoskeletal component of large myelinated axons, are heteropolymers that vary in subunit composition depending on neuron type and stage of development. In the mature CNS, neurofilaments contain four subunits, NF-H, NF-M, NF-L and α -internexin (Yuan *et al.* 2006) while, in the PNS, NF-H, NF-M and NF-L subunits are associated with a fourth intermediate filament protein, peripherin (Lee and Cleveland 1996). Each subunit

Received June 5, 2008; accepted June 16, 2008.

Address correspondence and reprint requests to Ralph A. Nixon, Nathan Kline Institute, New York University School of Medicine, 140 Old Orangeburg Rd., Orangeburg, NY 10962, USA. E-mail: mixon@nki.rfmh.org

Abbreviations used: ERK, extracellular signal regulated kinase; GSK, glycogen synthase kinase; GST, glutathione S transferase; JNK, c-Jun N-terminal kinase; MEK, mitogen activated protein kinase kinase; MS, mass spectometry; NF, neurofilament; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

has a highly conserved central rod domain flanked by a variable N-terminal head domain and a C-terminal tail domain that varies in length depending on the subunit (Nixon and Sihag 1991; Pant and Veeranna 1995). A regionally specialized neurofilament network serves as a structural framework for maintaining neuronal shape and axon caliber and provides a scaffold for organizing and regulating functions of proteins and organelles (Fuchs and Cleveland 1998).

Neurofilaments are extensively regulated by phosphorylation. Among the most phosphorylated proteins, NF-H and NF-M contain 52 and 5 serine/threonine residues, respectively in mouse and 44 and 13 serine/threonine residues in the NF-H and NF-M of human respectively, on their long carboxyl terminal tails which exist in conserved KS/TP motifs differing in flanking sequence: KSPXK and KSPXXXK (Shetty et al. 1993; Nixon and Sihag 1991; Pant et al. 2000). These sites and additional phosphorylation sites on the head domains of NF-L and NFM are regulated spatially and temporally within neurons (Sihag et al. 2007). The major phospho-epitopes on the NF-M and NF-H tail domains, recognized by the monoclonal antibodies SMI-31, SMI-34 and RT-97 (Probst et al. 1983; Sternberger and Sternberger 1983; Lawson et al. 1984), appear sequentially during neuronal maturation on axonal neurofilaments (Sanchez et al. 1996, 2000). Phosphorylation on these domains influences the susceptibility of neurofilaments to calcium activated neutral proteases, and modulates the kinetic behaviors of neurofilaments (Pant 1988; Nixon et al. 1994; Ackerley et al. 2000) and their interactions with other cytoskeleton elements and specific receptors (Carden et al. 1985; Nixon et al. 1986; Pant 1988; Hisanaga et al. 1991; Miyasaka et al. 1993). Appearance of the RT-97 phosphoepitope late in axon maturation is triggered by myelinating glial cells (Sanchez et al. 1996, 2000) and is associated with the elaboration of an extensive stationary neurofilament network essential for expansion of axonal caliber and proper electrical conductance (Gasser and Grundfest 1939; Nixon and Lewis 1986; Nixon and Logvinenko 1986). The increased abundance of RT-97 on neurofilament and the microtubule-associated protein tau in affected neuronal perikarya accompanies the pathological accumulation of these cytoskeletal proteins in neurodegenerative diseases (Anderton et al. 1982; Sternberger et al. 1985; Veeranna et al. 2004; Kesavapany et al. 2007). The identity and regulation of NF phospho-epitopes, however, has not been clarified.

Ser/Thr kinases have been implicated in the phosphorylation of KSP repeats on neurofilament tail domains, including cdk5 (Wang *et al.* 1992; Shetty *et al.* 1993), MAPKs such as ERKs (Roder *et al.* 1993; Veeranna *et al.* 1998), JNKs (Giasson and Mushynski 1997; Ackerley *et al.* 2000) and P38 kinases (Ackerley *et al.* 2004); and additional kinases, including glycogen synthase kinase $3-\beta$ (GSK3- β), Cdc2 and 115 kDa NF kinase (Xiao and Monteiro 1994; Starr *et al.* 1996; Sun *et al.* 1996; Bajaj and Miller 1997; Sasaki *et al.* 2002). Cdk5 phosphorylates KSPXK sites (Shetty *et al.* 1993), while ERK2 phosphorylates neurofilaments at both KSPXK and KSPXXXK repeats (Veeranna *et al.* 1998, 2004).

Here, we have investigated the structural determinants for generation of the RT-97 phospho-epitope. In addition, we modulated protein kinases pharmacologically and genetically to investigate their roles in generating the RT-97 phosphoepitopes in primary hippocampal neurons. The modulation of kinase activities revealed that cross-talk is extensive among signaling pathways that regulate neurofilament sidearm phosphorylation.

Materials and methods

Antibodies, peptides and other reagents

We obtained the following antibodies commercially: monoclonal antibodies to β-tubulin (Amersham Life Sciences, Chicago, IL, USA) SMI-31, and SMI 33 against phospho-and dephosphoepitopes, respectively, on NF-H and NF-M (Sternburger Monoclonal Inc. Lutherville, Baltimore MD, USA); Polyclonal antibodies against GSK3-B, ERK 1,2, P35 and cdk5 (SantaCruz Biotechnology Inc. CA, USA); phospho-ERK1,2 and phosphoindependent ERK1,2 phospho-mitogen activated protein kinase kinase 1,2 (pMEK 1,2), Phospho JNK 1,2 and Total JNK 1,2 and inactive GSK3-B (Cell Signaling, Boston, MA, USA); anti calnexin polyclonal antibody (Stressgen, Victoria, BC, Canada); anti-mouse and anti-rabbit secondary antibodies conjugated to alkaline phosphatase (Promega, Madison, WI, USA); or horseradish peroxidase conjugated anti-mouse and anti-rabbit secondary antibodies (Jackson Immunochemical Laboratories Baltimore MD, USA); or Alexa 488 and Alexa 568 (Invitrogen, Gaithersburg, MD, USA). A rabbit polyclonal antibody against NF-L was made in this laboratory. The RT-97 monoclonal antibody clone was a kind gift from Brian Anderton (Institute of Psychiatry London). Recombinant ERK2 and MEK1 proteins were kind gifts from Dr. N.G Ahn (University of Colorado, Boulder, CO, USA). Additional commercial reagents included microcystin LR (Calbiochem, San Diego, CA, USA), (gamma-32P) ATP (Perkin Elmer Life Sciences, Boston, MA, USA), ECL kit (Amersham Biotech, IL, USA), immobilon membranes (Millipore, Bedford, MA, USA), dialysis tubing (Spectrum, Los Angeles, CA, USA), myelin basic protein and histone H1 (Sigma Chemical Company, St. Louis, MO, USA), Synthetic peptides derived from the mouse NF-H tail domain sequence were custom synthesized at the Advanced Protein Technology unit at the Hospital for Sick Children (Toronto, On, Canada). Block Ace (Serotec, Raleigh, NC, USA). All cell culture reagents were purchased from Invitrogen. (Gaithersburg, MD, USA).

Primary neuronal cultures and treatment with kinase inhibitors

Primary hippocampal neurons were established from day-19 Sprague–Dawley rat embryos (Charles River Labs, NY, USA). Nineteen day timed pregnant rat was euthanized using CO2 and pups were removed, decapitated and hippocampal regions were dissected from cerebral cortices in Hibernate-E media (Brain Bits LLC, IL, USA). Dissociated hippocampal neurons were obtained by incubating the hippocampi in Hibernate-E containing 15 units/ mL of papain (Worthington Biochemicals, NJ, USA) for 15 min at 37°C before triturating in neurobasal medium containing 20% fetal bovine serum (Hyclone, UT, USA), DNAse (0.2 mg/mL) and 0.1 M MgSO₄. Undissociated neurons were removed from the cell suspension by passing the cell suspension through a 40 µm cell strainer (Fisher Scientific, NY, USA). Neurons were centrifuged at 200 g for 3 min at 20°C and the pellet was resuspended in neurobasal medium supplemented with B27, penicillin (100 U/mL), streptomycin (100 U/mL) and L-glutamine (0.5 mM, InVitrogen, NY, USA). Neurons were then plated at a density of 150 000 cells/mL on circular glass coverslips and 6well tissue culture dishes, coated with poly-d-lysine (50 µg/mL, Sigma, MO, USA), and incubated in a humidified atmosphere containing 5% CO2: 95% O2 at 37°C. The following drugs (at 10 µM), U-0126, roscovitine and SP600125 to specifically inhibit ERK1,2, cdk5, or JNKs respectively, were added to the media at 4 h, 24 h and 48 h after plating. Neurons were harvested after 72 h (3 days) in culture.

Immunocytochemistry of hippocampal neurons

Hippocampal neurons fixed in 4% paraformaldehyde were permeabilized for 20 min in 0.2% Triton X-100, blocked in 10% horse serum in phosphate-buffered saline (PBS) for 1 h, and incubated with primary antibodies (diluted in 4% horse serum/ phosphate buffered saline 0.05% Tween-20) against phosphospecific monoclonal antibody against NF-H (RT-97) or with polyclonal antibodies to p-ERK1,2, cdk5, p-MEK1,2 or p-JNK 1,2 (Cell signaling) for 1 h at 23°C. After three washes in blocking solution, the neurons were incubated with anti-rabbit and antimouse secondary antibodies, Alexa 488 and Alexa 568, respectively (Molecular Probes, Eugene, OR, USA). Secondary antibodies were diluted in the same buffer as the primary antibodies and incubated for 1 h at 23°C. Cells were washed and mounted on the cover slips and analyzed using a TCS software program on an Axiovert 200 M microscope (Zeiss, Germany).

Tissue extraction and immuno-precipitation of protein kinases

Brains from C57 BL/6 wild type mice aged 6 months or older were dissected and frozen in dry ice and stored at -80° C until further use. A separate set of spinal cords were harvested from C57 BL/6 wild type mice aged 1–15 days and frozen at -80° C until assayed for neurofilament proteins and for protein kinase activities and protein levels. Frozen brains were homogenized in a tissue extraction buffer (50 mM Tris/HCl pH 7.4 containing 100 mM Na Cl, 5 mM EDTA and 1% Triton X-100, 20 mM B-glycerophosphate, 20 mM sodium fluoride, 1 mM sodium vanadate, and a mixture of protease inhibitors containing 5 μ g/mL each of leupeptin, antipain and pepstatin (all from Sigma) in the ratio of 1 : 10 (W/V) using a handheld battery operated tissue homogenizer. The samples were centrifuged at 15 000 rpm (30 000 g) for 30 min.

Immunoprecipitation of cdk5 and ERK 1,2 from mouse brain was performed essentially as described earlier (Veeranna *et al.* 1996, 1998). In brief, protein A plus G agarose beads (60 μ L packed volume) were washed in 1X PBS three times, suspended in 500 μ L

of 1X PBS, and incubated at 23°C for 30 min with 60 μ L of primary antibodies to ERK 1,2 and supernatants obtained from brain homogenates (equivalent to 500 μ g of protein) were incubated with Protein A plus G agarose beads coupled to ERK 1,2 and cdk5 polyclonal antibodies for 2 h at 4°C. The beads were washed in buffer (50 mM Tris/HCl pH 7.4 containing 5 mM EDTA, 10 mM NaF, 10 mM β -glycerophosphate, and 0.01% Triton X-100) and suspended in 100 μ L of 1X kinase buffer.

Expression and purification of KSPXXXK and KSPXK fusion proteins

A rat NF-H tail fragment with 24 KSPXXXK repeats, and another fragment derived from human NF-H with 14 KSPXK repeats, both tagged to glutathione S transferase (GST) fusion protein were expressed and purified as described previously (Pant *et al.* 1997; Veeranna *et al.* 1998). Full length NF-H was expressed as described earlier (Takahashi *et al.* 1995).

Phosphorylation of Recombinant NF-H and NF-H fusion proteins

Phosphorylation of the bacterially expressed full length NF-H and NF-H tail domain GST fusion proteins was carried out as described earlier (Veeranna et al., 1995 and Veeranna et al. 1998) using the immuno-precipitates of cdk5, phospho ERK 1,2 or recombinant ERK2 or a cdk5/p25 complex. Briefly, 10 µL of the immunoprecipitates of ERK1,2, or cdk5 were mixed in a kinase reaction mixture containing 50 mM Tris/HCl, pH 7.5, with 1 mM each of EDTA, EGTA, dithiothreitol and orthovanadate, 1 µM microcystin LR, 5 µg of recombinant NF-H or KSPXK or KSPXXXK GST fusion protein and gamma (γ^{32} P) ATP (50 μ M) in a total volume of 100 μ L. The reaction was initiated by the addition of (γ^{32} P) ATP, incubated at 30°C for 2 h, and terminated by transferring an aliquot of 20 μ L of the kinase reaction mixture at 0, 0.5, 1, 1.5 and 2 h to a tube containing 5 $\,\mu L$ of 5X Laemmli buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis using RT-97 monoclonal antibody or gels were silver stained after SDS-PAGE and dried and subjected to autoradiography using Kodak X-ray film.

Purification and analysis of neurofilament triplet protein

The neurofilament triplets were prepared from mouse spinal cords and NF-H protein was purified on a DE-52 column using an FPLC system as described earlier (Veeranna *et al.*, 1995; Veeranna *et al.* 1998). NF-H and NF-M were further purified by SDS–PAGE using a 7% 20 × 20 cm preparative gel, followed by zinc staining (Dzandu *et al.* 1988) and electro-elution, dialysed against 0.1 M ammonium bicarbonate, and used as an antigen and to generate tryptic digests for further analysis by dot blot assay.

SDS-PAGE and western blot analysis

Protein concentration in each fraction was assayed by the BCA method (Pierce, IL, USA), and protein aliquots (10–20 µg) were loaded for each lane on a 7% or 10% minigel for electrophoresis unless otherwise indicated. Proteins were electro-transferred from gels to polyvinylidene difluoride or nitrocellulose membranes using a Genie blotter (Idea Scientific, Minneapolis, MN, USA) and blocked using 3% non-fat dry milk (Biorad) in Tris-buffered saline (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% Tween 20;

TBS-T) for at least 2 h. The membranes were incubated overnight at 4°C with primary antibodies at appropriate dilutions. Blots were developed with alkaline phosphatase-conjugated secondary antibodies using the chromogenic substrate BCIP/NBT (Promega) or with chemiluminescence based CDP star (Tropix, Bedford, MA, USA) phosphatase substrate or peroxidase conjugated secondary antibodies using the ECL kit (Amersham Biotech, Chicago, IL, USA).

Enzyme linked immunosorbent assay

The RT-97 IR against synthetic phosphopeptides was determined by direct enzyme linked immunosorbent assay (ELISA). Briefly, we added 100 µL of peptide solution, (peptides serially diluted in carbonate buffer (30 mM NaHCO3, 70 mM Na2 CO3, 0.05% NaN3 pH 9.6) ranging from 1 mM to 0.001 mM unless otherwise mentioned and incubated overnight at 4°C. Unbound peptides were removed by washing with PBS twice using a programmed ELISA washer MRW tm Dynex Inc. Chantilly VA, USA). The vacant sites on the polystyrene plates were quenched by adding 200 µL of blocking solution (1% Block Ace, 0.05% Na N3, in PBS pH 7.4) to each well and incubated at RT for 4-6 h. 100 µL aliquot of primary antibody (RT-97) of 1: 2000 dilution in antibody dilution buffer (3 mM NaH2PO4, 17 mM Na2HPO4, 2 mM EDTA, 400 mM NaCl, 1% bovine serum albumin pH 7.0) was added to each well and incubated overnight at 4°C. Unbound antibody was removed by washing twice with phosphate buffered saline containing Tween 20 (0.1%) and once with PBS using a programmed ELISA washer. A 100 µL aliquot of labeled secondary antibody (Goat anti mouse peroxidase conjugated IgG) was added to each well and incubated for 2 h at 23°C and unbound secondary antibody was removed by washing the plates with PBST twice and once with PBS using the ELISA washer and developed by addition of 100 µL of substrate solution (TMB 2-Component Micro well Peroxidase substrate kit provided by Kirkegaard and Perry Laboratories, Gaithersburg MD, USA). Incubation at RT was continued until the desired intensity of color developed. The reaction was stopped by adding 100 µL of 5.6% orthophosphoric acid and color was measured as optical density at 450 nm.

Mass spectrometric (MS) analysis of neurofilament phosphopeptides bound to RT-97 antibody

For MS analysis, an immunoaffinity ultrafiltration technique was employed (Wieboldt et al. 1997). NF-H (100°µg) was dissolved in 100 µL 8 M urea in 0.4 M ammonium bicarbonate, heated for 30 min at 50°C with 20 µL 0.45 M dithiothreitol. After cooling to RT, 20 µL 0.1 M iodoacetamide was added and allowed to react for 30 min. After dilution to 2 M urea/0.1 M ammonium bicarbonate, 5 µg trypsin (Promega) was added, and the reduced and alkylated protein was digested overnight at 37°C. An aliquot (25 µL) of the digest was mixed with 425 µL of 1 X PBS and 50 µL of the RT-97 antibody in a 30K Microcon ultrafiltration device (Millipore, Bedford, MA, USA) and incubated at 37°C for 2 h. The reaction mixture was spun down to 20 µL and washed with 500 µL of 1X PBS and eluted with 300 µL of 2% heptafluorobutyric acid. The released phosphopeptides were concentrated in a speedvac (Savant, Framingham, NY, USA) and analyzed by ESI/MS/MS as previously described (Jaffe et al. 1998a,b).

Results

Identification of the RT-97 phospho-epitope on mouse and human NF-H by mass spectrometry

To identify the RT-97 phosphoepitope of NF-H, we digested mouse NF-H with trypsin, which yielded phosphopeptides that bound to RT-97 antibody in a 30K immunoaffinity ultrafiltration device. Subsequent mass spectrometric analysis (Fig. 1a and b) identified two distinct KSPXK peptides bound to the RT-97 antibody (Fig. 1c and d). One of these, identified as KAKS*PVKEDIKPPAEAKS*PEK (789-809), is shown in Fig. 1(b). KSPXXXK peptides were not detected in this analysis because the KSP core of mouse NF-H containing these motifs is resistant to proteolysis conferred by endogenous phosphorylation (Elhanany et al. 1994). Therefore, we subsequently analysed tryptic digests of human NF-H that contains a structurally different KSP region completely cleaved by trypsin. Mass spectrometry of tryptic digests yielded multiple peptides with either KSPXK or KSPXXXK motifs that bound RT-97 (Fig. 1c and d).

Structural requirements for RT-97 phosphoepitope generation

To investigate the relative affinity of the RT-97 antibody for KSPXK and KSPXXXK motifs, we used synthetic phosphopeptides derived from the sequences of the mouse NF-H tail domain containing either KSPXK (amino acid residues 854-867) or KSPXXXK (amino acid residues 620-635) motifs (Fig. 2a). Based on the peptide structure prediction using a standard program, these two peptides differed in hydrophilicity, surface probability, chain flexibility turns, and alpha helices (Fig. 2a1 and a2). Although there are limitations in the reliability of these structural predictions, they are consistent with differences evident from sequence differences alone. The RT-97 immuno-reactivity of these peptides was analyzed either by ELISA (Fig. 2b) or dot blot assay (not shown). As shown in Fig. 2(b), a single phosphate on KSPXK peptides was necessary and sufficient for RT-97 IR. By contrast, RT-97 IR generation on the KSPXXXK repeat peptide required at least two phosphates and the RT-97 IR was 2 fold lower compared to that of the singly phosphorylated KSPXK peptide. These data suggested that the sequence of the peptide and the position of K(lysine) on the C-terminus of the peptides plays a key role in RT-97 IR generation.

We and others showed that tryptic digestion reduces RT-97 IR on NF-H *in vitro* (Fig. 3a) and in tissue sections (Ulrich *et al.* 1987). Since trypsin cleaves at the C-terminus of lysine (Keil-Dlouha *et al.* 1971; Olsen *et al.* 2004), the possibility exists that the N-terminal lysine may be important for RT-97 IR in addition to lysine in the C-terminal side of the peptide. To test this hypothesis, we generated phospho-variants of wild type (KSP) and mutant glycine serine proline (GSP)



Fig. 1 Identification of RT-97 epitope sites on NF-H. (a) Neutral Loss Chromatogram (-32.7) of the HPLC ESI/MS/MS analysis of Mouse NF-H digest bound to RT-97. (b) MS/MS spectrum of the triply charged diphosphopeptide eluting at 28.48 min (Fig. 2a) identified as KAKS*PVKEDIKPPEAK S*PEK. Most abundant doubly charged ser-

peptides derived from the mouse NF-H sequence, in which glycine replaced lysine in the KSP motifs (Fig. 3b). The data presented in Fig. 3(c) show that N-terminal lysine also affects the RT-97 IR. This replacement completely eliminated RT-97 IR on phosphopeptides containing either KSPXK or ies are labeled. (c) A schematic representation of NF-H showing the location of RT-97 bound KSP peptides in the tail domain of mouse and human NF-H. (d) Mouse and human NF-H tail domain sequence containing RT-97 binding peptides.

KSPXXXK motifs, thus explaining the effects of trypsin digestion on RT-97 IR (Fig. 3b and c) and establishing the importance of N-terminal lysine for RT-97 recognition. Because both N and C terminal lysines influence RT-97 IR, we analyzed RT-97 IR of a series of synthetic 8mer KSP



Fig. 2 RT-97 immunoreactivity of synthetic mouse NF-H tail phosphopeptides. (a) Schematic representation of mouse NF-H showing the sequences of synthetic phospho-peptides derived from mouse NF-H tail domain sequence. (a1) Representation of the predicted peptide structure of KSPXXXK peptide in (A). (a2) Representation of

phosphopeptides with N-terminal lysine with or without a Cterminal lysine varying in its position within the peptide. The presence and position of the C-terminal lysine significantly influenced RT-97 IR (Fig. 3d): The peptide K(S-P)AAVGS lacking a second lysine on the C-terminus was less RT-97 immunoreactive than the peptides, K(S-P)PAAVGK and K(S-P)PAKAVS, with C-terminal lysine. Among the latter, positioning of the second lysine as the 8th residue from the Nterminal lysine was less immunoreactive than positioning at the 5th residue. Moreover, further sequence extension at the C-terminal end of the KSPXK peptide (14 mer) (Fig. 3b), the predicted peptide structure of KSPXK peptide in (a). (b) Line graph of ELISA showing the RT-97 immunoreactivity against KSPXXXK and KSPXK peptides with and without phosphate (direct ELISAs were performed by coating the peptides onto ELISA plates).

without any additional phosphate, increased RT-97 IR suggesting that additional aspects of peptide structure influence affinity of RT-97.

Cdk5 and ERK 1,2 generate the RT-97 phospho-epitope with different specificities for KSP motifs

Among the various proline-directed kinases, ERK1,2, cdk5 and JNKs are known to be prominent in phosphorylating NF-H tail domain KSP motifs (Roder *et al.* 1993; Shetty *et al.* 1993; Giasson and Mushynski 1997; Veeranna *et al.* 1998). To examine RT-97 IR generation after phosphorylation by



Fig. 3 Trypsin abolishes RT-97 IR in vitro. (a) 20 µg of NF-H was trypsinized overnight as described in the text and a 2 μL aliquot of different dilutions was spotted on a nitrocellulose membrane as shown in the figure. Corresponding dilutions of NF-H without trypsinization also were spotted and the membrane was air dried at 23°C for 30 min and immunostained with RT-97 monoclonal antibody. (b) Schematic representation of mouse NF-H showing the sequences of synthetic phospho-peptides derived from mouse NF-H tail domain with and without replacement of lysine with glycine. (c) Bar graph of ELISA using synthetic KSPXXXK and KSPXK phospho-peptides with and without replacement of lysine with glycine. (d) Bar graph of ELISA using 100 μ M synthetic 8mer KSP phospho-peptides with and without C-terminal lysine at different positions as shown. Bar 1 represents a KSPXK 14mer peptide with a single phosphate and five lysine residues (Fig. 3b). Bar 2,3 and 4 represent 8mer KSP phospho-peptides with no second lysine residue K(S-P)PAAVGS (2), with second lysine at 8th position K(S-P)PAAVGK (3) and with second lysine at 5th position K(S-P)PAKAVS ($n = 4, p \le 0.001$).



Fig. 4 Phosphorylation of NF-H and NF-H tail domain fusion proteins by proline directed kinases. (a) Both cdk5 and ERK 1,2 phosphorylate recombinant NF-H and and generate the RT-97 phosphoepitope. Western blot analysis of full length bacterially expressed NF-H immunostained with RT-97 after it was phosphorylated using the immunoprecipitate (IP) of cdk5 as enzyme source (lane 2) and ERK 1,2 (lane 1) and lane 3 represents a 14 mer KSPXK repeat fusion protein derived from human NF-H tail sequence, immunostained with RT-97 after phosphorylation using immunoprecipitate of ERK1,2 and IP of cdk5 (lane 5) and lane 4 represents the fusion protein alone. (b) ERK2 phosphorylation KSPXXXK fusion protein elicits RT-97 IR: 10 µg of 24 KSPXXXK repeat fusion protein was phosphorylated with a mixture of recombinant ERK2 and MEK1 in a time course reaction ranging from 0-2 h (0, 0.5, 1, 1.5 and 2 h represented in lanes 1 through 5 respectively) in triplicate. Equal aliquots were removed from the reaction mixture and were electrophoresed on 7% SDS gels followed by electrotransfer onto nitrocellulose membrane in order to immunostain with RT-97 monoclonal antibody (lower panel). One gel was stained with silver to discern the protein (top panel) dried and subjected to autoradiography to visualize phosphorylation (middle panel).



Fig. 5 Western blot analysis of lysates of hippocampal neurons treated with kinase inhibitors. (a) 10 μ M each of U-0126 (lane-2), roscovitine (lane 3) and U-0126 together with Roscovitine (lane 4). Lane 1 represents the lysates from neurons treated with vehicle (DMSO). The neurons were treated for 72 h and lysed in MPER (Pierce) with added protease and phosphatase inhibitors. Quantities of

protein (25 µg/lane) were electrophoresced on an 8% SDS-Tris – glycine gel and immunoblot analysis was performed with antibodies against neurofilament kinases and phosphoneurofilament antibodies. (b) Relative band intensities of JNK1(p-46), (c) phosphoneurofilaments, (d) P-ERK 1,2, (e) p-JNK 2 (p-54), and (f) p-MEK1,2. (*p < 0.01, **p < 0.001)



Fig. 6 Inhibition of ERK 1,2 by U-0126 leads to enhanced RT-97 IR. The hippocampal neurons grown in culture, 4 h after plating on polylysine-coated glass coverslips were treated with vehicle (a, e, i) or with U-0126 (10 μ M) (b, f, j) or roscovitine (10 μ M) (c, g, k) or roscovitine together with U-0126 (d, h, l) for 72 h and fixed in 4% paraformalde-hyde and then subjected to double immunostaining using monoclonal

antibody RT-97 together with polyclonal antibodies to either pERK1,2 or pJNK. A mixture of anti-mouse and anti-rabbit secondary antibodies tagged to fluorophores Alexa 568 red and Alexa 488 green respectively, were used to develop and visualize the neurons in this and subsequent images of Figs 7 and 8 (scale bar 50 μ m).

ERK1,2 and cdk5, we purified bacterially expressed rat NF-H and GST fusion proteins carrying KSP repeats. ERK1, 2 immunoprecipitated from mouse brain generated the RT-97 epitope on GST fusion proteins derived from human NF-H containing 14 KSPXK repeats or GST fusion protein derived from rat NF-H containing 24 KSPXXXK repeats. By contrast, cdk5 generated the RT-97 epitope only on the KSPXK fusion protein in vitro, consistent with its more restricted sequence specificity (Fig. 4a). Both kinases immunoprecipitated from mouse brain were able to generate the RT-97 epitope on recombinant full length NF-H which contains both motifs (Fig. 4a). RT-97 IR paralleled the degree of phosphorylation of KSPXXXK fusion protein by recombinant ERK2 and was accompanied by a concomitant retardation in electrophoretic mobility of the phosphoprotein (Fig. 4b), as seen for full length NF-H after phosphorylation in vitro and in vivo.

Inhibition of ERK 1,2, JNK1,2 or cdk5 alter RT-97 immunoreactivity in hippocampal neurons

To investigate the regulation of the RT-97 phospho epitope *in vivo*, we used primary hippocampal neurons in culture and

modulated different kinase activities using specific inhibitors. We compared RT-97 IR by quantitative Western blot analysis in untreated hippocampal primary neurons in culture relative to those treated with the ERK 1,2 inhibitor U-0126 (10 µM), a cell permeable inhibitor of MEK, or with the cell permeable cdk5 inhibitor roscovitine (10 μ M), or with both inhibitors. Unexpectedly, U-0126 treatment, which significantly reduced pERK 1,2 levels (Fig. 5a and d), greatly increased RT-97 IR over control levels (Fig. 5a and c). This response was explained, however, by a concomitant increase in the levels of active JNK2 (Fig. 5a and e), a downstream target of phospho MEK1,2 (Adler et al. 2005), levels of which also rose after this treatment (Fig. 5f). and could reflect activation of a Raf or other MEKK that would not be inhibited by U-0126. Supporting this conclusion, roscovitine addition to U-0126 treated cells blocked JNK2 activation (Fig. 5a and e) and reduced RT-97 IR to close to normal baseline levels. Roscovitine treatment alone did not alter ERK or JNK phosphorylation and only modestly lowered RT-97 IR (Fig. 5a and c).

Confirming these Western blot studies, immunofluorescence labeling analyses of hippocampal neurons showed



Fig. 7 RT-97 immuno-reactivity and phospho-MEK 1,2 levels are augmented in hippocampal neurons treated with U-0126. Hippocampal neurons in culture following treatment with the MEK1,2 inhibitor, U-0126 (10 μ M) (b) for 72 h fixed in 4% paraformaldehyde along with

untreated control neurons (a) and immunostained using a polyclonal antibody raised p-MEK 1,2 and a monoclonal antibody RT-97 that recognizes the phosphorylated tail domain of NF-H.



Fig. 8 RT-97 immuno-reactivity is reduced in hippocampal neurons when both ERK 1,2 and JNKs are inhibited. The hippocampal neurons in culture following treatment with, the Mek1,2 inhibitor, U-0126 (10 μ M) together with JNK inhibitor SP 600125 (10 μ M) for 72 h (d,e), fixed in 4% paraformaldehyde along with untreated control neurons

(a,b) and immunostained using a polyclonal antibody raised against NF-L and a monoclonal antibody RT-97 that recognizes the phosphorylated tail domain of NF-H. Overlays are shown in (c) and (f) (scale bar 50 $\mu m).$

that U-0126 treatment decreased pERK 1,2 immunoreactivity (Fig. 6b) yet greatly increased RT-97 staining (Figs 6j and 7), which was accompanied by increased pJNK IR (Fig. 6f) and p MEK1,2 IR (Fig. 7). Roscovitine partially reversed the U-0126-induced increase in RT-97 labeling (Fig. 6k) and pJNK IR whereas, by itself, it only marginally reduced RT-97 IR compared to control. Similarly, SP600125, an inhibitor of JNKs (Bennett *et al.* 2001), markedly reduced RT-97 staining of processes from U-0126 – treated neurons but only modestly lowered RT-97 IR when administered alone. These data established the importance of both ERK 1,2 and JNKs in regulating the RT-97 epitope in neurons (Fig. 8d and e).

Importance of ERK-mediated phosphorylation of neurofilament tail domains during early postnatal brain development and in gene knock-out models

To investigate the functional role of specific protein kinases in RT-97 generation during brain development, western blot analyses were performed using whole homogenates of mouse spinal cord from wild-type mice at postnatal days 1, 3, 5, 7, and 15. These analyses revealed that RT-97 IR increases with maturation and strongly correlated with the rise in immunoreactivities of both pERK 1,2 and ERK 1,2 (Pearson r value of 0.99 and 0.95 respectively) (Fig. 9a and b). By contrast, the immunoreactivities of JNK 1,2 (r = 0.78), pJNK 1,2 (r = 0.24) and cdk5 (r = 0.11), did not show a significant correlation with RT-97 IR during this period of development (Fig. 9a and c).

Given that cdk5 inhibition only modestly lowered RT-97 IR levels in neurons, we analyzed its possible role further in vivo by investigating mice lacking P35, the protein activator of cdk5. The cdk5 activity of brain and spinal cord from P35 knockout mice (P35 KO) was 10-20% of wild type mice, suggesting that P35 is the major activator of cdk5 in these tissues (Ohshima et al. 2001). Despite markedly reduced cdk5 activity in these mice, phosphorylation at the RT-97 epitope was actually increased by 40% (p < 0.05) (Fig. 9d and e) in parallel to elevations in the levels of ERK 1,2 (Fig. 9d and e). JNK 46/54 levels remained unaltered in P35 KO mice (data not shown). The inactive form of GSK3- β , which is phosphorylated on serine -9, was elevated in the spinal cord of P35 KO mice (Fig. 9f and g) suggesting that its role in the regulation of phosphorylation at RT-97 epitope under these conditions was minimal. In cdk5 knockout mice, RT-97 IR was not significantly different from control levels (Fig. 9h) despite the total absence of cdk5. Active ERK1, 2 and JNK1 were present at normal levels that are sufficient to account for the normal levels of RT-97 IR seen in these mice. Interestingly we observed only active JNK p 46 although inactive isoforms of both p46 and p54 are present (Fig. 9h).



Fig. 9 Analysis of immunoreactivities of RT-97 and kinases in spinal cord of developing mice, p35 KO mice, and brains from cdk5 knockout mice: (a) *RT-97, ERK1,2 and pERK 1,2 immuno-reactivities rise in parallel during early postnatal development of mouse spinal cord:* Western blot analysis was carried out on whole homogenates of four sets of spinal cord from mice at postnatal days 1, 3, 5 and 15 using RT-97 monoclonal antibody and polyclonal antibodies to the neurofilament kinases, phospho and total ERK 1,2, JNK 1,2 and cdk5. Calnexin was measured to confirm equal protein loading. Blots were developed using an HRP based chemiluminescence (ECL kit from Amersham) (b and c): *Graphic representation of RT-97 and kinase immunoreactivities:* The films were scanned using HP Scan Jet 4070 and band densities were measured using NIH image J 1.37 V software, and analyzed using Graph Pad Prism-4 software. (d–h) *RT-97 IR is normal in mice lacking cdk5 and enhanced in P35 knock out mice:* (d) Western blot

Discussion

The appearance of the RT-97 phosphoepitope on NF-H and NF-M represents a functionally critical phosphorylation event that marks a late stage in the maturation of axons *in vivo* (Sanchez *et al.* 2000). This stage of maturation is associated with prolonged detachment of neurofilaments from the axonal transport machinery and marked expansion of axonal caliber in response to signals from myelinating glial cells (Sanchez *et al.* 2000). Whether the RT-97 phosphoepitope directly mediates these effects on neurofilament dynamic behaviors or plays a role after these events have occurred is not yet established. Here, we have established the molecular determinants of RT-97 epitope

analysis was carried out on whole homogenates of three sets of spinal cord from p-35 knock out and control mice using monoclonal antibodies to phospho-neurofilament H and polyclonal antibodies to neurofilament kinases ERK 1,2 and cdk5/p-35. Blots were developed using an HRP based chemiluminescence (ECL kit from Amersham) (e) The band densities presented in the bar graph were measured using NIH Image J 1.3 V software after scanning the films using HP Scan. Jet 4070. (f) Western blot analysis of samples used in Fig. 9(d), using polyclonal antibodies to inactive GSK3- β (phosphorylated at serine 9) and total GSK3- β . (g) The band densities presented in the bar graph were measured using NIH Image J 1.3 V after scanning the films using HP Scanjet 4070. (h) Western blot analysis of whole homogenates of brain from four sets of E-16.5 cdk5 knock out and control mouse embryos, using polyclonal antibodies against cdk5, P-ERK 1,2, ERK 1,2, P-JNK 1,2 and JNK 1,2 (*p < 0.05).

and defined the roles of specific protein kinases in its regulation.

Mass spectrometric analysis of human NF-H peptides isolated by RT-97 immunoaffinity ultrafiltration after tryptic digestion have demonstrated, for the first time, that RT-97 epitopes are generated by phosphorylation at both KSPXK and KSPXXXK motifs *in vivo*, extending the previous observation that tail domains of neurofilament subunits are phosphorylated *in vivo* (Elhanany *et al.* 1994; Pant and Veeranna 1995; Jaffe *et al.* 1998a,b). Our studies establish that flanking lysines at both the N and C-terminus are essential determinants for RT-97 generation, extending evidence that KSP motif phosphorylation generates RT-97 IR (Lee *et al.* 1988; Coleman and Anderton 1990). We also



Fig. 10 Schematic representation of cross-talk among kinases resulting in NF-H phosphorylation at RT-97 epitopes.

observed that positioning of the C-terminal lysine, which determined the relative specificity of cdk5 and ERK 1,2 for NF-H and NF-M, as previously shown (Shetty *et al.* 1993), influences the affinity of the RT-97 antibody for the phosphorylated KSP motif. The lower RT-97 IR exhibited by synthetic KSPXXXK phospho-peptide relative to that by KSPXK phospho-peptide parallels the immunogenicity of these two peptides and suggests the importance of peptide structure or conformation. The predicted structures of these peptides revealed clear differences providing a basis for the different antibody affinities.

RT-97 IR was generated on bacterially expressed NF-H and fusion proteins derived from the NF-H tail after phosphorylation on KSPXK motifs by cdk5, and phosphorylation on either KSPXP or KSPXXXK motifs by ERK 1,2. These results are consistent with the established consensus sequences for these kinases (Kemp and Pearson 1991; Kennelly and Krebs 1991). Selective inhibition of these kinases in hippocampal neurons in culture in our studies revealed that levels of RT-97 IR are regulated by extensive cross-talk among kinase pathways (Cowan and Storey 2003). U-0126, a specific inhibitor of MEK 1,2-mediated ERK phosphorylation, leads to an increase MEK1,2 phosphorylation by mechanisms that are not fully understood, as previously reported (Favata et al. 1998). Despite U-0126 suppression of ERK 1,2 activation (Veeranna et al. 2004), RT-97 generation was increased due to JNK2 activation. JNKs are known to phosphorylate KSP repeats (Giasson and Mushynski 1997) and in the presence of U-0126, MEK 1,2 has been reported to activate JNK2 in Xenopus oocytes (Adler et al. 2005). We observed a similar increase in RT-97 immunoreactivity after treating the mouse brain slices in culture with 10 μ M U-0126 (unpublished observation). Although MEK activation of JNK2 is the likely basis for the increased RT-97 IR after U-0126, we cannot exclude the possibility that stress induced by the inhibitor activates another upstream kinase or inactivates a phosphatase acting on the RT-97 epitope. Consistent with its role as a stress-activated protein kinase, JNK had relatively little activity in generating RT-97 IR in untreated cells but was activated in response to ERK inhibitor (SP600125) or by inhibiting cdk5 (Havlícek *et al.* 1997). Combined ERK 1,2 and JNK 2 inhibition markedly lowered RT-97 levels, nearly completely eliminating its presence in neurites and underscoring the importance of these two kinases as generators of RT-97 IR.

Previous studies have shown that overexpression of cdk5 and NF-H in non-neural cells generates RT-97 on the NF-H tail domain (Ackerley et al. 2000). We confirmed that cdk5 generates RT-97 on the NF-H tail derived GST fusion protein containing KSPXK repeats in vitro, however, surprisingly, cdk5 is not required for maintaining normal levels of RT-97 in neurons as evidenced by the presence of normal levels of RT-97 IR in cdk5 knock out mice. RT-97 IR in these mice is generated by other established NF kinases, via active ERK 1,2 and active JNK1,2 (Giasson and Mushynski 1997; Veeranna et al. 1998, Brownless et al. 2000). It should be noted that the intensity of RT-97 IR in E-16.5 embryo brains is much lower compared to that seen in P35 KO mouse spinal cord consistent with its developmental regulation (Sanchez et al. 2000). Further, RT-97 IR levels were actually increased in P35 KO mice where cdk5 activity is reduced by 80-90% (Ohshima et al. 2001; Takahashi et al. 2003). In these mice, rises in RT-97 levels coincided with increased ERK 1,2

activation (Sharma et al. 2002; Hallows et al. 2003; Zeng et al. 2006) while JNK activity remained unaltered, providing further evidence for the role of ERK 1, 2 in RT-97 generation. The elevated levels of the serine-9 phosphorylated inactive isoform of GSK3- β in the spinal cord of P35 KO mice suggest that its role in regulating the phosphorylation at RT-97 epitope under these conditions is minimal. GSK3-ß might play a role in other circumstances in regulating the phosphorilation of RT-97 epitope (Guidato et al. 1996). Moreover, cdk5 influences GSK3-β activity by altering the activity of protein phosphatase-1 through phosphorylation of inhibitor 1 and 2. (Plattner et al. 2006; Huang and Paudel 2000; Agarwal-Mawal and Paudel 2001; Morfini et al. 2004). Although cdk5 was modestly or minimally involved in regulating RT-97 IR on mouse NF-H, it is possible that its role is more important in regulating human NF-H because the majority of KSPs are of the KSPXK type, which are targets of cdk5 phosphorylation (Shetty et al. 1993).

Taken together, our results demonstrate that multiple proline-directed kinases, which cross-talk extensively, regulate the NF-tail domain phosphorylation that generates the RT-97 phosphoepitope (Fig. 10). The strong correlation between selective rises in RT-97 IR and pERK 1,2 and ERK 1,2 immuno-reactivities during postnatal development of mouse spinal cord and in P35 KO mice underscore the importance of this kinase in regulating the RT-97 epitope in vivo. However, the lack of correlation between RT-97 IR and immuno-reactivities of cdk5, JNKs and pJNKs does not exclude their possible role under other conditions. The age dependent increase in levels of the RT-97 epitope is consistent with our earlier work on the SMI-31 epitope in developing rat spinal cord and cerebellum (Bush and Gordon-Weeks 1994; Veeranna et al. 1997). Furthermore, RT-97 immunoreactivity is greatly influenced by the presence and positioning of flanking lysines and the structure of the peptide. These studies provide a foundation for understanding how phosphorylation leading to RT-97 epitope generation influences neurofilament function.

Acknowledgements

The authors thank Ramaswamy Neelakantan for useful discussions, Corrinne Peterhoff for assistance with figures, and Nicole Piorkowski for assistance with manuscript preparation. This work was supported by NIA AG05604, NIA P01A02219 and NIH intramural program.

References

- Ackerley S., Grierson A. J., Brownlees J., Thornhill P., Anderton B. H., Leigh P. N., Shaw C. E. and Miller C. C. (2000) Glutamate slows axonal transport of neurofilaments in transfected neurons. J. Cell Biol. 150, 165–176.
- Ackerley S., Grierson A. J., Banner S. et al. (2004) p38alpha stressactivated protein kinase phosphorylates neurofilaments and is

associated with neurofilament pathology in amyotrophic lateral sclerosis. *Mol. Cell Neurosci.* **26**, 354–364.

- Adler V., Qu Y., Smith S. J. *et al.* (2005) Functional interactiosn of Raf and MEK with Jun-N-terminal kinase (JNK) result in a positive feedback loop on the oncogenic Ras signaling pathway. *Biochemistry* 44, 10784–10795.
- Agarwal-Mawal A. and Paudel H. K. (2001) Neuronal Cdc2-like protein kinase (Cdk5/p25) is associated with protein phosphatase 1 and phosphorylates inhibitor-2. J. Biol. Chem. 276, 23712–23718.
- Anderton B. H., Breinburg D., Downes M. J., Green P. J., Tomlinson B. E., Ulrich J., Wood J. N. and Kahn J. (1982) Monoclonal antibodies show that neurofibrillary tangles and neurofilaments share antigenic determinants. *Nature* 298, 84–86.
- Bajaj N. P. and Miller C. C. (1997) Phosphorylation of neurofilament heavy-chain side-arm fragments by cyclin-dependent kinase-5 and glycogen synthase kinase-3alpha in transfected cells. J. Neurochem. 69, 737–743.
- Bennett B. L., Sasaki D. T., Murray B. W. et al. (2001) SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc. Natl Acad. Sci. USA 98, 13681–13686.
- Brownlees J., Yates A., Bajaj N. P., Davis D., Anderton B. H., Leigh P. N., Shaw C. E. and Miller C. C. (2000) Phosphorylation of neurofilament heavy chain side-arms by stress activated protein kinase-1b/Jun N-terminal kinase-3. J. Cell Sci. 113, 401–407.
- Bush M. S. and Gordon-Weeks P. R. (1994) Distribution and expression of developmentally regulated phosphorylation epitopes on MAP 1B and neurofilament proteins in the developing rat spinal cord. *J. Neurocytol.* 23, 682–698.
- Carden M. J., Schlaepfer W. W. and Lee V. M. (1985) The structure, biochemical properties, and immunogenicity of neurofilament peripheral regions are determined by phosphorylation state. *J. Biol. Chem.* 260, 9805–9817.
- Coleman M. P. and Anderton B. H. (1990) Phosphate-dependent monoclonal antibodies to neurofilaments and Alzheimer neurofibrillary tangles recognize a synthetic phosphopeptide. J. Neurochem. 54, 1548–1555.
- Cowan K. J. and Storey K. B. (2003) Mitogen-activated protein kinases: new signaling pathways functioning in cellular responses to environmental stress. J. Exp. Biol. 206, 1107–1115.
- Dzandu J. K., Johnson J. F. and Wise G. E. (1988) Sodium dodecyl sulfate-gel electrophoresis: staining of polypeptides using heavy metal salts. *Anal. Biochem.* 174, 157–167.
- Elhanany E., Jaffe H., Link W. T., Sheeley D. M., Gainer H. and Pant H. C. (1994) Identification of endogenously phosphorylated KSP sites in the high-molecular-weight rat neurofilament protein. *J. Neurochem.* 63, 2324–2335.
- Favata M. F., Horiuchi K. Y., Manos E. J. *et al.* (1998) Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J. Biol. Chem.* 273, 18623–18632.
- Fuchs E. and Cleveland D. W. (1998) A structural scaffolding of intermediate filaments in health and disease. *Science* 279, 514–519.
- Gasser H. S. and Grundfest H. (1939) Axondiameters in relation to the spike dimensions and conduction velocity in mammalian A fibers. *Am. J. Physiol.* **127**, 393–414.
- Giasson B. I. and Mushynski W. E. (1997) Study of proline-directed protein kinases involved in phosphorylation of the heavy neurofilament subunit. J. Neurosci. 17, 9466–9472.
- Guidato S., Tsai L. H., Woodgett J. and Miller C. C. (1996) Differential cellular phosphorylation of neurofilament heavy side-arms by glycogen synthase kinase-3 and cyclin-dependent kinase-5. *J. Neurochem.* 66, 1698–1706.
- Hallows J. L., Chen K., DePinho R. A. and Vincent I. (2003) Decreased cyclin-dependent kinase 5 (cdk5) activity is accompanied by redistribution of cdk5 and cytoskeletal proteins and increased

cytoskeletal protein phosphorylation in p35 null mice. *J. Neurosci.* **23**, 10633–10644.

- Havlícek L., Hanus J., Veselý J., Leclerc S., Meijer L., Shaw G. and Strnad M. (1997) Cytokinin-derived cyclin-dependent kinase inhibitors: synthesis and cdc2 inhibitory activity of olomoucine and related compounds. J. Med. Chem. 40, 408–412.
- Hisanaga S., Kusubata M., Okumura E. and Kishimoto T. (1991) Phosphorylation of neurofilament H subunit at the tail domain by CDC2 kinase dissociates the association to microtubules. *J. Biol. Chem.* 266, 21798–21803.
- Huang K. X. and Paudel H. K. (2000) Ser67-phosphorylated inhibitor 1 is a potent protein phosphatase 1 inhibitor. *Proc. Natl Acad. Sci.* USA 97, 5824–5829.
- Jaffe H., Veeranna and Pant H. C. (1998a) Characterization of serine and threonine phosphorylation sites in beta-elimination/ethanethiol addition-modified proteins by electrospray tandem mass spectrometry and database searching. *Biochemistry* **37**, 16211–16224.
- Jaffe H., Veeranna, Shetty K. T. and Pant H. C. (1998b) Characterization of the phosphorylation sites of human high molecular weight neurofilament protein by electrospray ionization tandem mass spectrometry and database searching. *Biochemistry* 37, 3931–3940.
- Keil-Dlouha V. V., Zylber N., Tong N. and Keil B. (1971) Cleavage of glucagon by alpha- and beta-trypsin. *FEBS Lett.* 16, 287–290.
- Kemp B. E. and Pearson R. B. (1991) Design and use of peptide substrates for protein kinases. *Methods Enzymol.* 200, 121–134.
- Kennelly P. J. and Krebs E. G. (1991) Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. J. Biol. Chem. 266, 15555–15558.
- Kesavapany S., Patel V., Zheng Y. L. et al. (2007) Inhibition of Pin1 reduces glutamate-induced perikaryal accumulation of phosphorylated neurofilament-H in neurons. *Mol. Cell. Biol.* 18, 3645–3655.
- Lawson S. N., Harper A. A., Harper E. I., Garson J. A. and Anderton B. H. (1984) A monoclonal antibody against neurofilament protein specifically labels a subpopulation of rat sensory neurones. *J. Comp. Neurol.* 228, 263–272.
- Lee M. K. and Cleveland D. W. (1996) Neuronal intermediate filaments. Annu. Rev. Neurosci. 19, 187–217.
- Lee V. M., Otvos L. J., Schmidt M. L. and Trojanowski J. Q. (1988) Alzheimer disease tangles share immunological similarities with multiphosphorylation repeats in the two large neurofilament proteins. *Proc. Natl Acad. Sci. USA* **85**, 7384–7388.
- Miyasaka H., Okabe S., Ishiguro K., Uchida T. and Hirokawa N. (1993) Interaction of the tail domain of high molecular weight subunits of neurofilaments with the COOH-terminal region of tubulin and its regulation by tau protein kinase II. J. Biol. Chem. 268, 22695– 22702.
- Morfini G., Szebenyi G., Brown H., Pant H. C., Pigino G., DeBoer S., Beffert U. and Brady S. T. (2004) A novel CDK5-dependent pathway for regulating GSK3 activity and kinesin-driven motility in neurons. *EMBO J.* 23, 2235–2245.
- Nixon R. A. and Lewis S. E. (1986) Differential turnover of phosphate groups on neurofilament subunits in mammalian neurons *in vivo*. *J. Biol. Chem.* 261, 16298–16301.
- Nixon R. A. and Logvinenko K. B. (1986) Multiple fates of newly synthesized neurofilament proteins: evidence for a stationary neurofilament network distributed nonuniformly along axons of retinal ganglion cell neurons. J. Cell Biol. 102, 647–659.
- Nixon R. A. and Sihag R. K. (1991) Neurofilament phosphorylation: a new look at regulation and function. *Trends Neurosci.* 14, 501–506.
- Nixon R. A., Quackenbush R. and Vitto A. (1986) Multiple calciumactivated neutral proteinases (CANP) in mouse retinal ganglion cell neurons: specificities for endogenous neuronal substrates

and comparison to purified brain CANP. J. Neurosci. 6, 1252-1263.

- Nixon R. A., Lewis S. E., Mercken M. and Sihag R. K. (1994) [32P]orthophosphate and [35S]methionine label separate pools of neurofilaments with markedly different axonal transport kinetics in mouse retinal ganglion cells *in vivo. Neurochem. Res.* **19**, 1445– 1453.
- Ohshima T., Ogawa M., Veeranna, Hirasawa M., Longenecker G., Ishiguro K., Pant H. C., Brady R. O., Kulkarni A. B. and Mikoshiba K. (2001) Synergistic contributions of cyclin-dependant kinase 5/p35 and Reelin/Dab1 to the positioning of cortical neurons in the developing mouse brain. *Proc. Natl Acad. Sci. USA* 98, 2764–2769.
- Olsen J. V., Ong S. E. and Mann M. (2004) Trypsin cleaves exclusively C-terminal to arginine and lysine residues. *Mol. Cell Proteomics* 3, 608–614.
- Pant H. C. (1988) Dephosphorylation of neurofilament proteins enhances their susceptibility to degradation by calpain. *Biochem. J.* 256, 665–668.
- Pant H. C. and Veeranna (1995) Neurofilament phosphorylation. *Bio-chem. Cell Biol.* 73, 575–592.
- Pant A. C., Veeranna, Pant H. C. and Amin N. (1997) Phosphorylation of human high molecular weight neurofilament protein (hNF-H) by neuronal cyclin-dependent kinase 5 (cdk5). *Brain Res.* 765, 259– 266.
- Pant H. C., Veeranna and Grant P. (2000) Regulation of axonal neurofilament phosphorylation. *Curr. Top. Cell. Regul.* **36**, 133–150.
- Plattner F., Angelo M. and Giese K. P. (2006) The roles of cyclindependent kinase 5 and glycogen synthase kinase 3 in tau hyperphosphorylation. J. Biol. Chem. 281, 25457–25465.
- Probst A., Anderton B. H., Ulrich J., Kohler R., Kahn J. and Heitz P. U. (1983) Pick's disease: an immunocytochemical study of neuronal changes. Monoclonal antibodies show that Pick bodies share antigenic determinants with neurofibrillary tangles and neurofilaments. *Acta Neuropathol.* **60**, 175–182.
- Roder H. M., Eden P. A. and Ingram V. M. (1993) Brain protein kinase PK40ERK converts TAU into a PHF-like form as found in Alzheimer's disease. *Biochem. Biophys. Res. Commun.* 193, 639–647.
- Sanchez I., Hassinger L., Paskevich P. A., Shine H. D. and Nixon R. A. (1996) Oligodendroglia regulate the regional expansion of axon caliber and local accumulation of neurofilaments during development independently of myelin formation. J. Neurosci. 16, 5095– 5105.
- Sanchez I., Hassinger L., Sihag R. K., Cleveland D. W., Mohan P. and Nixon R. A. (2000) Local control of neurofilament accumulation during radial growth of myelinating axons *in vivo*. Selective role of site-specific phosphorylation. *J. Cell Biol.* **151**, 1013–1024.
- Sasaki T., Taoka M., Ishiguro K., Uchida A., Saito T., Isobe T. and Hisanaga S. (2002) *In vivo* and *in vitro* phosphorylation at Ser-493 in the glutamate (E)-segment of neurofilament-H subunit by glycogen synthase kinase 3beta. *J. Biol. Chem.* 277, 36032–36039.
- Sharma P., Veeranna, Sharma M., Amin N. D., Sihag R. K., Grant P., Ahn N., Kulkarni A. B. and Pant H. C. (2002) Phosphorylation of MEK1 by cdk5/p35 down-regulates the mitogen-activated protein kinase pathway. J. Biol. Chem. 277, 528–534.
- Shetty K. T., Link W. T. and Pant H. C. (1993) cdc2-like kinase from rat spinal cord specifically phosphorylates KSPXK motifs in neurofilament proteins: isolation and characterization. *Proc. Natl Acad. Sci. USA* **90**, 6844–6848.
- Sihag R. K., Inagaki M., Yamaguchi T., Shea T. B. and Pant H. C. (2007) Role of phosphorylation on the structural dynamics and function of types III and IV intermediate filaments. *Exp. Cell Res.* 313, 2098– 2109.

- Starr R., Hall F. L. and Monteiro M. J. (1996) A cdc2-like kinase distinct from cdk5 is associated with neurofilaments. J. Cell Sci. 109, 1565–1573.
- Sternberger L. A. and Sternberger N. H. (1983) Monoclonal antibodies distinguish phosphorylated and nonphosphorylated forms of neurofilaments in situ. *Proc. Natl Acad. Sci. USA* 80, 6126– 6130.
- Sternberger N. H., Sternberger L. A. and Ulrich J. (1985) Aberrant neurofilament phosphorylation in Alzheimer disease. Proc. Natl Acad. Sci. USA 82, 4274–4276.
- Sun D., Leung C. L. and Liem R. K. H. (1996) Phosphorylation of the high molecular weight neurofilament protein (NF-H) by Cdk5 and p35. J. Biol. Chem. 271, 14245–14251.
- Takahashi M., Amin N., Grant P. and Pant H. C. (1995) P13suc1 associates with a cdc2-like kinase in a multimeric cytoskeletal complex in squid axoplasm. J. Neurosci. 15, 6222–6229.
- Takahashi S., Saito T., Hisanaga S., Pant H. C. and Kulkarni A. B. (2003) Tau phosphorylation by cyclin-dependent kinase 5/p39 during brain development reduces its affinity for microtubules. *J. Biol. Chem.* 278, 10506–10515.
- Ulrich J., Haugh M., Anderton B. H., Probst A., Lautenschlager C. and His B. (1987) Alzheimer dementia and Pick's disease: neurofibrillary tangles and Pick bodies are associated with identical phosphorylated neurofilament epitopes. *Acta Neuropathol.* **73**, 240–246.
- Veeranna, Shetty K. T., Link W. T., Jaffe H., Wang J. and Pant H. C. (1995) Neuronal cyclin-dependent kinase-5 phosphorylation sites in neurofilament protein (NF-H) are dephosphorylated by protein phosphatase 2A. J. Neurochem. 64, 2681–2690.
- Veeranna, Shetty K. T., Amin N., Grant P., Albers R. W. and Pant H. C. (1996) Inhibition of neuronal cyclin-dependent kinase-5 by

staurosporine and purine analogs is independent of activation by Munc-18. *Neurochem. Res.* 21, 629–636.

- Veeranna, Grant P. and Pant H. C. (1997) Expression of p67 (Munc-18), Cdk5, P-NFH and syntaxin during development of the rat cerebellum. *Dev. Neurosci.* 19, 172–183.
- Veeranna, Amin N. D., Ahn N. G., Jaffe H., Winters C. A., Grant P. and Pant H. C. (1998) Mitogen-activated protein kinases (ERK1,2) phosphorylate Lys-Ser-Pro (KSP) repeats in neurofilament proteins NF-H and NF-M. J. Neurosci. 18, 4008–4021.
- Veeranna, Kaji T., Boland B. *et al.* (2004) Calpain mediates calciuminduced activation of the ERK1,2 MAPK pathway and cytoskeletal phosphorylation in neurons: relevance to Alzheimer's disease. *Am. J. Pathol.* 165, 795–805.
- Wang F. I., Hinton D. R., Gilmore W., Trousdale M. D. and Fleming J. O. (1992) Sequential infection of glial cells by the murine hepatitis virus JHM strain (MHV-4) leads to a characteristic distribution of demyelination. *Lab. Invest.* 66, 744–754.
- Wieboldt R., Zweigenbaum J. and Henion J. (1997) Immunoaffinity ultrafiltration with ion spray HPLC/MS for screening small-molecule libraries. *Anal. Chem.* 69, 1683–1691.
- Xiao J. and Monteiro M. J. (1994) Identification and characterization of a novel (115 kDa) neurofilament-associated kinase. J. Neurosci. 14, 1820–1833.
- Yuan A., Rao M. V., Sasaki T. *et al.* (2006) Alpha-internexin is structurally and functionally associated with the neurofilament triplet proteins in the mature CNS. *J. Neurosci.* 26, 10006–10019.
- Zeng X., Overmeyer J. H. and Maltese W. A. (2006) Functional specificity of the mammalian Beclin-Vps34 PI 3-kinase complex in macroautophagy versus endocytosis and lysosomal enzyme trafficking. J. Cell Sci. 119, 259–270.