

Cdk5 regulates axonal transport and phosphorylation of neurofilaments in cultured neurons

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Summary

Phosphorylation has long been considered to regulate neurofilament (NF) interaction and axonal transport, and, in turn, to influence axonal stability and their maturation to large-caliber axons. Cdk5, a serine/threonine kinase homologous to the mitotic cyclin-dependent kinases, phosphorylates NF subunits in intact cells. In this study, we used two different haptenized NF subunits and manipulated cdk5 activity by microinjection, transfection and pharmacological inhibition to monitor the effect of Cdk5-p35 on NF dynamics and transport. We demonstrate that overexpression of cdk5 increases NF phosphorylation and inhibits NF axonal transport, whereas inhibition both

reduces NF phosphorylation and enhances NF axonal transport in cultured chicken dorsal-root-ganglion neurons. Large phosphorylated-NF 'bundles' were prominent in perikarya following cdk5 overexpression. These findings suggest that Cdk5-p35 activity regulates normal NF distribution and that overexpression of Cdk5-p35 induces perikaryal accumulation of phosphorylated-NFs similar to those observed under pathological conditions.

Key words: cdk5, p35, Neurofilaments, Axonal transport, Phosphorylation, Neurofibrillary pathology

Introduction

Mammalian neurofilaments (NFs), a major constituent of the axonal cytoskeleton, are 10 nm filaments composed of three polypeptide subunits, termed NF-H, NF-M and NF-L (for high, medium and low with respect to their molecular mass). NF subunits are among the most highly phosphorylated proteins in the nervous system. The C-terminal regions, or 'sidearms', of the subunits differ markedly from each other. Extensive phosphorylation of NF-H and NF-M C-terminal sidearms (which extend away from the filament backbone) initiates in cell bodies and continues during axonal transport, resulting in segregation of extensively phosphorylated NFs in axons, whereas hypophosphorylated NFs are largely confined to perikarya (reviewed in Pant and Veeranna, 1995; Julien and Mushynski, 1998). Phosphorylation has long been considered to regulate several aspects of NF dynamics, including rendering subunits more resistant to proteolysis, increasing NF spacing and therefore axonal caliber (Pant and Veeranna, 1995; Hirokawa et al., 1994; Nixon et al., 1994; Sanchez et al., 2000), dissociating NFs from the anterograde motor kinesin (Yabe et al., 2000), reducing the axonal transport rate (Jung et al., 2000; Jung and Shea, 1999; Lewis and Nixon, 1998; Zhu et al., 1998), and promoting NF-NF interactions leading to bundling within axons (Gou et al., 1998; Yabe et al., 2001a; Yabe et al., 2001b).

Kinases that phosphorylate the C-terminal regions of NF-H and/or NF-M include glycogen synthase kinase-3 α (GSK-3 α) and GSK-3 β , cyclin-dependent kinase5 (Cdk5), mitogen-

activated protein (MAP) kinase, casein kinase I and II, and stress-activated protein (SAP) kinases. These kinases phosphorylate NFs at distinct sites, leaving open the possibility that they mediate distinct aspects of NF dynamics (Pant and Veeranna, 1995; Julien and Mushynski, 1998).

Cdk5 is a serine/threonine kinase with close homology to the mitotic cyclin-dependent kinases (Lew et al., 1992; Meyerson et al., 1992). It plays a crucial role in brain development, neurite outgrowth, synaptic activity and neuronal migration (Gilmore et al., 1998; Li et al., 2000; Nikolic et al., 1996; Oshima et al., 1996), and regulates organelle distribution, including anterograde axonal transport (Paglini et al., 2001; Ratner et al., 1998; Smith and Tsai, 2002). Cdk5 is activated by binding the neuron-specific noncyclin molecule p35 (Dhavan and Tsai, 2001; Lee et al., 2000; Ko et al., 2001). Deregulation of Cdk5 activity by proteolytic conversion of p35 to p25 and the resultant overactivation of Cdk5 have been implicated in neurodegenerative diseases (Ahlijanian et al., 2000; Lee et al., 2000; Patrick et al., 1999; Patzke and Tsai, 2002; Smith and Tsai, 2002). Cdk5 phosphorylates the NF-H subunit (Bajaj et al., 1999; Grant et al., 2001; Lee et al., 2000; Pant et al., 1999; Sharma et al., 1999) and, in doing so, generates epitopes like those that accumulate in affected motor neurons in amyotrophic lateral sclerosis (Bajaj et al., 1999). These findings leave open the possibility that Cdk5 activity is involved in the normal distribution of NFs and the abnormal accumulation of phosphorylated-NFs (phospho-NFs) in perikarya under pathological conditions.

Materials and Methods

Establishment and maintenance of DRG neuronal cultures

Dorsal root ganglion (DRG) neurons were cultured from embryonic-day-12 chicken in Ham's F12 medium containing 10% fetal calf serum and 25 ng ml⁻¹ nerve growth factor (NGF) (Jung et al., 1998; Straube-West, 1996) and used within 3-5 days after culturing.

Purification of Cdk5 and p35, and cell-free phosphorylation of cytoskeletons

Cdk5 and its activator p35 were purified as described (Veeranna et al., 1995). To confirm Cdk5 activity, four freshly harvested embryonic chick brains were homogenized in 1% Triton X-100 in 50 mM Tris-HCl (pH 6.8) containing 5 mM EDTA, 1 mM PMSF and 50 µg ml⁻¹ leupeptin and Triton-X-100-insoluble cytoskeletons were sedimented by centrifugation at 15,000 g for 15 minutes at 4°C. Resuspended cytoskeletons (50 µg) were incubated for 30 minutes with or without Cdk5-p35 (20 µg each) and 0.1 mM [³²P]orthophosphate (Shea et al., 1990), then subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and autoradiography. Additional brain samples (200 µg) from adult murine brain were incubated as above with or without Cdk5 and p35 (25 µg each) without radiolabel and subjected to immunoblot analyses with either a phosphorylation-dependent monoclonal antibody against NF-H (RT97; 1:500 dilution) or a phosphorylation-independent antibody (R39; 1:1000 dilution) that reacts with all NF subunits (Jung et al., 1998).

Microinjection and transfection

Purified biotinylated NF-L subunits ('biotin-L'), prepared as described previously (Jung et al., 1998), were mixed 1:2 with 70 kDa fluorescein-conjugated dextran (Molecular Probes) to locate injected cells before microinjection of ~1 mg ml⁻¹ NF protein as described (Jung et al., 1998; Straube-West, 1996). For Cdk5 co-injection, one third of the injection buffer was replaced with 1 mg ml⁻¹ purified Cdk5 and 1 mg ml⁻¹ p35. Cells not injected with Cdk5-p35 received an equal volume of buffer instead so that all injected cells had identical levels of biotin-L and tracer. Injected cells were located under fluorescein optics and examined by phase-contrast microscopy; cells exhibiting any obvious trauma resulting from microinjection were excluded from further analyses. Biotinylated NF subunits are appropriate reporters to analyse the distribution of transporting NF subunits as used here, because they: (1) undergo transport into axonal neurites at a rate consistent with slow axonal transport; (2) are recovered sequentially from all NF subunit populations throughout perikarya and axonal neurites; and (3) ultimately localize with endogenous NF subunits in Triton-X-100-insoluble axonal NFs (Jung et al., 1998; Yabe et al., 2001a; Yabe et al., 2001b). Moreover, translocation of microinjected subunits into DRG axons is prevented by the antimicrotubule drug nocodazole (Jung et al., 1998), confirming that microinjected subunits undergo active transport and that no significant contribution to the transport of biotinylated subunits into neurites is derived from subunit diffusion and/or injection pressure. Microinjected neurons were localized with the fluorescent tracer.

DRG neurons were transfected with a construct GFP-M, encoding rat NF-M fused with green fluorescent protein (GFP) (Yabe et al., 2001a; Yabe et al., 2001b); additional neurons were co-transfected with a mixture of GFP-M and constructs encoding Cdk5 and p35. Transfection was carried out using lipofectamine according to the manufacturer's instructions. Briefly, DNA constructs (2 µg) were resuspended in 100 µl serum-free Dulbecco's modified Eagle's medium (DMEM), then combined with 8 µl lipofectamine that had been resuspended in 100 µl serum-free DMEM and allowed to equilibrate for 45 minutes at room temperature (rt). Following equilibration, 800 µl of serum-free DMEM was added, and this

mixture was then added to cultures. After incubating cultures for 3.5 hours, an additional 1 ml of DMEM containing 10% serum was added (without removing the DNA:lipofectamine mixture) and incubation continued for 24 hours, after which the medium was replaced with 2 ml fresh DMEM containing 10% serum. Cultures were first viewed at this time, because previous studies (Yabe et al., 2001a; Yabe et al., 2001b) demonstrated that 18-24 hours were required for transfected cells to accumulate sufficient GFP-M for visualization. Successfully transfected cells were located by GFP fluorescence. For co-transfection, 2 µl each of constructs encoding GFP-M, Cdk5 and p35 were combined in the above initial mixture.

The assembly competence of biotin-L and GFP-NF-M have been reported (Jung et al., 1998; Yabe et al., 2001a; Yabe et al., 2001b). To confirm the assembly competence of biotin-L and GFP-NF-M in the present study, additional cultures were extracted before fixation as described. Briefly, cultures were rinsed with PBS (pH 7.4), then extracted with 1% Triton X-100 in 50 mM Tris-HCl (pH 6.8) containing 5mM EDTA, 1 mM PMSF and 50 µg ml⁻¹ leupeptin for 15 minutes before fixation with 4% paraformaldehyde as described (Jung et al., 1998). To provide sufficient signal for immunoblot analyses, GFP-NF-M was also expressed in NB2a/d1 neuroblastoma cells as described (Yabe et al., 2001a,b).

Transfected neurons received roscovitine, a specific inhibitor of Cdk5 (Meijer et al., 1997), for the final 2 hours of incubation; microinjected neurons received roscovitine immediately following injection.

Immunofluorescence and immuno-EM

Neurons were fixed and processed for biotin immunoreactivity with a rabbit polyclonal anti-biotin antibody (1:100 dilution in Tris-buffered saline), monoclonal antibodies (Sternberger Monoclonals, Bethesda, MD) directed against phosphorylated epitopes (SMI-31) and nonphospho-epitopes (SMI-32) shared by NF-H and NF-M, a monoclonal antibody (RT97) directed against developmentally delayed phosphorylated epitopes of NF-H (Anderton et al., 1982) or a monoclonal anti-NF-H antibody (generous gift of Virginia Lee University of Pennsylvania, PA, USA), followed by either goat anti-rabbit or goat anti-mouse secondary antibody conjugated to Texas Red at a 1:150 dilution (Sigma). Microinjected neurons were fixed 2 hours after injection. Transfected neurons were fixed 24 hours after transfection (see above). Cultures were fixed without extraction for densitometric analyses. In experiments designed to monitor the nature and extent of incorporation of exogenous NFs into filamentous profiles, cultures were extracted with Triton X-100 as described (Yabe et al., 2001a; Yabe et al., 2001b) and/or under conditions that induce 'splaying' of individual NF profiles (Brown, 1997).

For immuno-electron microscopy (immuno-EM), cells were extracted with 1% Triton X-100 at 4°C 18 hours after injection, fixed in 4% glutaraldehyde at 4°C, rinsed, incubated (1 hour, rt) with 1:100 dilutions of anti-biotin, rinsed three times and then incubated with secondary antibodies conjugated to 5 nm or 10 nm colloidal gold particles. Samples were then dehydrated, embedded in resin, sectioned and stained with uranyl acetate by conventional methods (Yabe et al., 2001a; Yabe et al., 2001b).

Microscopic and densitometric analyses

Images of microinjected and transfected neurons were captured under rhodamine and fluorescein optics, respectively, along with phase-contrast optics, using a Photometrix CoolSnap digital camera mounted on an Olympus IMT-2 epifluorescent microscope. The distribution of biotin-L was also examined by immuno-EM in a Phillips 300 electron microscope and via immunofluorescence. Distribution of endogenous NF-H and NF-L was carried out by immunofluorescence using the above epifluorescence microscope or a Zeiss Laser Scan microscope equipped with a 488 nm argon laser

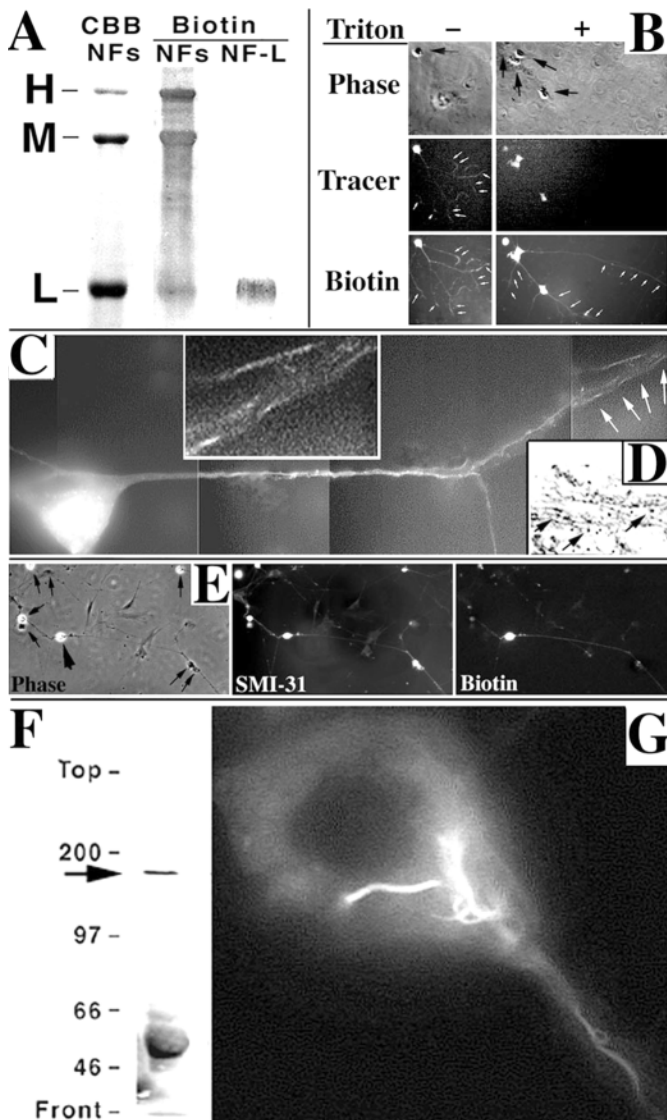


Fig. 1. Characterization of exogenous NF subunits and their distribution in DRG neurons. (A) Coomassie Blue staining (CBB) following SDS-gel electrophoresis of NFs isolated from bovine spinal cords, and immunoblot analysis of this preparation (NFs) following biotinylation and of chromatographically separated NF-L as indicated. (B) Representative DRG neuron 2 hours after microinjection with a mixture of biotin-L and fluorescein-conjugated tracer, followed by extraction with Triton X-100 and immunostaining with an anti-biotin antibody followed by a Texas-Red-conjugated secondary antibody. Notice the retention of biotin-L along axons (arrows), indicating its incorporation into Triton-X-100-insoluble structures. (C) A second microinjected neuron processed for anti-biotin immunoreactivity following extraction under conditions that induce splaying of NFs, more clearly revealing individual filamentous profiles. (D) Region of a DRG axon extracted with Triton X-100 and processed for immuno-EM (directed against biotin) 2 hours after injection. Notice the association of colloidal gold particles with filamentous profiles (arrows). (E) Representative DRG neurons (arrows), interspersed with non-neuronal cells, fixed and processed for SMI-31 immunoreactivity 3 days after plating. One neuron (large arrowhead) was microinjected 2 hours before fixation with biotin-L. Neurons in culture have expressed and transported endogenous NFs by this time, as shown by perikaryal and axonal SMI-31 immunoreactivity. The single microinjected neuron also displays prominent perikaryal and axonal biotin immunoreactivity. (F) Immunoblot probed with an antibody (SMI-32) against non-phosphorylated epitopes common to NF-H and NF-M, of material immunoprecipitated from a homogenate of a NB2a/d1 culture that had been transfected the previous day with GFP-M. Notice the appearance of an SMI-32-immunoreactive band of ~172 kDa (arrow), which corresponds to the expected migratory position of NF-M (~145 kDa) conjugated to GFP (~30 kDa) (Yabe et al., 2001a,b). (G) Perikaryon and proximal axonal neurite of a cell transfected with GFP-M the previous day. Notice the incorporation of GFP-M into filamentous profiles.

and a 543 nm helium-neon laser. Densitometric analyses of multiple neurons (10-50) from two or more experiments and of radiolabeled bands in autoradiographs was carried out with NIH Image. The percentage of biotin-L or GFP-M that had been transported into axons was calculated as $\{(\text{densitometric level in axon}) \div [(\text{densitometric level in axon}) + (\text{densitometric level in perikaryon})]\}$.

Technical considerations

Positive identification of microinjected neurons was achieved by including fluorescein-conjugated tracer in all reaction mixtures. Positive identification of transfected cultures was achieved by checking the appearance of fluorescence caused by expression of GFP-M. By contrast, when cultures were transfected with a combination of GFP-M, Cdk5 and p35, we did not attempt to confirm whether or not individual neurons were transfected with all three vectors. Rather, we scored all neurons expressing GFP-M in cultures that had been transfected with GFP-M alone and in cultures transfected with a mixture of GFP-M, Cdk5 and p35. It remains probable that some of the neurons, even though exposed to a mixture of all three vectors, were in fact not transfected with, or did not express, Cdk5 or p35. Therefore, although our transfection results are statistically significant, they probably caused us to underestimate the full effect of Cdk5 and p35 on NF axonal transport and organization.

However, because we only scored neurons expressing GFP-M, we nevertheless remain confident of our results.

We did not attempt to quantify any alterations induced by Cdk5-p35 in axonal phospho-NF immunoreactivity. Increased perikaryal phospho-NF immunoreactivity was obvious even by visual inspection, and this visual impression was supported densitometric analyses. However, the influence of Cdk5-p35 on axonal phospho-NF immunoreactivity was not obvious. We suspect that this is because Cdk5-p35 both decreases NF transport and increases NF phosphorylation, exerting opposing influences on levels of phospho-NFs with axons; i.e., a decrease in NF transport diminishes the total axonal NF content and therefore might also diminish phospho-NFs, whereas an increase of overall NF phosphorylation might increase axonal NF phosphorylation despite an overall decrease in total NF levels.

Because our intent was to achieve maximal Cdk5 activity, we always injected a combination of Cdk5 and p35, and transfected neurons with constructs expressing both Cdk5 and p35, and did not manipulate Cdk5 and p35 individually. Previous studies have demonstrated that overexpressing Cdk5 or p35 individually is effective but generates activities that lie between control values and those achieved by a simultaneous overexpression of both Cdk5 and p35³²⁻³⁵.

Finally, for simplicity, we refer to translocation of biotinylated and GFP-tagged subunits from perikarya into axons as 'axonal transport'. Although we have demonstrated that simple diffusion does not account for a detectable portion of any translocation of subunits into neurites (Jung et al., 1998), we nevertheless do not imply that all aspects of such translocation in our cultured neurons necessarily constitute bona fide axonal transport as would be observed in situ.

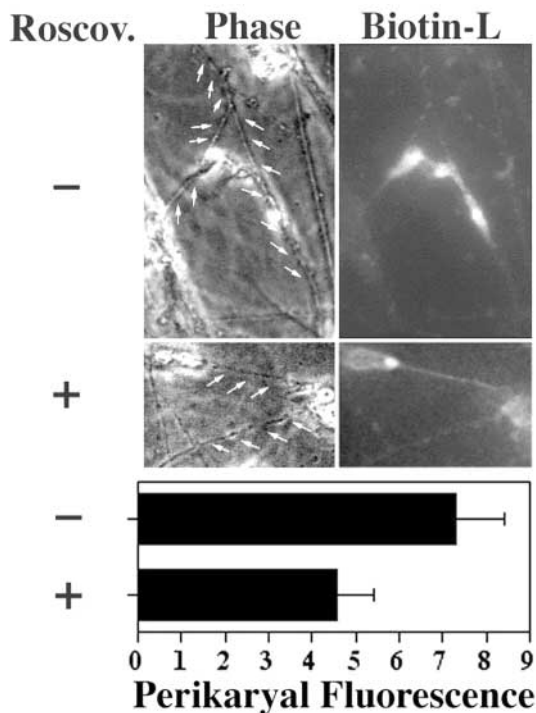


Fig. 2. Roscovitine increases the translocation of biotin-L into axons. DRG neurons were injected with a mixture of biotin-L and fluorescein-conjugated tracer. Following a 2 hour incubation, cells were fixed and processed for biotin immunoreactivity. Injected cells were located by fluorescein tracer and the distribution of biotin-L (arrows) was then monitored. Micrographs present images of representative neurons, arrows denote axons. The accompanying graph presents a densitometric analyses of the proportion of biotin-L (mean densitometric value \pm standard error of the mean) in perikarya from multiple neurons. Notice the marked decrease in levels of biotin-L in in perikarya of neurons treated with roscovitine, indicative of increased translocation into axons.

Results

We set out to determine whether the NF kinase Cdk5 regulates the distribution of NFs in neurons. To accomplish this, we injected DRG neurons with biotin-L, which allowed us to monitor the distribution of newly introduced NF subunits and to compare it with the steady-state NF population (Fig. 1), and subjected such cultures to up- and downregulation of Cdk5-p35. As in our previous studies (Jung et al., 1998; Yabe et al., 2001a), biotin-L assembled into Triton-X-100-insoluble filamentous structures throughout perikarya and axons within hours after injection, confirming the assembly competence of biotin-L (Fig. 1). To monitor whether or not Cdk5 regulates NF transport, cultures were treated with the Cdk5 inhibitor roscovitine following microinjection of biotin-L. The extent of biotin-L translocation was monitored as an index of NF transport, by comparing the ratio of biotin-L in axons to that in the respective perikarya. For these experiments, cultures were not extracted with Triton X-100 so that we could quantify total biotin-L, rather than Triton-X-100-insoluble biotin-L only. This was important because small individual NFs, including those undergoing axonal transport, can be extracted by Triton X-100 (not shown) and can therefore lead to an underestimation of the level of biotin-L in axons and/or

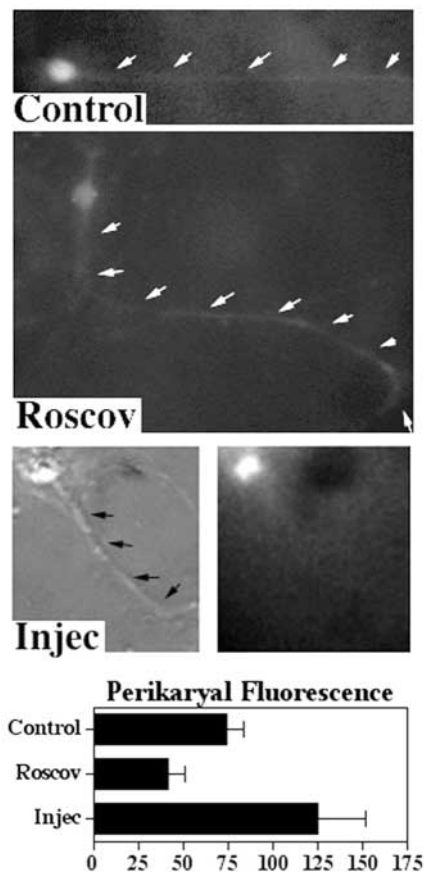


Fig. 3. Modulation of Cdk5-p35 activity regulates phospho-NF levels in neuronal perikarya. Micrographs present representative DRG neurons treated for 2 hours with 100 μ M roscovitine or microinjected with Cdk5-p35 (together with fluorescein-conjugated tracer) as indicated. Following a 2 hour incubation, cultures were fixed and processed for immunofluorescence with RT97 without extraction. Axons are indicated by arrows. The corresponding phase-contrast image is also presented for the microinjected neuron to facilitate visualization. The accompanying graph presents the mean (\pm standard deviation) relative perikaryal fluorescence for 10 to 50 cells. Notice the reduction in perikaryal RT97 immunoreactivity by roscovitine and the increase in RT97 immunoreactivity in cells injected with Cdk5-p35.

perikarya. Treatment with roscovitine increased the transport of biotin-L into axons, as evidenced by an increased ratio of biotin immunoreactivity in axons to that in perikarya in cultures treated with roscovitine (Fig. 2). Roscovitine also decreased phosphorylated NF epitopes in perikarya (Fig. 3) suggesting that, as shown in other cell types (Sharma et al., 1999), Cdk5 regulated NF phosphorylation in DRG neurons. To corroborate these data obtained with roscovitine and because it remains possible that roscovitine inhibits one or more additional kinases, we next upregulated Cdk5 activity by microinjecting a mixture of bacterially-expressed, purified Cdk5 and its activator p35, along with biotin-L. The activity of this Cdk5-p35 mixture was confirmed by its ability to carry out *in vitro* phosphorylation of dephosphorylated NF-H and NF-M subunits from chicken brain (Fig. 4). Microinjection of active Cdk5-p35 into cultured DRG neurons increased perikaryal NF phospho-epitopes (Fig. 3). Also, transport of

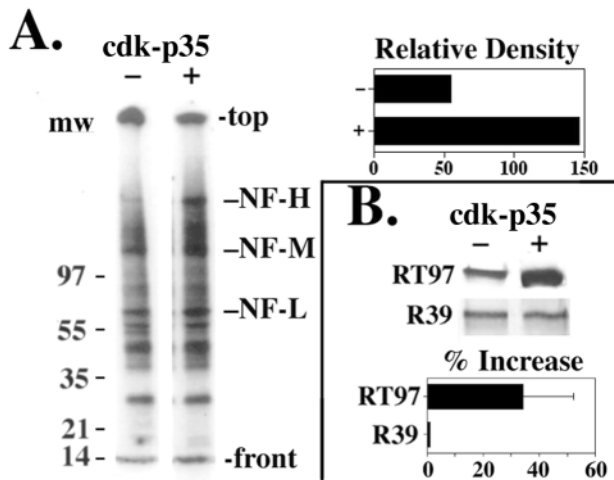


Fig. 4. Purified Cdk5-p35 phosphorylates NF subunits in cell-free analyses. (A) Autoradiographic analyses following SDS-gel electrophoresis of Triton-X-100-insoluble cytoskeletons from day 17 embryonic chicken brains incubated for 2 hours with [32 P]orthophosphate with or without Cdk5 and p35. Migratory position of NF-H, NF-M and NF-L are indicated. The accompanying graph presents the relative density of 200 kDa NF-H incubated for 2 hours with [32 P]orthophosphate with or without Cdk5 and p35. Notice the marked increase in radiolabel associated with NF subunits and, in particular, that associated with NF-H following incubation with Cdk5-p35. (B) Immunoblot analyses of 100 μ g Triton-X-100-insoluble cytoskeletons incubated for 2 hours at 30°C \pm 25 μ g Cdk5 and 25 μ g p35 probed with RT97 and R39 that reacts with all NF subunits regardless of phosphorylation state (Jung et al., 1998) as an index of total NF-H; only the 200 kDa region of immunoblots is presented. The accompanying graph shows the percentage increase in immunoreactivity for each antibody following incubation with Cdk5-p35. Notice the specific increase in phospho-NF (RT97) immunoreactivity.

biotin-L into axons was markedly reduced in neurons that had been injected with both Cdk5-p35 and biotin-L, as shown by retention of increased levels of biotin-L in perikarya (Fig. 5). These data demonstrate that Cdk5 activity regulates NF phosphorylation and axonal transport.

In a second approach to monitor the impact of Cdk5-p35 activity on NF dynamics, we tested the consequences of cdk/p35 overexpression on NF subunits that were expressed following transfection rather than microinjection. DRG neurons were transfected with a construct that expressed GFP-conjugated NF-M (GFP-M) and was also assembly competent in cultured neurons (Fig. 1) (Yabe et al., 2001a; Yabe et al., 2001b). Additional neurons were transfected with a mixture of

constructs expressing Cdk5 and p35, or with a mixture of GFP-M, Cdk5 and p35. Transfection with Cdk5-p35 alone increased NF phospho-epitopes of endogenous subunits in perikarya (Fig. 6). Comparison of total GFP-M levels in the axons and perikarya of transfected cells [as carried out for microinjected neurons (above)] revealed that transport of GFP-M into axons was markedly reduced in cultures that had been co-transfected with Cdk5-p35. Conversely, treatment with roscovitine enhanced transport of GFP-M into axons of neurons in cultures that had been transfected with GFP-M alone (Fig. 7).

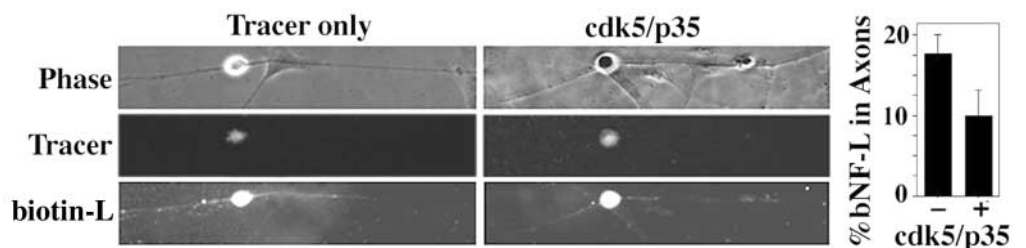
We next considered potential mechanisms by which increased Cdk5-p35 activity might inhibit the translocation of NF subunits into neurites. Because NF phosphorylation has been reported to promote NF-NF association (for a review, see Pant and Veeranna, 1995), we examined whether or not overexpression of Cdk5-p35 can promote aberrant NF-NF associations in DRG perikarya, a result that could in turn inhibit their transport (Shea and Yabe, 2000). The fate of endogenous and exogenous NF subunits in perikarya, before and after overexpression of Cdk5-p35, was therefore observed more closely. We extracted neurons with Triton X-100 to visualize filamentous profiles; high-magnification images obtained following Triton X-100 extraction revealed that both endogenous and exogenous NF subunits in perikarya of neurons overexpressing Cdk5-p35 were organized into relatively thick fibrous structures (Fig. 8). By contrast, NF subunits in perikarya of neurons that had not been transfected with Cdk5-p35 were diffuse, punctate or organized into fine filamentous profiles that exhibited a substantially thinner caliber than those in neurons that overexpressed Cdk5-p35 (Fig. 8). Immunostaining of extracted cells with RT97 revealed that these perikaryal NF bundles displayed extensively phosphorylated NF epitopes (Fig. 8), suggesting that overexpression of Cdk5-p35 induced precocious bundling of NFs in perikarya, which might interfere with their transport (Shea and Yabe, 2000).

Discussion

NFs are the most highly phosphorylated proteins in myelinated axons (Pant and Veeranna, 1995; Julien and Mushynski, 1998). The function(s) of NF phosphorylation and of the kinases responsible are not fully resolved. However, NF phosphorylation has classically been considered to regulate NF transport, perhaps by fostering increased NF-NF interactions and/or dissociating NFs from their transport vector (for reviews, see Grant and Pant, 2000; Julien and Mushynski, 1998; Shea and Yabe, 2000; Shea and Flanagan, 2001). Previous studies demonstrated that Cdk5 can phosphorylate NF

Fig. 5. Microinjection of Cdk5-p35 decreases translocation of biotin-L into axons. Micrographs present representative DRG neurons injected with a mixture of biotin-L and fluorescein-conjugated tracer with and without Cdk5-p35, then processed for biotin immunoreactivity 2 hours after

injection without extraction. The accompanying graph shows the densitometric analyses of the percentage of biotin-L in axons from multiple neurons. Notice the marked reduction in axonal transport of biotin-L in neurons that have been microinjected with Cdk5-p35.



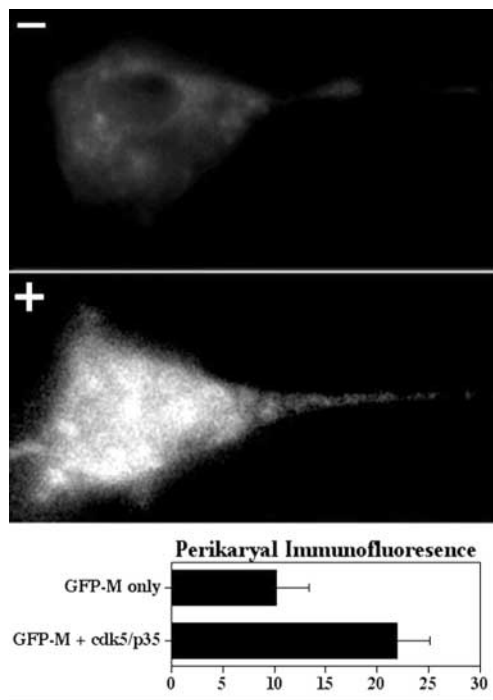


Fig. 6. Overexpression of Cdk5-p35 increases NF phospho-epitopes in perikarya. Panels present DRG neurons 24 hours after transfection with a construct expressing GFP-M in the absence (-) or presence (+) of constructs that express Cdk5 and p35, followed by immunofluorescence analyses with RT97 followed by Texas-Red-conjugated secondary antibody. Transfected cells were localized by GFP fluorescence (under fluorescein optics) and RT97 immunoreactivity was then imaged under rhodamine optics. The graph shows the mean percentage of RT97 immunoreactivity (\pm standard deviation, expressed in arbitrary densitometric units) that had translocated into axons. Notice that perikaryal RT97 immunoreactivity (the result of increased levels of phosphorylated NF-H) was significantly ($P < 0.05$) increased in response to the overexpression of Cdk5-p35.

subunits in human neuroblastoma cells (Sharma et al., 1999) and that Cdk5 regulates axonal transport of vesicular cargo (Paglini et al., 2001; Ratner et al., 1998). Here, we present data that indicate that Cdk5 directly or indirectly regulates NF transport in cultured neurons. A similar conclusion was reached following treatment of neurons with roscovitine, which increased the transport of NF-H sidearms, expressed following transfection, into axons of cultured cortical neurons (Ackerley et al., 2003). Treatment of DRG cultures with roscovitine also accelerated NF axonal transport, but NF axonal transport can also be decreased when increasing Cdk5-p35 activity by microinjecting or transfecting a mixture of Cdk5, p35 and biotin-L [an experiment not carried out in the studies of Ackerley et al. (Ackerley et al., 2003)].

Although it seems clear that alterations in Cdk5 activity change both NF C-terminal phosphorylation and NF-axonal transport, these changes might result from independent actions of Cdk5. The reduced NF axonal transport in response to overexpression of Cdk5-p35 and the enhanced NF transport following the inhibition of Cdk5 activity might be a result of changes in overall transport, caused by Cdk5-mediated phosphorylation of motor proteins or of linker proteins that

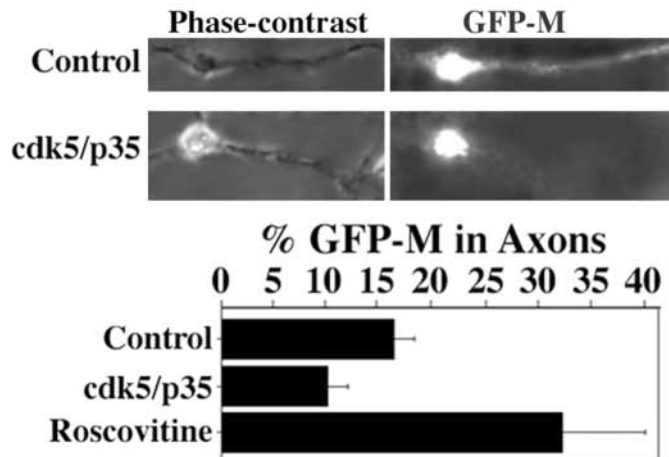


Fig. 7. Modulation of Cdk5-p35 activity regulates transport of GFP-M into axons. DRG neurons 24 hours after the transfection with a construct expressing GFP-M, in the absence (-) or presence (+) of constructs expressing Cdk5 and p35 are shown. The graph shows the mean percentage of GFP fluorescence (\pm standard error of the mean, arbitrary densitometric units) that had translocated into axons in cultures transfected with GFP-M alone, GFP-M plus Cdk5-p35 or GFP-M followed by 100 μ M roscovitine for the final 2 hours of incubation, before visualization. Notice that transport of GFP-NF-M into axons was significantly ($P < 0.05$) reduced in cultures transfected with Cdk5-p35 and significantly enhanced ($P < 0.05$) in cultures treated with roscovitine.

maintain association between the motor proteins and their cargo. A similar mechanism was suggested to contribute to the faster transport of NF-H-sidearm constructs that mimic the dephosphorylated state compared with NF-H-sidearm constructs that mimic the phosphorylated state (Ackerley et al., 2003). Previous studies demonstrated that hypophosphorylated subunits preferentially purified with a microtubule-motor preparation and that kinesin was preferentially associated with the front of the transporting wave of NF subunits (Yabe et al., 2000). Moreover, incubation of this motor-protein preparation with MAP kinase both increased the C-terminal phosphorylation of NF-H and selectively prevented newly phosphorylated subunits from further purifying with kinesin, whereas less phosphorylated subunits continued to do so (Yabe et al., 2000). As considered above for Cdk5, these phenomena could nevertheless be due to independent MAP-kinase-mediated phosphorylation events of NF-H and kinesin and/or linker protein(s). The combined and perhaps sequential actions of several kinases, including MAP kinase, Cdk5 and others, might mediate the C-terminal phosphorylation of NF and the dissociation of NFs from their transport vector in situ. In this regard, a second NF kinase, GSK-3B, regulates the association of cargo with kinesin (Paglini et al., 2001). Thus, although our findings implicate Cdk5 in the regulation of NF dynamics, these data cannot be extrapolated to indicate that Cdk5 is the major kinase regulating NF transport and phosphorylation. Such an interpretation would require comparative studies of the impact of up- and downregulation of all candidate NF kinases. In this regard, our data are derived from overexpression (by transfection and/or microinjection) or drastic inhibition (by roscovitine) of Cdk5-p35. Increased

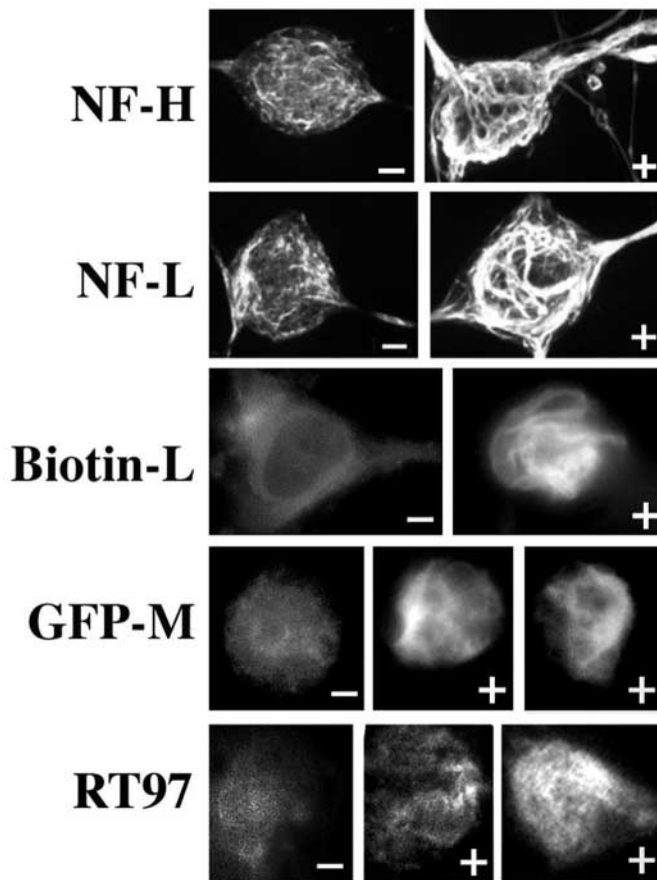


Fig. 8. Overexpression of Cdk5-p35 increases NF bundling in perikarya. DRG neurons before (–) and 24 hours after (+) transfection with constructs expressing Cdk5 and p35 are shown in all panels (first two panels have no +/- label). Neurons in panels 'NF-H' and 'NF-L' were immunostained with antibodies directed against these subunits to reveal the distribution of endogenous NFs with (right) and without (left) overexpressing Cdk5-p35. Panels labeled 'Biotin-L' and 'GFP-M' show representative perikarya of neurons 2 hours after microinjection of biotin-L with (+) or without (–) Cdk5-p35 (followed by immunostaining with anti-biotin) or 24 hours following transfection with a construct expressing GFP-M with (+) or without (–) Cdk5-p35. Notice that perikarya of neurons overexpressing Cdk5-p35 displayed thick filamentous profiles containing endogenous subunits, microinjected subunits and subunits expressed following transfection. By contrast, endogenous and exogenous subunits in perikarya of neurons not overexpressing Cdk5-p35 were diffuse, punctate or present as relatively fine filaments that exhibited a much smaller caliber than those of cells overexpressing Cdk5-p35. Panels labeled 'RT97' show representative neurons transfected with GFP-M with (+) or without (–) Cdk5-p35 then immunostained with RT97 to reveal the distribution of NF phospho-epitopes in perikarya. Notice the increase in perikaryal RT97 immunoreactivity in perikarya of neurons transfected with Cdk5-p35 and the association of RT97 with the resultant thick filamentous profiles, indicating that perikaryal NF bundles contain phospho-NFs.

Cdk5 activity is related to neurodegeneration (Ahlijanian et al., 2000; Lee et al., 2000; Patrick et al., 1999; Patzke and Tsai, 2002). It remains to be determined whether or not physiological levels of Cdk5 activity mediate phosphorylation-dependent NF-NF associations leading to NF bundling that

normally occur only in axons. A recent study reported that the deletion of the C-terminal sidearms of NF-H did not alter the axonal transport rate of NFs, leading the investigators to challenge the long-standing hypothesis that NF-H-sidearm phosphorylation regulates NF axonal transport (Rao et al., 2002). If this is indeed the case, the changes observed in translocation of NF subunits into axons following an alteration of Cdk5 activity as observed in the present study must be due to an effect other than NF-H sidearm phosphorylation. In contrast to the study of Rao et al. (Rao et al., 2002), however, other transgenic studies have supported a role for NF-H sidearms in NF transport. For example, deletion of the entire NF-H molecule accelerates NF transport, whereas increased NF-H expression retards NF transport (Collard et al., 1995; Marszalek et al., 1996; Zhu et al., 1998).

Moreover, in mice in which either the entire NF-H molecule or its sidearm has been deleted, NF-M sidearm phosphorylation undergoes a compensatory increase to the extent that it displays the phosphorylation-dependent developmentally delayed phospho-epitopes, which are usually only detected on NF-H (Rao et al., 2002; Sanchez et al., 2000; Tu et al., 1995; Zhu et al., 1998). Such compensatory changes in NF-M after modulation of NFH expression might affect NF transport following the deletion of NF-H and/or the NF-H sidearm. Finally, isolated NF-H sidearm constructs engineered to mimic the dephosphorylated state underwent faster transport in cortical neurons than did similar constructs engineered to mimic the phosphorylated state (Ackerley et al., 2003). This latter result is consistent with previous studies of the distribution of endogenous NFs that collectively support a role for NF phosphorylation in the regulation of axonal transport (Jung et al., 2000; Jung and Shea, 1999; Lewis and Nixon, 1998; Zhu et al., 1998 and refs. therein). Further studies will be necessary to resolve the discrepancy raised by the studies of Rao et al. (Rao et al., 2002) regarding the nature and extent to which phosphorylation of NF-H and NF-M sidearms regulate NF axonal transport (Shea et al., 2003).

A related, but yet unanswered question is why extensive NF phosphorylation is usually observed only in axons, despite the fact that NF subunits, and the necessary kinases, must be synthesized in perikarya (Pant and Veeranna, 1995). This segregation of extensively phosphorylated NF subunits in axons could be achieved by concentrating candidate kinases and/or requisite cofactors in axons rather than perikarya. However, *in situ* inhibition of phosphatase activity with okadaic acid (OA)-induced precocious accumulation of phospho-epitopes (shown by RT97 immunoreactivity) in retinas and proximal regions of axons – areas in which it is not normally detected – indicates that the responsible kinase(s) are also present and active in these regions (Jung and Shea, 1999). This finding is supported by our observation that the perikarya NF bundles that were induced following Cdk5-p35 overexpression displayed NF phospho-epitopes. OA treatment also increased NF phosphorylation and bundling in axonal neurites of NB2a/d1 cells, which are inversely correlated with axonal transport rate (Yabe et al., 2001a; Yabe et al., 2001b). Notably, OA inhibits protein phosphatase 2A (Bialojan et al., 1988; Ishihara et al., 1999), which dephosphorylates sites that have been phosphorylated by the NF C-terminal kinase Cdk5 (Sharma et al., 1999; Veeranna et al., 1995). Therefore, OA probably exerts at least a portion of its effects on NF

phosphorylation and axonal transport by sustaining Cdk5-mediated NF phosphorylation. In addition this, OA also sustains MAP kinase activity in cultured neurons (Shea and Dider, 1997). Because MAP kinase dissociates NFs from the anterograde motor protein kinesin (Yabe et al., 2000) and regulates transport of NFs in axonal neurites of differentiated neuroblastoma cells (Shea, 2001), OA could also impair NF transport by sustaining MAP kinase activity. Consistent with this line of reasoning, in situ treatment with the calcineurin inhibitor cyclosporin A fostered perikaryal phospho-NF accumulation (Tanaka et al., 2001). These and other findings (DeWaegh et al., 1992) indicate that NF transport is regulated by the interplay of kinases and phosphatases. The thick bundles of phospho-NFs observed in neuronal perikarya following the overexpression of Cdk5-p35 might be derived from precocious NF phosphorylation of the nature and extent that normally induces NF bundling in axons (Yabe et al., 2001a; Yabe et al., 2001b), perhaps resulting from precocious phosphorylation-mediated NF dissociation from their transport vector (Yabe and Shea, 2000). Consistent with the findings of the present study was an increase in NF immunoreactivity in apical dendrites of triple-transgenic mice, expressing Cdk5, p35 and human tau (Van den Haute et al., 2001). However, the extent to which Cdk5 activity contributed to NF displacement under these conditions is not fully resolved, because overexpression of human tau alone impairs NF axonal transport (Stamer et al., 2002). Assigning further functional roles to Cdk5 in mediating NF phosphorylation under normal and neurodegenerative conditions will contribute to an understanding of the regulation of NF axonal transport, axonal caliber axonal maturation and axonal degeneration.

References

- Ackerley, S., Thornhill, P., Grierson, A. J., Brownlee, J., Anderton, B. H., Leigh, P. N., Shaw, C. E. and Miller C. J. (2003). Neurofilament heavy chain side-arm phosphorylation regulates axonal transport of neurofilaments. *J. Cell Biol.* **161**, 489-495.
- Ahlijanian, M. K., Barrezueta, N. X., Williams, R. D., Jakowski, A., Kowsz, K. P., McCarthy, S., Coskran, T., Carlo, A., Seymour, P. A., Burkhardt, J. E., et al. (2000). Hyperphosphorylated tau and neurofilament and cytoskeletal disruptions in mice overexpressing human p25, an activator of cdk5. *Proc. Natl. Acad. Sci. USA* **97**, 2910-2915.
- 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. S., Al-Sarraj, S. T., Leigh, P. N., Anderson, V. and Miller, C. J. (1999). Cyclin dependent kinase 5 (cdk5) phosphorylates neurofilament heavy (NF-H) chain to generate epitopes for antibodies that label neurofilament accumulations in amyotrophic lateral sclerosis (ALS) and is present in affected motor neurons in ALS. *Neuro-Psychopharm. Biol. Psych.* **23**, 833-850.
- Bialojan, C., Ruegg, J. C. and Takai, A. (1988). Effects of okadaic acid on isometric tension and myosin phosphorylation of chemically skinned guinea-pig taenia coli. *J. Physiol.* **398**, 81-95.
- Brown, A. (1997). Visualization of single neurofilaments by immunofluorescence microscopy of splayed axonal cytoskeletons. *Cell Motil. Cytoskel.* **38**, 133-145.
- Collard, J.-F., Cote, F. and Julien, J.-P. (1995). Defective axonal transport in a transgenic mouse model of amyotrophic lateral sclerosis. *Nature* **375**, 61-64.
- Dhavan, R., and Tsai, L. H. (2001). A decade of cdk5. *Nat. Rev. Mol. Cell Biol.* **2**, 749-759.
- DeWaegh, S. M., Lee, V. M. and Brady, S. T. (1992). Local modulation of neurofilament phosphorylation, axonal caliber, and slow axonal transport by myelinating Schwann cells. *Cell* **68**, 451-463.
- Gilmore, E. C., Ohshima, T., Goffinet, A. M., Kulkarni, A. B. and Herrup, K. (1998). Cyclin-dependent kinase5-deficient mice demonstrate novel developmental arrest in cerebral cortex. *J. Neurosci.* **18**, 6370-6377.
- Gou, J. P., Gotow, T., Jamney, P. A. and Letierrier, J. F. (1998). Regulation of neurofilament interactions in vitro by natural and synthetic polypeptide sequences within the heavy neurofilament subunit NF-H: Neurofilament crossbridging by antiparallel sidearm overlapping. *Med. Biol. Eng. Comp.* **36**, 371-387.
- Grant, P. and Pant, H. C. (2000). Neurofilament protein synthesis and phosphorylation. *J. Neurocytol.* **29**, 843-872.
- Grant, P., Sharma, P. and Pant, H. C. (2001). Cyclin-dependent protein kinase 5 (Cdk5) and the regulation of neurofilament metabolism. *Eur. J. Biochem.* **268**, 1534-1546.
- Hirokawa, N., Glicksman, M. A. and Willard, M. B. (1984). Organization of mammalian neurofilament polypeptides within the neuronal cytoskeleton. *J. Cell Biol.* **98**, 1523-1536.
- Ishihara, T., Hong, M., Zhang, B., Nakagawa, Y., Lee, M. K., Trojanowski, J. Q. and Lee, V. M. (1999). Age-dependent emergence and progression of a tauopathy in transgenic mice overexpressing the shortest human tau isoform. *Neuron* **24**, 751-762.
- Julien, J.-P. and Mushynski, W. E. (1998). Neurofilaments in health and disease. *Prog. Nucleic Acid. Res. Mol. Biol.* **61**, 1-23.
- Jung, C. and Shea, T. B. (1999). Regulation of neurofilament axonal transport by phosphorylation in optic axons in situ. *Cell Motil. Cytoskel.* **43**, 230-240.
- Jung, C., Yabe, J., Wang, F.-S. and Shea, T. B. (1998). Neurofilaments are present within axonal neurites before incorporation into Triton-insoluble structures. *Cell Motil. Cytoskel.* **40**, 44-58.
- Jung, C., Yabe, J. T. and Shea, T. B. (2000). C-terminal phosphorylation of the heavy molecular weight neurofilament subunit is inversely correlated with neurofilament axonal transport velocity. *Brain Res.* **856**, 12-19.
- Lee, M. S., Rosales, J. L., Tang, D. and Wang, J. H. (2000). Neurotoxicity induces cleavage of p35 to p25 by calpain. *Nature* **405**, 360-364.
- Lewis, S. E. and Nixon, R. A. (1988). Multiple phosphorylated variants of the high molecular mass subunit of neurofilaments in axons of retinal cell neurons: Characterization and evidence for their differential association with stationary and moving neurofilaments. *J. Cell Biol.* **107**, 2689-2701.
- Lew, J., Winkfein, R. J., Paudel, H. K. and Wang, J. H. (1992). Brain proline-directed protein kinase is a neurofilament kinase which displays high sequence homology to p34^{cdc2}. *J. Biol. Chem.* **267**, 25922-25926.
- Li, B. S., Zhang, L., Gu, J., Amin, N. D. and Pant, H. C. (2000). Integrin alpha(1) beta(1)-mediated activation of cyclin-dependent kinase 5 activity is involved in neurite outgrowth and human neurofilament protein H Lys-Ser-Pro tail domain phosphorylation. *J. Neurosci.* **20**, 6055-6062.
- Ko, J., Humbert, S., Bronson, R. T., Takahashi, S., Kulkarni, A. B., Li, E., Tsai, L. H. (2001). p35 and p39 are essential for cdk5 function during neurodevelopment. *J. Neurosci.* **21**, 6758-6771.
- Marszalek, J. R., Williamson, T. L., Lee, M. K., Xu, Z. S., Hoffman, P. N., Becher, M. W., Crawford, T. O. and Cleveland, D. W. (1996). Neurofilament subunit NF-H modulates axonal diameter by selectively slowing neurofilament transport. *J. Cell Biol.* **135**, 711-724.
- Meijer, L., Borgne, A., Mulner, O., Chong, J. P., Blow, J. J., Inagaki, N., Inagaki, M., Delcros, J. G. and Moulinoux, J. P. (1997). Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2 and cdk5. *Eur. J. Biochem.* **243**, 527-536.
- Meyerson, M., Enders, G. H., Wu, C. L., Su, L. K., Gorka, C., Nelson, C., Harlow, E. and Tsai, L. H. (1992). A family of human cdc2-related protein kinases. *EMBO J.* **11**, 2909-2917.
- Nikolic, M., Dudek, H., Kwon, Y. T., Ramos, Y. F. and Tsai, L. H. (1996). The cdk5/p35 kinase is essential for neurite outgrowth during neuronal differentiation. *Genes Dev.* **10**, 816-825.
- Nixon, R. A., Paskevich, P. A., Sihag, R. K. and Thayer, C. Y. (1994). Phosphorylation on carboxyl terminus domains of neurofilament proteins in retinal ganglion cell neurons in vivo: influences on regional neurofilament accumulation, inter-filament spacing and axonal caliber. *J. Cell Biol.* **126**, 1031-1046.
- Ohshima, T., Ward, J. M., Huh, C. G., Longenecker, G., Veeranna, Pant, H. C., Brady, R. O., Martin, L. J. and Kulkarni, A. B. (1996). Targeted disruption of the cyclin-dependent kinase 5 gene results in abnormal corticogenesis, neuronal pathology and perinatal death. *Proc. Natl. Acad. Sci. USA* **93**, 11173-11178.
- Paglini, G., Peris, L., Diez-Guerra, J., Quiroga, S. and Caceres, A. (2001). The Cdk5-p35 kinase associates with the Golgi apparatus and regulates membrane traffic. *EMBO J.* **2**, 1139-1144.
- Pant, H. C. and Veeranna (1995). Neurofilament phosphorylation. *Biochem. Cell Biol.* **73**, 575-592.

- Pant, A. C., Veeranna, Pant, H. C. and Amin, N.** (1999). Phosphorylation of human high molecular weight neurofilament protein (hNF-H) by neuronal cyclin-dependent kinase 5 (cdk5). *Brain Res.* **765**, 259-266.
- Patrick, G. N., Zukerberg, L., Nikolic, M., de la Monte, S., Dikkes, P., Tsai, L. H.** (1999). Conversion of p35 to p25 deregulates cdk5 activity and promotes neurodegeneration. *Nature* **402**, 615-622.
- Patzke, H. and Tsai, L.-H.** (2002). Cdk5 sinks into ALS. *Trends Neurosci.* **25**, 8-10.
- Rao, M. V., Garcia, M. L., Miyazaki, Y., Gotow, T., Yuan, A., Mattina, S., Ward, C. M., Calcutt, N. A., Uchiyama, Y., Nixon, R. A. and Cleveland, D. W.** (2002). Gene replacement in mice reveals that the heavily phosphorylated tail of neurofilament heavy subunit does not affect axonal caliber or the transit of cargoes in slow axonal transport. *J. Cell Biol.* **158**, 681-693.
- Ratner, N., Bloom, G. S. and Brady, S. T.** (1998). A role for cyclin-dependent kinases(s) in the modulation of fast axonal transport: Effects defined by olomoucine and the APC suppressor protein. *J. Neurosci.* **18**, 7717-7772.
- 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.
- Sharma, M., Sharma, P. and Pant, H. C.** (1999). CDK-5-mediated neurofilament phosphorylation in SHSY5Y human neuroblastoma cells. *J. Neurochem.* **73**, 79-86.
- Shea, T. B.** (2001). Neurofilament phosphorylation and axonal transport. *J. Neurocytol.* **29**, 873-877.
- Shea, T. B. and Didier, M.** (1997). Biphasic effects of phosphatase inhibition on accumulation of tau phospho-isoforms in cultured cerebellar neurons. *Neurosci. Res. Commun.* **22**, 39-44.
- Shea, T. B. and Flanagan, L.** (2001). Kinesin, dynein and neurofilament transport. *Trends Neurosci.* **24**, 644-648.
- Shea, T. B. and Yabe, J. T.** (2000). Occam's razor slices through the mysteries of neurofilament axonal transport: Can it really be so simple? *Traffic* **1**, 522-523.
- Shea, T. B., Sihag, R. K. and Nixon, R. A.** (1990). Dynamics of phosphorylation and assembly of the high molecular weight NF subunit in NB2a/d1 neuroblastoma. *J. Neurochem.* **55**, 1784-1792.
- Shea, T. B., Jung, C. and Pant, H. C.** (2003). Does C-terminal phosphorylation regulate neurofilament transport? Recent evidence suggests that it does. *Trends Neurosci.* **26**, 397-400.
- Smith, D. S. and Tsai, L. H.** (2002). Cdk5 behind the wheel: a role in trafficking and transport? *Trends Cell. Biol.* **12**, 28-36.
- Straube-West, K., Loomis, P. A., Opal, P. and Goldman, R. D.** (1996). Alterations in neuronal intermediate filament organization, functional implications and the induction of pathological changes related to motor neuron disease. *J. Cell Sci.* **109**, 2319-2329.
- Stamer, K., Vogel, R., Thies, E., Mandelkow, E. and Mandelkow E.-M.** (2002). Tau blocks traffic of organelles, neurofilaments, and APP vesicles in neurons and enhances oxidative stress. *J. Cell Biol.* **156**, 1051-1063.
- Tanaka, T., Veeranna, Ohshima, T., Rajan, P., Amin, N. D., Cho, A., Sreenath, T., Pant, H. C., Brady, R. O. and Kulkarni, A. B.** (2001). Neuronal cyclin-dependent kinase 5 activity is critical for survival. *J. Neurosci.* **21**, 550-558.
- Tu, P.-H., Elder, G., Lazzarini, R. A., Nelson, D., Trojanowski, J. Q. and Lee, V. M.-Y.** (1995). Overexpression of the human NFM subunit in transgenic mice modifies the level of endogenous NFL and the phosphorylation state of NFH subunits. *J. Cell Biol.* **129**, 1629-1640.
- Van den Haute, C., Spittaels, K., van Dorpe, J., Lasrado, R., Vandezande, K., Laenen, I., Geerts, H. and van Leuven, F.** (2001). Coexpression of human cdk5 and its activator p35 with human protein tau in neurons in brain of triple transgenic mice. *Neurobiol. Dis.* **8**, 32-44.
- Veeranna, Shetty, K. T., Link, W. T., Jaffe, H., Wang, J., 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.
- Yabe, J. T., Chan, W. and Shea, T. B.** (2000). Phospho-dependent association of neurofilament proteins with kinesin in situ. *Cell Motil. Cytoskel.* **42**, 230-240.
- Yabe, J. T., Chan, W. K.-H., Chylinski, T. M., Pimenta, A. F. and Shea, T. B.** (2001a). Neurofilaments consist of distinct populations that can be distinguished by C-terminal phosphorylation, bundling and axonal transport rate in growing axonal neurites. *J. Neurosci.* **21**, 2195-2205.
- Yabe, J. T., Chylinski, T. M., Wang, F.-S., Pimenta, A. F., Kattar, S. D., Linsley, M.-D., Chan, W. K.-H. and Shea, T. B.** (2001b). The predominant form in which neurofilament subunits undergo axonal transport varies during axonal initiation, elongation and maturation. *Cell Motil. Cytoskel.* **48**, 61-83.
- Zhu, Q., Lindenbaum, M., Levavasseur, F., Jacomy, H. and Julien, J.-P.** (1998). Disruption of the NF-H gene increases axonal microtubule content and velocity of neurofilament transport: relief of axonopathy resulting from the toxin β' -iminodipropionitrile. *J. Cell. Biol.* **143**, 183-193.