DIVERGENT AND CONVERGENT ROLES FOR KINASES AND PHOSPHATASES IN NEUROFILAMENT DYNAMICS

Sangmook Lee¹, Harish C. Pant² and Thomas B. Shea¹

¹Center for Cellular Neurobiology and Neurodegeneration Research
Department of Biological Sciences
University of Massachusetts Lowell
Lowell, MA 01854

²Cytoskeletal Protein Regulation Section, NIH, NINDS, Bethesda, MD 20892

S Lee 978-934-2890 sangmook_lee@uml.edu
H Pant 301-402-2124 PantH@ninds.nih.gov
TB Shea 978-934-2881 thomas_shea@uml.edu

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ABSTRACT

C-terminal neurofilament (NF) phosphorylation mediates cation-dependent associations leading to incorporation into the stationary axonal cytoskeleton. Multiple kinases phosphorylate NF-H C-terminal domains, including cyclin-dependent protein kinase 5 (cdk5), mitogen-activated protein kinase (MAPk), casein kinase 1 and 2 (CK1, CK2), glycogen synthase kinase 3 beta (GSK). The respective contributions of these kinases have been confounded since they phosphorylate multiple substrates in addition to NFs and display extensive interaction. Herein, differentiated NB2a/d1 cells were transfected with constructs expressing green fluorescent protein-tagged NF-H, isolated NF-H sidearms and NF-H lacking the distal-most 187 amino acids. Cultures were treated with roscovitine, PD98059, lithium, D4476, tetraBromoBenzoleTriazole, and calyculin, which are active against cdk5, MKK1, GSK, CK1, CK2, and protein phosphatase 1 (PP1), respectively. Sequential phosphorylation by cdk5 and GSK mediated NF-NF associations. MAPk downregulated GSK and CK1 activated PP1, both of which promoted axonal transport and restricted NF-NF associations to axonal neurites. MAPk and cdk5, but not CK1 and GSK, inhibited NF proteolysis. These findings indicate that phosphorylation of NFs by the proline-directed kinases MAPk and cdk5 counterbalance the impact of phosphorylation of NFs by the non-proline-directed CK1 and GSK.

Key words: neurofilament, kinase, phosphatase, axonal stability, cytoskeleton
INTRODUCTION

Mammalian neurofilaments (NFs) consist of 3 subunits, termed NF-H, NF-M and NF-L (corresponding to heavy, medium and light according to their molecular mass) (Nixon and Shea, 1992; Pant and Veeranna, 1995), along with α-internexin and peripherin (Yuan et al., 2006; Yuan et al., 2012). NF-H and NF-M C-terminal phosphorylation fosters divalent cation-dependent NF-NF interactions that mediate the formation of the stationary cytoskeleton, that provides support to the axon (Nixon, 1998; Yabe et al., 2001b; Shea and Lee, 2011, 2013). Extensively phosphorylated NFs are normally segregated within axons via a complex interaction of kinases and phosphatases, disruption of which fosters aberrant accumulation of NF spheroids within perikarya and proximal axons resembling those of ALS (Pant and Veeranna, 1995; Julien and Mushynski, 1998; Sihag et al., 2007).

Kinases regulating NF dynamics include cyclin-dependent protein kinase 5 (cdk5), mitogen-activated protein kinase (MAPk), casein kinase 1 and 2 (CK1, CK2), glycogen synthase kinase 3 alpha and beta (GSK3α, GSK3β); p38 kinase, c-jun terminal kinase (JNK) (Guan et al., 1991; Link et al., 1992; Guidato et al., 1996; Bajaj and Miller, 1997; Giasson and Mushynski, 1997; Veeranna et al., 1998; Bajaj et al., 1999; Li et al., 1999; Ackerley et al., 2000; Ackerley et al., 2003; Kesavapany et al., 2003; Waetzig and Herdegen, 2003; Chan et al., 2004; Barry et al., 2007; DeFuria and Shea, 2007; Perrot et al., 2008; Holmgren et al., 2012). Phosphatases that regulate NF dynamics include protein phosphatase 1, 2A and 2B (PP1, PP2A, PP2B) (Shea et al., 1993; Saito et al., 1995; Strack et al., 1997; Jung and Shea, 1999; Gong et al., 2003; Veeranna et al., 2011).

Elucidation of the respective contribution of these kinases has been confounded since they mediate multiple, integral roles in neuronal homeostasis (Macciòni et al., 2001; Galletti et al., 2009; Pucilowska et al., 2012; Shukla et al., 2012; Pan et al., 2013), are activated independently of NF dynamics, and display considerable crosstalk. For example, MAPk downregulates GSK (Ding et al., 2005). GSK activates MAPk activity while cdk5 inhibits MAPk (Noh et al., 2012) (Zheng et al., 2007). CK1-mediated phosphorylation primes several GSK3β substrates (Hagen and Vidal-Puig, 2002; Harwood, 2002; Wang et al., 2002; Hergovich et al., 2006) and regulates cdk5 activity (Liu et
Finally, phosphatases dephosphorylate NFs and also positively and negatively regulate NF kinases (Goldberg, 1999; Adams et al., 2005).

To surmount these difficulties, we utilized NB2a/d1 cells, which express and phosphorylate all NF subunits and establish a stationary phase within axonal neurites. These cells are readily transfected, allowing selective manipulation of kinases and phosphatases, and can be grown in bulk to allow a combination of immunofluorescent analyses and immunological analyses of cellular fractions. This reductionist approach elucidated divergent and convergent roles for CK1, GSK3β, MAPk, cdk5 and PP1 in NF dynamics.
RESULTS

NFs containing extensively phosphorylated NF-H are expressed by and incorporated into the cytoskeleton of differentiated NB2a/d1 cells (Fig. 1A,B). In efforts to identify which kinase(s) were predominantly responsible for generation of commonly-studied NF phospho-epitopes, we overexpressed the known NF kinases cdk5, MAPk, GSK and CK1. Increased activity of cdk5 and MAPk have previously been demonstrated within these cells following transfection with p25 and constitutively-active M KK1 (Chan et al., 2004; Shea et al., 2004). We confirmed increased activity of GSK and CK1 following transfection and inhibition following lithium and D4476 treatment (Fig. 1C,D). Homogenates of cells overexpressing these kinases were subjected to immunoblot analyses. Since NB2a/d1 cells contain robust levels of phospho- and nonphospho-NFs (Fig. 1B), in order to facilitate detection of any changes in epitopes following selective kinase activation, total aliquots loaded onto gels were restricted such that the levels in non-transfected cells were barely detectable (Fig. 1E). These analyses indicated that the SMI-31, SMI-34 and RT97 epitopes were predominantly generated by MAPk. By contrast, the RMO-24 epitope was predominantly generated by GSK (Fig. 1E).

To investigate further the potential roles of phosphorylation of NFs by these kinases on NF phosphorylation, we transfected cells with GFP-tagged NF constructs, which facilitated monitoring levels and distribution of newly-expressed NFs independently of differentially-phosphorylated endogenous NFs. In addition, expression of GFP-tagged constructs allowed us to more closely monitor the influence of kinase manipulation on NF dynamics without interference of endogenous NFs.

CK1 mediates the major retardation of NF-H migration on SDS-gels

Phosphorylation alters NF-H migration on SDS gels from 160kDa to 200kDa (Nixon and Shea, 1992). To determine the responsible kinase(s), we treated cells expressing GFP-H with the above kinase inhibitors plus the casein kinase 2 (CK2) inhibitor tBBT. The bulk of GFP-H migrated at 195kDa (Fig. 2), corresponding to hypophosphorylated (160kDa) NF-H fused to 35kDa GFP; GFP-H was also detected at 260kDa (extensively phosphorylated NF-H fused to GFP (Lee et al., 2011). Phospho-H immunoreactivity was concentrated at the 260kDa isoform for untreated and lithium, PD98059 and roscovitine-treated homogenates. D4476 increased 195kDa phospho-dependent reactivity and reduced 260kDa phospho-dependent reactivity (Fig. 2). D4476 did not alter GFP-H distribution, indicating that this shift in phospho-reactivity was not due to altered migration of total NF-H.
This finding suggests that (1) phosphorylation events mediated by CK1 are critical for inducing the migratory shift of NF-H to 200kDa, (2) phosphorylation events mediated by MAPk, cdk5 and GSK are not sufficient to induce this migratory shift, and (3) SMI-31, SMI-34 and RT97 phospho-epitopes can be generated in the absence of the migratory shift.

**Cdk5 and GSK mediate relatively minor changes in NF migration on SDS-gels**

The broad band observed for extensively-phosphorylated NF-H on SDS gels can be resolved into multiple phospho-dependent isoforms on low acrylamide gels (Lewis and Nixon, 1988). GFP-H, and its resultant phospho-isoforms, also appear as a relatively broad band; e.g., the phospho-isoform migrating at 260kDa spans approx. 10kDa (e.g., Fig. 3A). To examine further the impact of the above kinases on NF-H migration, we transfected cells with constructs expressing GFP-tagged C-terminal sidearms, including site-directed mutants in which the serine residues of cdk5 consensus sequences had been replaced by aspartates (asp) or alanines (ala), termed GFP-Hasp and GFP-Hala, along with GFP-H wt (i.e., the normal NF-H sidearm) to mimic permanently phosphorylated or nonphosphorylated states (Lee et al., 2011). These site-specific mutations allowed monitoring of potential impact of cdk5 on isoform migration. In addition, these “tail only” constructs were more sensitive to subtle migratory shifts than were full-length constructs (e.g., Fig. 3B).

SMI-31 and RT97 in wt sidearms resolved on low acrylamide SDS-gels into two distinct isoforms migrating at approximately 200 and 205kDa. RMO-24 immunoreactivity was exclusively associated with the 205kDa isoform and a 210kDa isoform; Fig. 3C). The sidearm construct with ala substitutions revealed a unique isoform migrating at approx. 190kDa, coupled with the loss of the SMI-31/RT97-reactive 200kDa isoform (Fig. 3D). RMO-24 immunoreactive isoforms were not affected. This suggests that the migratory shift of this isoform from 190 to 200kDa is mediated by cdk5, since the ala substitution specifically blocked cdk5-mediated phosphorylation. Migration of the 205kDa isoform was not affected, suggesting that its migration was not dependent upon cdk5 activity (Fig. 3D). No difference was detected between migration of GFP-Hasp and GFP-Hwt sidearms, consistent with the presence of endogenous cdk5 activity in NB2a/d1 cells (Lee et al., 2011; Shea et al., 2004).
The majority of RMO-24 immunoreactivity was derived from GSK, since RMO-24 immunoreactivity was reduced by 61 ± 6% following lithium treatment; the 210kDa RMO-24-reactive isoform was more severely depleted, and the 205kDa isoform was relatively increased (Fig. 3E). This pattern of reduction in isoform reactivity was also observed following probing of additional replicas with a polyclonal antibody (R39) directed against all NFs regardless of phosphorylation state. These findings indicated that GSK played a role in shifting the migration of the 205kDa isoform to 210kDa. By contrast, the 210kDa isoform was not depleted in asp sidearms (Fig. 3E). Since asp sidearms were mutated exclusively at cdk5 consensus sites, this finding indicated that cdk5-mediated phosphorylation played a role in generation of the 210kDa isoform in addition to that of GSK. PD98059 treatment also depleted the RMO-24-reactive 210kDa isoform, coupled with an increase in the 205kDa isoform, suggesting that MAPk-mediated phosphorylation also contributes to generation of the 210kDa form.

While the major shift in migration of NF-H on SDS-gels apparently mediated by CK1 (Fig. 2), these findings demonstrate that generation of the full range of NF-H isoforms involves MAPk, cdk5 and GSK. In contrast to other phospho-dependent NF antibodies, immunoreactivity of NF-H with RMO-24 in non-transfected cells is difficult to detect by immunoblot analyses. However, when the stacking gel was retained for transfer to nitrocellulose, RMO-24 immunoreactivity was observed within the stack, suggesting that the majority of endogenous RMO-24-reactive NF-H isoforms are associated with SDS-resistant complexes (Fig. 3F). This possibility was confirmed by treatment of cytoskeletal preparations with 8M urea to dissociate NF complexes (Marston and Hartley, 1990), which eliminated RMO-24 immunoreactivity within the stack, increased immunoreactivity associated with the anticipated migratory position for extensively phosphorylated NF-H. Notably, some SMI-31 and SMI-34 reactivity was observed within this aggregate (Fig. 3F). This is also consistent with detection of the nearly all RMO-24 immunoreactivity within in the axonal NF bundle in immunofluorescent analyses (Fig. 1).

**Multiple kinases contribute to NF bundling**

Axonal neurites contain a centrally-situated bundle of closely-opposed NFs that undergo transport and turnover more slowly than the surrounding individual NFs (Fig. 1A). Bundled NFs can be separated from surrounding individual NFs by sedimentation over sucrose, indicating that they are physically associated (Kushkuley et al., 2009). NF-NF associations leading to bundling are mediated by cation-dependent cross-bridging of phospho-NFs (Kushkuley et al., 2009; Yabe et al., 2001a). To investigate
which kinase(s) mediate critical events that promote NF-NF associations, we compared the impact of kinase overexpression or inhibition on intracellular GFP-H distribution, GSK activity and, to a lesser extent, cdk5 and MAPk activity increased NF-NF associations, while, by contrast, CK1 activity decreased NF-NF associations (Fig. 4C, D).

We also examined the impact of kinase activity in cell-free analyses (Fig. 4E). Individual NFs recovered from spinal cord were conjugated with rhodamine and incubated with and without purified kinases, after which we quantified the percentage of NFs that were associated with ≥3 other NFs (Kushkuley et al., 2009). In these cell-free analyses, we were also able to treat isolated NFs with multiple kinases; this was not practical to attempt within intact cells, since such would require transfection with multiple kinase constructs. In cell-free analyses, neither MAPk nor GSK promoted NF-NF associations individually or in combination. Consistent with prior studies (Kushkuley et al., 2009), cdk5 induced an approximate 40% increase in NF-NF associations. By contrast, cdk5 and GSK induced NF-NF associations for approximately 90% of NFs, indicating a synergistic impact of these kinases on NF bundling. Notably, incubation with cdk5, GSK and MAPk attenuated the extent of NF-NF associations observed following incubation with cdk5 and GSK to the level observed following incubation with cdk5 alone. This was consistent with the possibility that MAPk interfered specifically with the impact of GSK, and not that of cdk5, on NF-NF associations.

**Phosphorylation by cdk5 and MAPk prevent NF Proteolysis**

C-terminal phosphorylation inhibits NF proteolysis (Grant et al., 2001; Pant and Veeranna, 1995). We therefore compared the relative contribution of these NF kinases to protection against proteolysis. To accomplish this, we treated cells with the pharmacological inhibitors roscovitine, PD98059, lithium and D4476, none of which altered GFP-H levels in isolation (Fig. 5A). However, combined treatment with roscovitine and PD98059 significantly reduced total GFP-H and RT97-reactive NF-H (Fig. 5A). By contrast, reduction was not observed following treatment with individually or in combination. Moreover, overexpression of GSK or CK1 could not prevent GFP-H depletion following combined treatment with roscovitine and PD98059 (Fig. 5B). These findings indicate that phosphorylation events mediated by cdk5 and MAPk, but not by GSK or CK1, protect NFs from proteolysis. Consistent with recent studies (Rao et al., 2012), phospho-NFs within bundles were more resistant to proteolysis than
were NFs in homogenates (Fig. 5C). Cdk5 and MAPk may therefore maintain a critical level of NFs, which would indirectly promote NF bundling.

**MAPk mediates anterograde NF transport by inhibiting GSK-mediated NF bundling**

MAPk activity is required for transport of NFs into and along axons (Chan et al., 2004) but the responsible mechanism remains unclear. Notably, MAPk inactivates GSK (Ding et al., 2005). Herein, we demonstrate that GSK participates in NF bundling, while MAPk attenuated GSK-mediated bundling (Fig. 4). Since NFs that undergo bundling are withdrawn from the transporting pool (Shea and Lee, 2011), we considered that the mechanism by which MAPk mediates NF transport may be by inhibiting GSK-mediated NF-NF interactions.

If this were indeed the case, we considered that inhibition of NF transport by treatment with PD98059 coupled with overexpression of GSK may have an additive effect. To test this possibility, we first confirmed that MAPk phosphorylated GSK at ser 9 (Fig. 6A). Overexpression of GSK in which ser9 was mutated to ala (“GSKala,” which cannot be inactivated by phosphorylation and is therefore constitutively active) inhibited NF transport into and along axonal neurites (Fig. 6B). Treatment with PD98059 (which inhibits MAPk activity) inhibits NF transport and fosters perikaryal NF accumulation (Chan et al., 2004) (see also Fig. 6C for representative image). However, when cells overexpressing GSKala were treated with PD98059, we observed an increase in perikaryal NF bundles (Fig. 6C). These findings indicate that MAPk promotes NF axonal transport by inhibiting GSK activity, and in doing so, may preclude inappropriate perkaryal NF bundling.

**GSK potentiates cdk5-induced NF bundling**

Overexpression of cdk5 induces the accumulation of phospho-NF bundles within perikarya (Shea et al., 2004). Given the above demonstration of a critical role for GSK in perikaryal NF accumulation, we questioned whether or not GSK participated in cdk5-induced perikaryal NF accumulation. To investigate this possibility, we overexpressed GFP-H along with p25 or GSK, and treated cells with lithium or roscovitine (active against GSK and cdk5, respectively) prior to harvest. Both p25 and GSK overexpression increased the percentage of cells with perikaryal NF bundles. However, treatment with lithium inhibited p25-induced perikaryal NF accumulation, and treatment with roscovitine inhibited perikaryal NF accumulation induced by GSK (Fig. 6D). These findings indicate that a combination of
cdk5 and GSK activity is required to induce perikaryal NF accumulations, and are consistent with our demonstration that GSK potentiated cdk5-induced NF-NF associations in cell-free analyses (Fig. 4).

**CK1 inhibits NF phosphorylation within perikarya by maintaining phosphatase activity**

The above results (Fig. 4) indicated that CK1 apparently inhibited NF bundling. This finding seemed counterintuitive, since CK1 activity was responsible for the shift in NF-H migration on SDS gels attributed to extensive phosphorylation (Fig. 2), and bundles contain the bulk of extensively phosphorylated NFs (Kushkuley et al., 2009; Yabe et al., 2001a). We therefore examined the levels and distribution of total (GFP-H) and RT97-reactive NF-H within total homogenates and bundle fractions derived from cells following overexpression of CK1 and treatment with D4476 (a pharmacological inhibitor active against CK1) (Fig. 7A). Visual inspection, confirmed by densitometric analyses, revealed that overexpression of CK1 statistically reduced total RT97 immunoreactivity ($p<0.01$, ANOVA), while treatment with D4476 statistically increased total RT97 immunoreactivity ($p<0.01$, ANOVA). Overexpression of CK1 decreased the amount of total and RT97-reactive NF-H within bundles (trend towards significance, $p<0.06$, ANOVA) while treatment with D4476 increased the amount of total and RT97-reactive NF-H within bundles (trend towards significance, $p<0.08$, ANOVA). Immunofluorescent analyses demonstrated an increase in axonal and perikaryal RT97 reactivity following D4476 treatment (Fig. 7B).

Since CK1 activates protein phosphatase 1 (PP1) (Henry and Killilea, 1993), we probed whether or not CK1 regulated NF dynamics via modulation of PP1 activity. D4476 treatment significantly ($p<0.05$) reduced PP1 but not PP2a activity in perikarya and axonal neurites (Fig. 7C). Calyculin, a pharmacological agent active against PP1, fostered the formation of SDS-resistant high-molecular weight material that was reactive with RT97 but not the nonphospho-NF antibody SMI-32 (Fig. 7D) and increased RT97 immunoreactivity within perikarya and axonal neurites (Fig. 7E). These findings indicate that PP1 activity regulates NF-H C-terminal phosphorylation, including restricting phospho-NF accumulation within perikarya, and that CK1 may restrict NF phosphorylation by activation of PP1.

MAPk, cdk5, CK1 and GSK each contribute to axonal NF bunding
The above findings highlight that the activity of MAPk, cdk5, CK1 and GSK exert divergent roles on NF dynamics, including inhibition of proteolysis (MAPk and cdk5; Fig. 5), restriction of NF phosphorylation within perikarya and promotion of NF transport into axonal neurites (MAPk and CK1; Fig. 6,7) and promotion of bundling (cdk5, GSK; Fig. 6,7). Since the collective impact of these diverse functions would foster increased in phospho-NFs within axonal neurites, we considered that increased activity of each of these kinases would contribute either indirectly or directly to the establishment and maintenance of axonal NF bundles. In support of this notion, overexpression of each of these kinases both increased the percentage of neurites displaying NF bundles and cdk5, CK1 and GSK each increased the association of GFP-H with bundles (Fig. 8A,B). These findings confirm that each of these kinases contributes directly or indirectly to the incorporation of NFs into axonal bundles.

The distal portion of the NF-H C-terminal is essential for bundling

To address further the role of GSK in bundling, we compared incorporation into the bundle-enriched fraction of full-length GFP-H with incorporation of GFP-H in which the terminal 187 amino acids (the region of the C-terminal sidearm reported to be essential for bundling (Chen et al., 2000)) were deleted (“HΔ187”). Cells expressing HΔ187 generated prominent GFP-reactive species migrating at approximately 150kDa on SDS-gels, which corresponds to the anticipated migratory position of 115kDa NF-H (i.e., lacking the terminal 187 amino acids) fused to GFP. Additional slower-migrating GFP-reactive species were observed between 155-175kDa (Fig. 8C) that displayed prominent immunoreactivity with antibodies directed against phospho-dependent NF C-terminal epitopes (RT97, SMI34, and SMI31), confirming retention of these epitopes within the proximal portion of the sidearm. Retardation of migration of phospho-reactive HΔ187 isoforms demonstrates its ability to undergo phospho-mediated conformation alterations that foster retardation of full-length NF-H migration on SDS-gels (Pant and Veeranna, 1995; Shea and Chan, 2008).

GFP-HΔ187 co-assembled with the endogenous NF network as evidenced by its distribution within the cytoskeleton and co-localization with filamentous profiles (Fig. 8D). Despite deletion of the portion of the NF-H sidearm purported to mediate NF-NF bundling (i.e., the distal-most 187 amino acid residues of the C-terminal sidearm (Chen et al., 2000)), GFP-HΔ187 was recovered within bundles both in cellular fractionation and immunofluorescent analyses (Fig. 5B,C), which was mediated by C-terminal
crosslinking among endogenous (full-length) NF-H co-assembled into the same NFs as shown previously (Kushkuley et al., 2009; Lee et al., 2011).

We next compared the influence of overexpression of GSK and CK1, since the majority of NF-H consensus sites for these kinases exist within the distal-most 187 amino acids (Chen et al., 2000; Hollander and Bennett, 1992; Hollander et al., 1996; Sasaki et al., 2002; Shaw et al., 1997), on bundling of GFP-H and GFP-HΔ187 (Fig. 8E). In the absence of kinase overexpression, both GFP-H and GFP-HΔ187 displayed an identical relative distribution within bundles versus the surrounding axoplasm. However, overexpression of GSK or CK1 each increased the relative amount of GFP-H that was associated with axonal bundles, but did not alter the association of GFP-HΔ187 within bundles.

These findings suggested that GSK- and CK1-mediated phosphorylation of sites within the distal 187 amino acid residues of the NF-H C-terminal tail played a critical role in NF bundling.
DISCUSSION

Key phosphorylation events foster NF-NF associations that generate the stationary phase. Neurons are faced with the task of preventing or eliminating those events within perikarya, which would otherwise result in accumulation of perikaryal spheroids of phospho-NFs characteristic of conditions such as ALS, yet promoting these events within axons, without which the developing axon will not undergo stabilization (Julien and Mushynski, 1998; Pant and Veeranna, 1995; Shea and Lee, 2011; Shea and Lee, 2013). The findings presented herein elucidate divergent roles for NF kinases and phosphatases that encompass axonal transport and establishment/maintenance of the stationary phase. These functions were mediated in part by direct phosphorylation of NFs, but also by interactions among kinases and phosphatases.

We demonstrated herein that GSK activity is essential for NF-NF interactions leading to incorporation of NFs into the stationary phase, which is readily viewed in NB2a/d1 cells and cultured neurons as tightly-associated, “bundled” NFs (Kushkuley et al., 2009; Yabe et al., 2001a; Yuan et al., 2009a). However, GSK-induced bundling was not restricted to axonal neurites but also occurred within perikarya. We demonstrated herein that MAPk, activity of which is essential for transport of NFs into and along axonal neurites of NB2a/d1 cells (Chan et al., 2004), promotes NF axonal transport by inhibiting GSK activity; increasing MAPk activity increased GSK phosphorylation at ser-9 (which inactivates GSK (Sutherland et al., 1993)) and prevented GSK-induced NF-NF association, while inhibition of MAPk activity potentiated GSK-induced inhibition of axonal transport and accumulation of NF bundles within perikarya.

CK1 activated PP1, which was essential for translocation of NFs out of perikarya and into axonal neurites; inhibition of CK1 activity or direct inhibition of PP1 fostered accumulation of phospho-NF immunoreactivity and NF bundles within perikarya. This finding suggest that the normal segregation of extensive phosphorylation and resultant NF bundling to axons is achieved by maintaining a relatively higher ratio of phosphatase/kinase activity within perikarya than within axonal neurites. This possibility is consistent with the rapid de novo accumulation of phospho-NF immunoreactivity within retinal ganglion cell perikarya and proximal axons in situ following inhibition of PP2A (Jung and Shea, 1999).
The above findings demonstrate essential roles for MAPk and CK1 in promotion of NF transport out of perikarya. We did not determine whether or not MAPk and CK1 mediated continued transport of NFs along axonal neurites by their respective inhibition of GSK and activation of PP1. However, transporting NFs enter and leave the stationary cytoskeleton (Kushkuley et al., 2009; Lewis and Nixon, 1988; Nixon and Logvinenko, 1986; Trivedi et al., 2007; Yabe et al., 2001a; Yuan et al., 2009b)). This finding, coupled with increased phospho-NFs along axons following inhibition of PP1 and PP2A (herein and Shea et al., 1993) suggest the presence of ongoing cycles of NF phosphorylation/dephosphorylation within axons.

In addition to regulation of PP1 activity, CK1 mediated NF-H migratory shift on SDS-gels from 160kDa to 200kDa. Herein, we observed prominent RT97 immunoreactivity with the entire range of NF-H isoforms, including the 160kDa isoform, following CK1 inhibition. This was unexpected, since RT97 immunoreactivity, which is generated by MAPk (Veeranna et al., 2008), is normally associated with the most highly phosphorylated NF-H isoforms, migrating at ≥200kDa (Jung and Shea, 1999; Veeranna et al., 2008; Yabe et al., 2000). However, prior studies have not simultaneously manipulated CK1 along with proline-directed cdk5 and MAPk. Since CK1 is constitutively active, CK1-mediated phosphorylation events responsible for the shift of NF-H from 160 to 200kDa would occur constitutively independently of MAPk activity; while MAPk manipulation alters RT97 immunoreactivity, this immunoreactivity would routinely be detected on isoforms already phosphorylated by CK1. This line of reasoning is supported by the observation of 200kDa NF-H within perikarya and axonal hillocks, while RT97 is normally restricted to the axonal shaft (Jung and Shea, 1999; Sanchez et al., 2000; Yabe et al., 2000).

The use of SDS-gels containing a relatively low acrylamide content revealed an array of NF-H isoforms similar to those observed in optic pathway (Lewis and Nixon, 1988). Site-directed mutagenesis of cdk5-specific phosphorylation sites induced minor alterations in migration of NF-H isoform on SDS-gels. GSK activity induced RMO-24 reactivity and the appearance of the slowest-migrating NF isoform, corresponding to the 210kDa isoform in optic pathway (Lewis and Nixon, 1988). Inhibition of MAPk depleted this slowest-migrating isoform, indicating that a combination of MAPk and GSK activity generated this isoform.
Phosphorylation inhibits NF proteolysis (Pant and Veeranna, 1995). Our findings provided further insight into this regulation by demonstrating that phosphorylation by cdk5 and MAPk, and not by CK1 and/or GSK, mediated this protection. We observed increased NF-H proteolysis following simultaneous inhibition of cdk5 and MAPk but not GSK or CK1, and overexpression of GSK and CK1 failed to prevent NF-H proteolysis following inhibition of cdk5 and MAPk. These findings indicate that phosphorylation of the KSP domains provides protection against proteolysis. Since the terminal 187 amino acid residues, which contains the majority of the CK1 and GSK consensus sequences, is essential for bundling (Chen et al., 2000), one speculation arising from the differential impact of these kinases on proteolysis and bundling is that the terminal 187 amino acid residues, rather than the KSP-rich regions, may mediate the cation-dependent crosslinking that incorporates NFs into bundles (Kushkuley et al., 2009), leaving the more proximal KSP-rich region, or a portion of it, exposed to potential protease activity. If this were indeed the case, dephosphorylation of the KSP-rich region could allow proteolysis of bundled NFs, which may provide a means for localized remodeling of the cytoskeleton, including that required axonal branching (Xie et al., 2006).

Comparison of GFP-H within axonal neurites and bundles indicated that CK1, GSK, and cdk5 all increased bundling of NFs, indicating that once NFs were within axons, all of these kinases contributed to the establishment and maintenance of the stationary phase. However, our systematic overexpression and inhibition highlighted that these kinases regulated an hierarchical series of events encompassing proteases and phosphatases. GSK-mediated NF bundling, required prior phosphorylation of those NFs by cdk5. MAPk-mediated downregulation of GSK activity within perikarya was essential to restrict segregation of bundling within axonal neurites. Like MAPk, CK1 also promoted NF transport into axonal neurites by restricting the accumulation of extensive phosphorylation within perikarya, although CK1 mediated this by activation of PP1 rather than inhibition of GSK. In addition, inhibition of NF proteolysis by cdk5 and MAPk also likely contributed to bundling by maintaining sufficient concentration of axonal NFs. Promotion of NF transport into axons by MAPk and CK1, inhibition of NF proteolysis by MAPk and cdk5, and prior NF phosphorylation at least by cdk5 represent mechanisms by which these kinases could contribute to GSK-induced NF bundling within axons. These findings collectively indicate that the impact on NF dynamics of the non-proline-directed kinases CK1 and GSK are functionally regulated by the proline-directed NF kinases MAPk and cdk5.
A limitation of these analyses is that we did not dephosphorylate NFs prior to examination of the impact of kinase manipulation. Accordingly, we cannot completely exclude the possibility that prior phosphorylation events contributed to the changes observed following manipulation of individual kinases. Unfortunately, experimental NF dephosphorylation results in rapid proteolysis within cells (Pant and Veeranna, 1995) and under cell-free conditions due to NF-associated proteases (Kushkuley et al., 2009). A further limitation is that we were unable to examine the consequences of simultaneous overexpression of multiple kinases. We have routinely manipulated kinases in cells co-transfected with GFP-H or GFP–M by utilizing 2x of the respective kinase construct versus the GFP-NF construct. To examine the consequence of multiple kinases would require successful triple transfection (i.e., GFP-H plus two or more kinase constructs), which is impractical.

The kinases studied herein have multiple substrates beyond NFs. As such, we cannot exclude the possibility that at least some of the impact of kinase/phosphatase manipulation on NFs as seen hererin was derived by indirect effects on other cellular pathways. For example, while NF phosphorylation clearly provides resistance to proteolysis (Pant and Veeranna, 1995), we cannot exclude the possibility that manipulation of kinase/phosphatase activities also suppressed activity of proteolytic enzymes themselves, which would further contribute to increased NF levels. Similarly, while phosphorylation promotes NF bundling, and in doing so removes NFs from the transporting pool (Shea and Lee, 2011; Kushkuley et al., 2009), kinases examined herein also directly and indirectly impact kinesin itself (Morfini et al., 2002). We therefore cannot completely exclude the possibility that at least some of the alterations in NF distribution following kinase/phosphatase manipulations were derived at least in part from disruptions in overall axonal transport. This likelihood is reduced, however, by our prior demonstration that manipulation of MAPk and cdk5 by the same methodologies utilized herein did not alter axonal transport of tau (also dependent upon kinesin) in these cells (Moran et al., 2005; Dubey et al, 2007). Notably, even if the kinases and phosphatases studied herein mediate some of their impact on NFs by modulation of proteolytic and transport systems, it does not diminish the overall conclusion that the collective impact of these divergent kinase activities increases the amount of phospho-NFs within axonal neurites, which in turn contributes to establishment and maintenance of the stationary phase.

C-terminal NF phosphorylation does not directly interfere with NF axonal transport, but indirectly interferes with transport by allowing some NFs to withdraw from the transporting pool to establish the
stationary cytoskeleton, which is essential for axonal maturation. This avoids the need for any alteration in the transport system itself to foster axonal maturation. Notably, this allows continued activity of transport systems within axons. Using the same transport battery throughout neuronal differentiation and maturation simplifies these dynamics, allows stabilization of proximal axonal regions while distal regions are still elongating, and ultimately allows transition into a maintenance system that can repair/replace damaged or worn out NFs at any locus along the axon.

The kinases studied herein share pivotal roles in neuronal homeostasis. Any requirement to modulate their activity to foster NF transport and assembly of the stationary phase would impact multiple essential neuronal pathways. However, since extensively phosphorylated NFs essentially undergo self-assembly to form the stationary phase within axons, there is no apparent requirement for a developing neuron to modulate the activity of these kinases nor their corresponding phosphatases for establishment or repair of the stationary phase. Intermediate filaments (IFs) provide mechanical strength to cells and mediate the formation of tissues (Bertaud et al., 2010; Fuchs, 1994). Perhaps nowhere can the need for long-term, stable structural support be more critical than in axons, which, once synaptogenesis has occurred, remain in place for the lifetime of the individual (Shea and Lee, 2013). The interplay of kinase and phosphatase activities on NF dynamics as demonstrated herein demonstrate how this class of IFs can establish a long-lasting, supportive macrostructure polarity during development without the need to alter activity of participating kinases or that of the overall transport system.

**MATERIALS and METHODS**

**Differentiation** Mouse NB2a/d1 neuroblastoma cells were cultured in DMEM containing 10% fetal bovine serum and differentiated with 1mM dibutyryl cyclic AMP (dbcAMP) (Yabe et al., 1999). For
simplicity and clarity of writing, translocation of NFs into and along axonal neurites of these cells is referred to as “axonal transport.”

**Expression of NF Constructs** Cells were transfected using LipofectAMINE (Invitrogen, Carlsbad, CA) with constructs expressing GFP-tagged full-length NF-H (GFP-H), plasmids expressing GFP-tagged isolated NF-H sidearm (i.e., lacking the rod domain; “GFP-Hwt,”) GFP-tagged sidearms in which the serines in C-terminal cdk5 consensus sites were mutated to aspartates (“GFP-Hasp”) or alanines (“GFP-Hala”) to mimic permanently phosphorylated or nonphosphorylated states (Ackerley et al., 2003; Lee et al., 2011), and GFP-tagged rat NF-H lacking the distal-most C-terminal 187 amino acids (GFP-HΔ187). GFP-HΔ187 was prepared from the above full-length GFP-tagged NF-H construct. Since there is a unique AccI site in the C-terminal region of rod domain of NF-H (Chen et al., 2000), the sequence between Acc I and the last KSP motif was amplified by PCR using the following set of primers: 5’-AGAGTCGCGAAAGTGAACACGGATGCT-3’ and 5’-CTGAGGATCTAAGGGGACTTCCTTC-3’. Amplified PCR fragments were digested with AccI and BamHI, for which consensus sites were included in the primers, then cloned into the prepared template plasmid digested with the same enzymes. A ≥70% transfection efficiency for GFP-tagged NF constructs is attained under these conditions (Chan et al., 2004).

**Manipulation of kinase activities** Cells were transfected with a plasmid expressing constitutively-active mouse GSK3β (“GSK,” generous gift from Dr. Chris Miller), mouse CK1-α (“CK1,” Origene, Rockville, MD), constitutively-active MKK1 (upstream activator of MAPk) (Li et al., 1999), MAPk, and p25 (the truncated and constitutively-active form of the cdk5 activator p35 (Patrick et al., 1999). Additional cultures were treated with the pharmacological inhibitors roscovitine (20µM), PD98059 (10µM), lithium (10mM), D4476 (100µM), and tetraBromoBenzoleTriazole (tBBT; 60µM), which are active against cdk5, MKK1, GSK, CK1 and CK2 respectively (Cheng et al., 1983; Dudley et al., 1995; Meijer et al., 1997; Rena et al., 2004; Sarno et al., 2001). Since the downstream impact of manipulation of MKK1 on NFs is alteration of p42/44 MAP kinase, we refer to MAPk manipulation at points for simplicity of writing. For co-transfection with kinases and GFP-H, ≥2x of kinase constructs versus GFP-H were utilized (1µg and 0.5µg respectively); insuring that cells displaying GFP were cotransfected with the kinase construct (Chan et al., 2004; Shea et al., 2004).
Fractionation

Cells were homogenized in 50mM Tris-HCl (pH 6.8) containing 1% Triton X-100, 5mM EDTA, 1mM PMSF and 50µg/ml leupeptin and centrifuged (15,000 x g; 15min). The resulting pellet was defined as the Triton-insoluble cytoskeleton, and the resulting supernatant was defined as the Triton-soluble fraction. Spinal cord NFs from adult C57BL6 mice of both genders (sacrificed in accordance with the approval of our Institutional Animal Care and Use Committee) were resuspended in 0.1M MES (pH6.8) containing 1mM MgCL2, 1mM EGTA, 1mM PMSF and 50 µg/ml leupeptin and labeled with rhodamine as described (Kushkuley et al., 2009; Wagner et al., 2003).

To obtain fractions enriched in bundled NFs, additional homogenates were layered over the same buffer containing 1M sucrose, and centrifuged 15,000 x g; 15min). Bundled NFs sedimented through the sucrose cushion; “individual” NFs (not contained within bundles) were recovered at the sucrose interface (Kushkuley et al., 2009; Yabe et al., 2001a). Protein concentration was determined by BCA assay (Thermo scientific).

Cell-free NF manipulations

Individual NFs obtained as described above were incubated ± purified kinases (Kushkuhley et al., 2009) for 2hr at 37°C with 0.1µg/µl cdk5 and its activator p35, MAPk, and/or GSK (Upstate Biochemicals, Lake Placid, NY). The % of NFs aligned with ≥3 other NFs were quantified via fluorescence microscopy (Kushkuley et al., 2009). Closely-aligned NFs typically splay apart at their ends, facilitating detection of multiple NFs (Kushkuley et al., 2009).

Electrophoresis and Immunoblot analysis

Samples were normalized according to total protein, subjected to SDS-gel electrophoresis and transferred to nitrocellulose. The bundled fraction was solubilized with 8M urea for electrophoresis. Membranes were blocked with 5% BSA and 5mM sodium fluoride in Tris-buffered saline containing 0.1% Tween-20 for 1hr then incubated overnight at 4°C with antibodies directed against GFP (1:1000, Invitrogen) and antibodies directed against NF phospho-epitopes [RT97 (generous gift of B. Anderton, Institute of Psychiatry, UK), SMI-34 and SMI-31 (Covance; Dedham, MA), RMO-24 (Invitrogen)], nonphospho-epitopes (SMI-32) and an antibody directed against NFs regardless of phosphorylation state
Membranes were washed with the same buffer then incubated with alkaline phosphatase-conjugated secondary antibodies for 1hr at room temperature and developed using a NBT/BCIP substrate kit (Promega, Madison, WI). Immunoreactive species were quantified in digitized images of replicas using Image J; the background signal from an adjacent, identically-sized area in the identical lane was subtracted from each reactive species (Yabe et al., 2001a; Yabe et al., 1999). All samples to be compared were electrophoresed on the same gel, transferred to nitrocellulose and visualized simultaneously.

**Immunofluorescence**

Cells grown on poly-L-Lysine-treated coverslips were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) for 10 minutes at room temperature, rinsed 2x in PBS (5min/rinse), and blocked for 30min in PBS containing 1% bovine serum albumin (BSA) and 2% normal goat serum. Cultures were then incubated overnight at 4°C in PBS containing 1% BSA and a 1:500 dilution anti-GFP or R39, or 1:100 dilutions of RT97, SMI34, SMI-31, SMI-32, and RMO-24. Cultures were rinsed 3x with PBS, incubated for 30min at 37°C in PBS containing 1% BSA and a 1:300 dilutions of appropriate secondary antibodies.

**Monitoring of intracellular NF distribution**

NF distribution was quantified using Image J (http://rsb.info.nih.gov/ij). Translocation of NF constructs into and along axonal neurites was quantified as an index of axonal transport. The shaft of axonal neurites was divided into 10 equivalent segments excluding the hillock and the growth cone, and the % GFP within each segment was calculated relative to the total GFP within the axonal shaft (Yabe et al., 2001b). In additional studies, axonal neurites (>25 for each condition) were divided into 3 equivalent segments (defined as proximal, central and distal) excluding the hillock and growth cone. The translocation rate was determined by dividing the GFP intensity in the distal fragment by that of central fragment within each neurite (Chan et al., 2004). To monitor the extent of incorporation of GFP-tagged NFs into axonal NF bundles, GFP intensity of the bundle and that of the area adjacent to the bundle were quantified within the central neurite segment for >20 cells for each condition (Chan et al., 2004; Chan et al., 2005).
Kinase and Phosphatase Assays

GSK activity was assayed by monitoring phosphorylation of GSK at ser9 (which inactivates GSK) and phosphorylation of the GSK substrate beta-catenin at Ser33/37/Thr41. Homogenates of cells ± GSK overexpression or lithium treatment treated with 10mM lithium were subjected to electrophoresis, transferred to nitrocellulose and probed with antibodies directed against total GSK, GSK that had been phosphorylated at ser9, total beta-catenin, and beta-catenin phosphorylated at Ser33/37/Thr41 (Cell Signaling), and anti-tubulin antibody DM1A as a loading control. Since beta-catenin is degraded once phosphorylated by GSK (Ding et al., 2005), a decrease in phospho-beta catenin (also be reflected in total beta-catenin levels) provides an index of GSK activity.

CK1 activity was monitored by casein gel zymology (Cheng and Louis, 1999). Lysates from cells incubated for 4 hours ± 100µM D4476 were separated on a 12% polyacrylamide gel polymerized in the presence of 1mg/ml dephosphorylated casein. After electrophoresis, SDS was removed from the gel by rinsing twice for 30min with 50mM Tris-HCl (pH 8.0) containing 20% isopropanol. The gel was washed 2x for 30 min in 50mM Tris-HCl containing 5mM 2-mercaptoethanol. Electrophoresed proteins were denatured by incubating the gel 2x for 30min in the above buffer containing 6M guanidine/HCl, then renatured by 15 hours washing at 4°C in 4 changes of the above buffer containing 0.05% Tween 40. The gel was equilibrated in 10mM Tris-HCl (pH 7.5) containing 10mM MgCl2 for 30min at room temperature. Casein phosphorylation was carried out by incubation of the gel in 10mM Tris-HCl (pH 7.5) containing 10mM MgCl2, 25µM ATP and 2.5 µCi /ml, [γ-32P] ATP for 2h at room temperature followed by washing with 5% trichloroacetic acid/1% sodium pyrophosphate until no radioactivity was detected in the wash. The gel was dried and exposed to X-ray film.

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**Figure Legends**

**Fig. 1: NF phospho-epitopes of NB2a/d1 cells and responsible kinases**  
Panel A: Representative differentiated cells probed with antibodies directed against phospho- (RT97, SMI-31, SMI-34, RMO-24) and nonphospho (SMI-32) epitopes as indicated. Note the centrally-situated NF bundle along neurites (arrows).  
Panel B: Nitrocellulose replicas of homogenates probed with the above antibodies; migration of NF-H and NF-M are indicated.  
Panel C: Transfection with a construct expressing GSK significantly (p<0.05) increased overall GSK levels (which increases net GSK activity (Wagner et al., 1996)), and decreased levels of the GSK substrate beta catentin (proteolyzed following GSK-mediated phosphorylation). Lithium significantly increased levels of GSK phosphorylated at ser9 (which inactivates GSK (Lamarre and Desrosiers, 2008) and prevented degradation of phospho- and total beta-catentin. Beta-tubulin (probed with antibody DM1A) was included as a loading control. The accompanying graphs present the mean ± standard error for 3 immunoblots for total and phospho GSK (pser9-GSK) and 2 for phospho- (p-) and total beta-catentin.  
Panel D: CK1 overexpression increased, while the CK1 inhibitor D4476 decreased, casein phosphorylation versus untreated controls. A representative gel zymograph is presented along with a nitrocellulose replica of an aliquot of the same sample probed with anti-tubulin antibody DM1A as a loading control. The accompanying graph presents the mean ± standard error of casein phosphorylation versus untreated controls for 3 such gels.  
Panel E: Nitrocellulose replicas of homogenates overexpressing the indicated kinases, probed with the above NF antibodies and a polyclonal antibody (R39) that reacts with NFs regardless of phosphorylation state as a loading control. Note overexpression of MKK1 increased SMI-31, SMI-34 and RT97 immunoreactivity, while GSK increased RMO 24. All samples probed with the same antibody in each panel are from the same gel.

**Fig. 2: CK1 mediates the retardation of NF-H migration on SDS-gels.**  
Panels A,B: Nitrocellulose replicas of homogenates transfected 24hr previously with a construct expressing GFP-H and treated for the final 2hr of incubation with the indicated inhibitors, along with homogenates of untreated control cells. Migration of extensively and hypo-phosphorylated NF-H fused to GFP (260 and 195kDa) are indicated. Note only D4476 inhibited GFP-H migration. The accompanying graph presents densitometric analyses of 195kDa H; not all conditions in panels A and B are included. D4476 treatment did not alter total levels of the 195kDa isoform, but increased 195kDa phospho-immunoreactivity versus all other conditions (asterisks; p<0.01; ANOVA with post hoc analyses). Values represent mean % immunoreactivity (± standard error) versus untreated homogenates.

**Fig. 3: Multiple kinases generate NF-H isoforms**
Nitrocellulose replicas of thin SDS-gels of homogenates expressing GFP-H (A, B), GFP-tagged C-terminal sidearms (C-F) or no NF-H construct (G) ± treatment with lithium, PD98059 or urea, and probed with anti-GFP SMI-31, SMI-34, RT97 or R39 (directed against total NFs).

Panel A: GFP-H migrates over a range of 195-260kDa, which corresponds to the migratory range of NF-I (160-210kDa) plus the 35kDa GFP. Phospho-epitopes are concentrated within the slowest-migrating isoforms.

Panel B: Nitrocellulose replicas probed with RT97 of homogenates expressing full-length GFP-Hwt, GFP Hasp or GFP-ala, or their respective GFP-H sidearm as indicated. As anticipated, sidearm-only construct migrate faster on SDS-gels.

Panel C: SMI-31 and RT97-reactive isoforms in GFP-Hwt sidearms resolved on thin SDS-gels into isoforms migrating at approx. 200 and 205kDa. RMO-24 immunoreactivity was associated with isoform migrating at 205 and 210kDa.

Panel D: Nitrocellulose replicas of homogenates expressing GFP-Hwt, GFP-Hasp or GFP-Hala sidearm probed with the indicated antibodies. Cells expressing the GFP-Hala sidearm lack the 200kDa isoform but display a 190kDa isoform reactive with SMI-31 and RT97 but not RMO-24.

Panel E: Nitrocellulose replicas of homogenates expressing GFP-Hwt or GFP-Hasp sidearms ± lithium treatment probed with RMO-24 or R39. Lithium depleted the 270kDa isoform and increased the 205kDa isoform in GFP-Hwt sidearms but did not affect the GFP-Hasp sidearm.

Panel F: PD98059 treatment depleted the RMO-24-reactive 210kDa isoform and increased the 205kDa isoform.

Panel G: Nitrocellulose replicas of homogenates from non-transfected cells ± treatment with 8M urea; the stacking gel was retained for transfer to nitrocellulose; clear NF-H isoforms are only detected following treatment of homogenates with urea prior to electrophoresis. This aggregated material is reactive with SMI-31 and SMI-31; however, clear isoforms from approx. 210 to 200kDa (arrows) are detectable in the absence of urea.

**Fig. 4:** Kinase activities promote NF bundling. Panel A: nitrocellulose replicas of endogenous NF recovered in total cytoskeletons (Pellet) or remaining in the Triton-soluble supernatant (Super), and NFs that sedimented through a 0.8M sucrose cushion (Pellet, enriched in bundled NFs) or remaining at the buffer sucrose interface (Super, enriched in individual, non-bundled NFs). Panel B: a representative epifluorescent image of a cell expressing GFP-H. Note GFP-H distributes throughout cells and is incorporated into the bundle (arrows). Panel C: nitrocellulose replicas, probed with anti-GFP, of homogenates (H) and bundle-enriched
fractions (B) from cells transfected 24hr previously with GFP-H constructs alone (–) or co-transfected with constructs expressing p25, MKK1, GSK and CK1. Panel D: the % change in the ratio of bundled/individual NF in cells transfected with the indicated NF kinase constructs versus those not transfected with kinase constructs values represent the mean ± the range from 2 independent experiments; incubation with GSK, p25 or MKK increased NF-NF associations vs untreated NFs (p<0.05, ANOVA). CK1 decreased NF associations versus untreated NF and NFs incubated under all other conditions (asterisk; p<0.03; ANOVA with post-hoc analyses) Panel E: quantification of rhodamine-conjugated spinal cord NFs incubated for 2hr ± kinases. Incubation with cdk5 increased NF-NF associations (asterisk; p<0.05 vs untreated NFs; Student’s t test); incubation with cdk5-GSK further increased NF-NF associations (double asterisk; p<0.05 vs cdk5 alone; Student’s t test). Incubation with cdk5, GSK and MAPk prevented the GSK-mediated increase beyond that observed with cdk5 alone (p<0.05 vs. untreated NFs; asterisk). Insets present a nitrocellulose replica of this preparation probed with SMI 31, depicting H and M phospho-epitopes, and representative epifluorescent images of NFs from these preparations.

Fig. 5: cdk5 and MAPk protect NFs against proteolysis. Panel A: Nitrocellulose replicas of homogenates (H) and bundle-enriched fractions (B) from cells treated with roscovitine (Ros), PD98509 (PD) or both, along with untreated controls (Untreat). The accompanying graphs present the total GFP-H (H + B present under each condition; values represent mean immunoreactivity ± standard error from 2-3 experiment for each condition. Note the specific reduction in GFP-H and RT97 immunoreactivity for cells treated with Ros + PD (asterisks; p<0.03 and 0.01 versus all other conditions; ANOVA with post-hoc analyses). Panel B Nitrocellulose replicas probed with anti-GFP or non-phospho-specific SMI32 of homogenates (H) from cell treated with Ros + PD ± over expression of GSK or CK1 along with untreated controls. The accompanying graph presents the immunoreactivity present under each condition. Note reduction in levels following treatment with Ros + PD (asterisks; p<0.02; ANOVA), and that overexpression of GSK or CK1 did not compensate for this treatment (p<0.19 for Ros+PD vs Ros+PD+GSK or CK1; ANOVA). Panel C: Nitrocellulose replica probed with SMI-34 or RT97 of homogenates (H) and bundle-enriched fractions (B) derived from cells treated as in panel B. The accompanying graphs present the total immunoreactivity present under each condition. A in panel B, treatment with Ros+PD reduced phospho-NF immunoreactivity in homogenates in the presence or absence of GSK or CK1 (asterisk; p<0.01; ANOVA). Note increased depletion in homogenates versus bundle following treatment with Ros + PD ± overexpression of GSK or CK1 (asterisks between graphs; p<0.01 ANOVA).
**Fig. 6: GSK-mediated NF-NF associations require cdk5 and are inhibited by MAPk**

**Panel A:** Nitrocellulose replicas of homogenates from cells ± overexpression of MKK1, probed with an antibody directed against GSK phosphorylated at ser9 (p-GSK) and homogenates expressing GFP-H ± PD98059 treatment and probed with RMO24. Beta-actin was utilized as loading control. The accompanying graph presents p-GSK and actin levels. Note overexpression of MKK1 increased p-GSK.

**Panel B:** The left-most graph presents quantification of GFP-H along axonal neurites (n=19 for each condition. A single asterisk indicates a significant increase (p<0.05) in GFP in segments of cells expressing GSKala vs untreated cells; a double asterisk indicates a significant decrease (p<0.05). Values represent the mean % GFP (± standard error) in each of 10 axonal segments. The right-most graph presents the % of total GFP with axonal neurites; GSK overexpression + PD98059 treatment significantly (p<0.05) reduced overall GFP-H levels.

**Panel C:** Representative cells expressing GFP-H ± GSKala co-expression and PD98059 treatment. Not increased perikaryal and decreased neuritic GFP immunoreactivity following PD98059 treatment, an perikaryal NF bundles (arrows) in PD98049-treated cells expressing GSKala. PD98059 + GSKala treatment increased the % of cells containing perikaryal NF bundles (asterisk; p<0.05, ANOVA, p<0.01 for PD98059 + GSKala vs PD98059 alone; p<0.06 for PD98059 + GSKala vs. untreated). Values represent mean % of cells (± standard error; n≥20 cells/condition).

**Panel D:** Overexpression of GSK or cdk5 increased perikaryal NF bundles (p<0.001; ANOVA) Post hoc analyses via Turkey-Kramer test for Differences between Means and Fischer LSD each revealed that perikaryal bundling in cells overexpressing GSK and in cells overexpressing cdk5 differed from that in untreated cell (p<0.001 and <0.001, respectively; asterisks). Roscovitine prevented the GSK-mediated increase, while lithium prevented the cdk5-mediated increase; values for these conditions remained statistically identical to that of untreated cells (ANOVA with above post-hoc analyses). Values represent the mean percentage of cells (± standard error) containing perikaryal NF bundles.

**Fig. 7: CK inhibits NF phosphorylation by activation of PP1**

**Panel A:** Nitrocellulose replicas of total homogenates (H) and bundle fractions (B) from cells expressing GFP-H ± CK1 overexpression or D4476 treatment. The accompanying graphs present the total cellular immunoreactivity (and, separately, the amount within H and B as indicated. Values represent the mean ± standard deviation. A double asterisk indicates a significant decrease, while a single asterisk indicates...
significant increase, in GFP-H or RT97-reactive NF-H versus untreated cells (p<0.05, ANOVA with post-ho analyses). Panel B: Representative image of RT97 immunoreactivity ± D4476 treatment. Arrows denote the perikaryon. Panel C: D4476 reduced PP1 activity preferentially in soma (asterisk; p<0.01; Student’s t test) but not PP2a activity. Panel D: Immunoblot analyses of cells ± calyculin treatment probed with RT97 and SMI-32. Calyculin induced formation of SDS-resistant aggregates of phospho-NFs unable to penetrate the stacking gel. Panel E: Cells expressing GFP-H ± calyculin treatment probed with RT97. Note calyculin treatment induced the accumulation of phospho-NFs within perikarya and reduced phospho-NFs within neurites (arrowheads).

Fig. 8: Multiple NF kinases contribute to NF bundling

Panel A: Representative images of the hillock and axonal neurite of cells expressing GFP-H ± GSK3β overexpression. Panel B: The % of cells displaying GFP-labeled NF bundles and the relative densitometric intensity of GFP within bundles vs. the surrounding axoplasm for cells expressing GFP-H ± kinases overexpression. The % cells with bundles was increased following overexpression of all kinases (p<0.05 ANOVA). The ratio of GFP in bundles vs axoplasm was increased (p<0.05) following overexpression of all kinases except MKK1. Values represent the mean (± standard error) from multiple cells from duplicate experiments.

Panel C: Upper row presents nitrocellulose replicas of Triton-soluble (S) and insoluble (I) fractions from cells expressing GFP-H and Δ187 probed RT97, SMI-34 and SMI-31. The lower row presents nitrocellulose replicas of initial homogenates (H) and bundle (B) fractions from cells expressing NF-H or HAΔ187. Migratory positions of each fusion protein are indicated along the right of each panel. Fractions from cells expressing GFP-tagged full-length NF-H displayed prominent GFP-reactive species migrating at approximately 195-250kDa on SDS-gels, corresponding to the migratory position of NF-H fused to 30-35kDa GFP. Fractions from cells expressing HAΔ187 displayed a prominent 150kDa GFP-reactive species, corresponding to the migratory position of the NF-H fragment fused to GFP; additional slower-migrating GFP-reactive species were observed between 155-175kDa. Note HAΔ187 retained immunoreactivity with all antibodies and incorporated into the cytoskeleton and the bundle-enriched fraction, indicating co-assembly with endogenous NFs. Minor additional immunoreactive species correspond to endogenous NF-H (160-205kDa) and NF-M (125-140kDa).

Panel D: Representative cells overexpressing GFP-H and HAΔ187 and immunostained with RT97. Note the distribution along axons, including their association with the centrally-situated NF bundle. Inserts: higher magnification of axonal neurites depicting filamentous profiles.
Panel E: representative images of the central region of axonal neurites of cells expressing GFP-tagged full length NF-H or HΔ187, with and without overexpression of CK1 as indicated. The accompanying graph depicts the ratio of GFP intensity within bundle versus the surrounding axoplasm calculated as described in Methods. This ratio was identical for NF-H and HΔ187 in the absence of kinase overexpression. GSK or CK overexpression increased this ratio for NF-H but not for HΔ187; asterisk indicates $p < 0.05$ (Student’s $t$ test).
A

GFP

RT97

Relative Density

Untreated
Roscovitine
PD98059
Both

B

Ros + PD
GSK
CKI

Relative Density

Ros + PD + GSK

C

Ros + PD
GSK
CKI

Relative Density

Untreated
Ros + PD
GSK
Ros + PD + GSK
CKI
Ros + PD + CKI

RT97

SMI-34

Bundle

 Relative Density

Relative Density