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# Phosphorylation of the Neurofilament Heavy Subunit by Stress-Activated Protein Kinase.

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September 2000

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **Master's of Science**

## Abstract

Stress-activated protein kinases (SAPKs) were previously implicated in the phosphorylation of the neurofilament heavy subunit (NFH). This study presents direct evidence that stress-induced phosphorylation of NFH in both differentiated PC12 cells and cultured sensory neurons is inhibited by CEP-1347 (KT7515), a specific inhibitor of SAPK activation. In addition, long-term treatment of unstressed sensory neurons with CEP-1347 decreased the phosphorylation state of NFH in neurites. CEP-1347 differentially inhibited the activation of various SAPK isoforms in neuronal cell body and neurite fractions. Specifically, activation of a 55 kDa SAPK isoform in the neurite fraction was highly sensitive to CEP-1347 inhibition, with an $IC_{50}$ of about 0.02 μM, while $IC_{50}$ values for other SAPK species were at least 10-fold higher. The data indicate that SAPKs are involved in both constitutive phosphorylation of axonal NFH and stress-induced phosphorylation of perikaryal NFH in cultured sensory neurons and that SAPKs in the two neuronal compartments are activated by different signaling pathways.

## Résumé

Les protéines kinases activées par le stress (SAPK) ont déjà été impliquées dans la phosphorylation de la chaine lourde des neurofilaments (NFH). L'étude qui suit démontre que dans des cellules PC12 différenciées et dans des cultures de neurones sensorielles, la phosphorylation de NFH causée par le stress est inhibée par CEP-1347 (KT7515), un inhibiteur spécifique de l'activation de SAPK. De plus, le traitement à long terme de neurones sensorielles non-stressées avec CEP-1347 diminue le niveau de phosphorylation de NFH dans les axones. L'inhibition par CEP-1347 variait selon les isoformes de SAPK situées dans les corps cellulaires neuronaux et les axones. Plus précisément, l'activation de l'isoforme de 55 kDa de la SAPK axonale était nettement sujette à l'inhibition par CEP-1347, avec une $CI_{50}$ d'environ 0,02 μM. Par contre, la $CI_{50}$ des autres isoformes de SAPK était au moins dix fois plus élevée. Les données indiquent que les SAPK sont impliquées et dans la phosphorylation constitutive de la NFH axonale, et dans la phosphorylation de la NFH induite par le stress dans le corps cellulaire des neurones sensorielles en culture. Les données indiquent aussi que les SAPK des deux compartiments neuronaux sont actvées par des voies de signalisation différentes.

## Acknowledgements

The work presented here is my own. Some results presented here (Figs. 3-1, 3-6 and 3-7) have been submitted to the Journal of Neurochemistry for publication.

I would like to thank Cephalon for the gift of CEP-1347 (KT7515) and Dr. Woodgett for the gift of the GST-cJun construct.

I would like to thank Dr. Eric Athlan for generously sharing his extensive technical expertise and for translating the abstract into French. Dr. Almazan also provided many helpful constructive criticism with respect to the presentation of the figures for which I am grateful. I would also like to thank Dr. Janice Robertson for helping me with immunocytochemical experiments. She has been a terrific source of support and encouragement for me throughout my Master's project.

Above all, I would like to thank Dr. Walter Mushynski. As my supervisor, he has provided me with an excellent learning environment, unending patience, a fun lab atmosphere and all the encouragement and support I required. I feel very fortunate to have had the opportunity to learn from him.

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## List of Abbreviations

| | |
|---|---|
| ASK | apoptosis signal-regulating kinase |
| ATF-2 | activating transcription factor-2 |
| BSA | bovine serum albumin |
| C-terminal | Carboxy terminal |
| CDK | cyclin dependent kinase |
| CI | N-acetyl-Leu-Leu-norleucinal |
| CK | casein kinase |
| DLK | dual-leucine zipper kinase |
| DRG | dorsal root ganglion |
| DTT | dithiothreitol |
| ECL | enhanced chemiluminescence |
| EDTA | ethylenediaminetetraacetic acid |
| EGTA | ethyleneglycol-bis-(β-aminoethylether)N,N'-tetraacetic acid |
| ERK | extracellular signal-regulated kinase |
| GCK | germinal center kinase |
| GCKR | germinal center kinase-related kinase |
| GLK | germinal center-like kinase |
| GSK | glycogen synthase kinase |
| GST | glutathione S-transferase |
| HGK | HPK/GCK-like kinase |
| HRP | horseradish peroxidase |
| IF | intermediate filament |
| IL | interleukin |
| JIP | JNK/SAPK interacting protein |
| JNK | cJun N-terminal kinase (also called SAPK) |
| JNKK1 | JNK/SAPK kinase-1 (also known as SEK1/MKK4) |
| JNKK2 | JNK/SAPK kinase-2 (also known as MKK7) |
| kDa | kilodalton |
| KHS | kinase homologous to STE20/SPS-1 |
| KRS | kinase responsive to stress or kinase responsive to ras |
| KSP | lysyl-seryl-prolyl |
| MAPK | mitogen-activated protein kinase |
| MAPKK | mitogen-activated protein kinase kinase |
| MAPKKK | mitogen-activated protein kinase kinase kinase |
| MAPKAP | MAPK-activated protein |
| MEK | MAPK/ERK kinase |
| MEKK | MEK kinase |
| MKK | MAPK kinase |
| MKP | MAP kinase phosphatase |
| MLK | mixed-lineage kinase |
| MP-1 | MEK partner-1 |
| MTK | MAP three kinase |
| N-terminal | amino terminal |
| NIK | Nck interacting kinase |

| | |
|---|---|
| NF | neurofilament |
| NFL | neurofilament light subunit |
| NFM | neurofilament mid-sized subunit |
| NFH | neurofilament heavy subunit |
| NGF | nerve growth factor |
| PAGE | polyacrylamide gel electrophoresis |
| PAK | p21-activated kinase |
| PBS | phosphate-buffered saline |
| PKA | protein kinase A/ cyclic AMP-dependent protein kinase |
| PKC | protein kinase C |
| PP2A | protein phosphatase-2A |
| PTK | see SPRK |
| PVDF | polyvinylidene fluoride |
| SAPK | Stress-activated protein kinase |
| SAPKα | Stress-activated Protein kinase-α/ cJun N-terminal kinase 2 (JNK2) |
| SAPKβ | Stress-activated Protein kinase-β/ cJun N-terminal kinase 3 (JNK3) |
| SAPKγ | Stress-activated Protein kinase-γ/ cJun N-terminal kinase 1 (JNK1) |
| SDS | sodium dodecyl sulfate |
| SEK1 | SAPK/ERK kinase (also known as MKK4/JNKK1) |
| SEM | standard error of the mean |
| SOD | superoxide dismutase |
| SPRK | SH3 domain-containing, proline-rich kinase |
| TAK | TGF-β-activated kinase |
| TBS | Tris-buffered saline |
| Triton | Triton X-100 |
| TNF | tumor necrosis factor |
| ZPK | leucine-zipper protein kinase |

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# Chapter 1: Introduction

## Introduction

Neurofilaments (NFs) are intermediate filaments (IFs) that contribute to the structural scaffolding of most neurons. NFs are especially important constituents of large myelinated axons where they are the most abundant cytoskeletal structure. Since perturbations in NF expression, organization, axonal transport and phosphorylation are observed in neurodegenerative disorders, such as amyotrophic lateral sclerosis (ALS), Alzheimer's disease and Parkinson's disease, it has been postulated that NFs may play a role in the development of these pathologies (Trojanowski and Lee, 1994; Julien and Mushynski, 1998; Morrison et al., 1998). In addition, animal models of diabetic neuropathy in sensory neurons exhibit abnormalities in NF axonal transport, and phosphorylation (Fernyhough et al., 1999).

## The Intermediate Filament Superfamily

NFs are members of the IF superfamily (reviewed in Fuchs and Weber, 1994). The term "intermediate filament" derives from their diameter of 10 nm, which is intermediate between those of thicker filaments (microtubules, 25 nm; myosin thick filaments, 16 nm) and the thin actin filaments (~6nm). IFs are widely expressed in eukaryotic cells and are relatively abundant in the cells in which they are expressed. Although the IF content of most cells represents around 1% of the total cell protein, certain cell types, such as epidermal keratinocytes and neurons, express relatively large amounts of IFs (up to 85% of total protein in keratinocytes) (Fuchs and Cleveland, 1998). There are five types of IF proteins which are classified based on gene intron position and amino acid sequence homology in the α-helical domain. Type I and type II IF proteins are represented by the acidic and basic keratins, respectively. The type III group of IF proteins includes vimentin, glial fibrillary acidic protein, desmin and peripherin. NF proteins, α-internexin and nestin are in the type IV group, while nuclear lamins make up the type V IF proteins (reviewed in Fuchs and Weber, 1994).

IF proteins have a common domain organization. They all contain a central, highly conserved, α-helical "rod" domain of approximately 310 amino acids. The rod

domain is flanked by globular amino (N)-terminal "head" and carboxy (C)-terminal "tail" regions of variable length and primary structure. The α-helical domain displays a heptad repeat pattern in which apolar amino acid residues are located at the first and fourth positions of consecutive blocks of seven amino acids. These apolar residues form a hydrophobic seam enabling IF proteins to associate in parallel and form coiled-coil dimers through hydrophobic interactions. The central rod domain can be further divided into α-helical segments referred to as: helix 1A, 1B, 2A, 2B. The most highly conserved sequences in the rod domain are those in the initial segment of helix 1A and at the end of helix 2B. A monoclonal antibody that binds the latter sequence universally recognizes IF proteins (Pruss et al., 1981). Intercalated between the α-helices are three non-helical linker sequences, L-1, L1-2 and L-2, which contain Pro or Gly residues. However, NFM and NFH lack the L-1 linker region, and thus contain an uninterrupted α-helical stretch in helix I (see Fig. 1-1). This may in part explain why these two NF subunits cannot form homopolymers or heteropolymers with each other (Balin et al., 1991; Balin and Lee, 1991; Ching and Liem, 1993; Lee et al., 1993).

IF proteins can be post-translationally modified by phosphorylation and/or glycosylation. Phosphorylation occurs at Ser and, to a lesser extent, at Thr residues in the head and tail domains (reviewed in Ku et al., 1996) but not in the rod domains (Steinert et al., 1982). IF phosphorylation is thought to affect many processes including IF assembly, organization, subcellular localization, interactions with other proteins, and degradation (Ku et al., 1996).

**Neurofilaments**

NFs are the major components of large myelinated axons and are found in most neurons. NFs are composed of three subunits (Hoffman and Lasek, 1975) referred to as heavy (NFH), medium (NFM) and light (NFL) (reviewed in (Lee and Cleveland, 1996). In humans, these subunits have predicted sizes of 110 (Lees et al., 1988), 102 (Myers et al., 1987) and 62 (Julien et al., 1987) kDa for NFH, NFM and NFL, respectively, while those of the rat are 115 (Chin and Liem, 1990), 95 (Napolitano et al., 1987), and 63 (Julien et al., 1987; Chin and Liem, 1989) kDa, respectively. NFs are the most abundant IFs in most central neurons and some peripheral neurons. In view of their widespread

expression in the nervous system and based on the finding that neurons expressing high somatodendritic levels of NFs appear to be selectively vulnerable in Alzheimer's disease, ALS and Parkinson's disease (reviewed in Morrison et al., 1998), NFs have been the focus of studies aimed at defining their potential role in disease mechanisms (Julien and Mushynski, 1998).

*Neurofilament Assembly*

As with other IF proteins, NF subunits form coiled-coil dimers as the first step in assembly (Balin et al., 1991; Balin and Lee, 1991; Ching and Liem, 1993; Lee et al., 1993; Athlan and Mushynski, 1997). Tetramers are then formed through antiparallel, staggered association between two dimers (Steinert et al., 1982) and subsequent linear and lateral associations lead to the formation of protofilaments, protofibrils, and ultimately, IFs.

Transfection studies have demonstrated that NFs are obligate heteropolymers requiring NFL and NFM or NFH for assembly (Ching and Liem, 1993; Lee et al., 1993). Heterodimers composed of NFL/NFM or NFL/NFH as well as NFL homodimers form *in vitro* and *in vivo*. However, NFM and NFH can neither homodimerize nor form heterodimers together (Athlan and Mushynski, 1997).

Transfection studies with truncated NFM and NFL have demonstrated that the C-terminal tail domains of these two subunits are not required for filament assembly (Ching and Liem, 1993) although one study found that the NFL tail domain is indispensable (Gill et al., 1990). Similar studies with deletion mutants of the NFH and NFM head domains have shown that this domain is required along with the rod domain for co-assembly with NFL (Lee et al., 1993) . The head domain may also be involved in promoting lateral associations between protofilaments (Heins et al., 1993). Together, these studies indicate that the head domain of NF subunits, and possibly the tail domain of NFL, are important for NF assembly.

Transgenic mice lacking one or more NF proteins have provided a definitive insight into which NF subunits are important for NF formation *in vivo*. Studies of gene knockout mice lacking either NFH or NFM have demonstrated that NFs will form with the two remaining subunits (Elder et al., 1998; Rao et al., 1998; Zhu et al., 1998; Jacomy

et al., 1999), although lack of both NFM and NFH, or NFL alone blocks NF formation (Zhu et al., 1998; Jacomy et al., 1999). The indispensable role of NFL in NF formation was also demonstrated in Japanese quail with a nonsense mutation in the NFL gene. The absence of functional NFL in these birds is paralleled by a lack of NFs (Ohara et al., 1993).

*Neurofilament Phosphorylation*

NF proteins are phosphorylated both on their N-terminal head domain and C-terminal tail domain, although the latter is more extensively phosphorylated in NFM and NFH (for reviews, see Nixon and Sihag, 1991; Pant and Veeranna, 1995; Julien and Mushynski, 1998). Abnormal patterns of NF phosphorylation are seen in neuropathological conditions such as ALS, Parkinson's disease and Alzheimer's disease (for reviews, see Lee and Cleveland, 1994; Williamson et al., 1996; Julien and Mushynski, 1998). Aberrant NF phosphorylation is also seen in toxic neuropathies induced by acrylamide (Gold et al., 1988) and β,β'-iminodipropionitrile (IDPN) (Gold et al., 1991). In rat models of diabetes, abnormal NF phosphorylation has been observed in spinal chord (Pekiner and McLean, 1991), sciatic nerve (Terada et al., 1998), and in the perikaryon of sensory neurons (Fernyhough et al., 1999). An understanding of the components involved in normal and aberrant NF phosphorylation as well as their effects on NF homeostasis may therefore be important for the elucidation of pathogenic mechanisms underlying these disorders.

*Head domain phosphorylation*

The N-terminal head domains of NFL and NFM are phosphorylated at multiple sites (see Table 1-1 and Fig. 1-1). Several phosphorylation sites in the head domain of NFL have been identified: Serines-12, -27, -33 and -51 in porcine NFL (Gonda et al., 1990); Serine-55 in murine NFL (Sihag and Nixon, 1991); Serines-26 and -57 in bovine NFL (Nixon et al., 1989); and Serines-2, -12, -41, -49, -43, and -55 in rat NFL (Giasson et al., 1996; Cleverley et al., 1998). Head domain phosphorylation has also been observed at several Ser residues in NFM (Sihag and Nixon, 1990; Cleverley et al., 1998; Sihag et al.,

1999), although phosphorylation sites in the head domain of NFH have not yet been characterized.

*Protein kinases and protein phosphatases implicated in head domain phosphorylation*

Several second messenger-dependent kinases have been implicated in phosphorylation of the head domains in NFM and NFL. Ser-2 and -55 in NFL were demonstrated to be phosphorylated by protein kinase A (PKA) (Sihag and Nixon, 1991; Giasson et al., 1996) and three additional Ser residues in the NFL head domain may also be targeted by PKA (Cleverley et al., 1998). Protein kinase C (PKC) is also thought to be involved in phosphorylating the head domain of NFL at Ser-12, -27, -33 and -51 (Gonda et al., 1990). PKA and PKC are also putative head domain kinases for NFM (Giasson and Mushynski, 1998; Sihag et al., 1999).

In addition to PKA and PKC, rho-kinase has also been implicated in head domain phosphorylation of NFL. Rho-kinase, a target of the small GTP-binding protein, rho, has been shown to phosphorylate Ser-26 and -57 in NFL (Hashimoto et al., 1998).

Although several kinases have been shown to phosphorylate the head domains of NFL and NFM, much less is known about the phosphatases that dephosphorylate these sites. Removal of NFL head domain phosphates was shown to involve protein phosphatase-2A (PP2A) (Sacher et al., 1992; 1994; Giasson et al., 1996) and the phosphorylation state of Ser-2 and -55 in rat NFL is thought to depend on the antagonistic actions of PKA and PP2A (Giasson et al., 1996). PP2A also appears to be involved in removal of head domain phosphates in NFM (Giasson and Mushynski, 1998). This extensive involvement of PP2A no doubt reflects its intimate association with NFs (Saito et al., 1995).

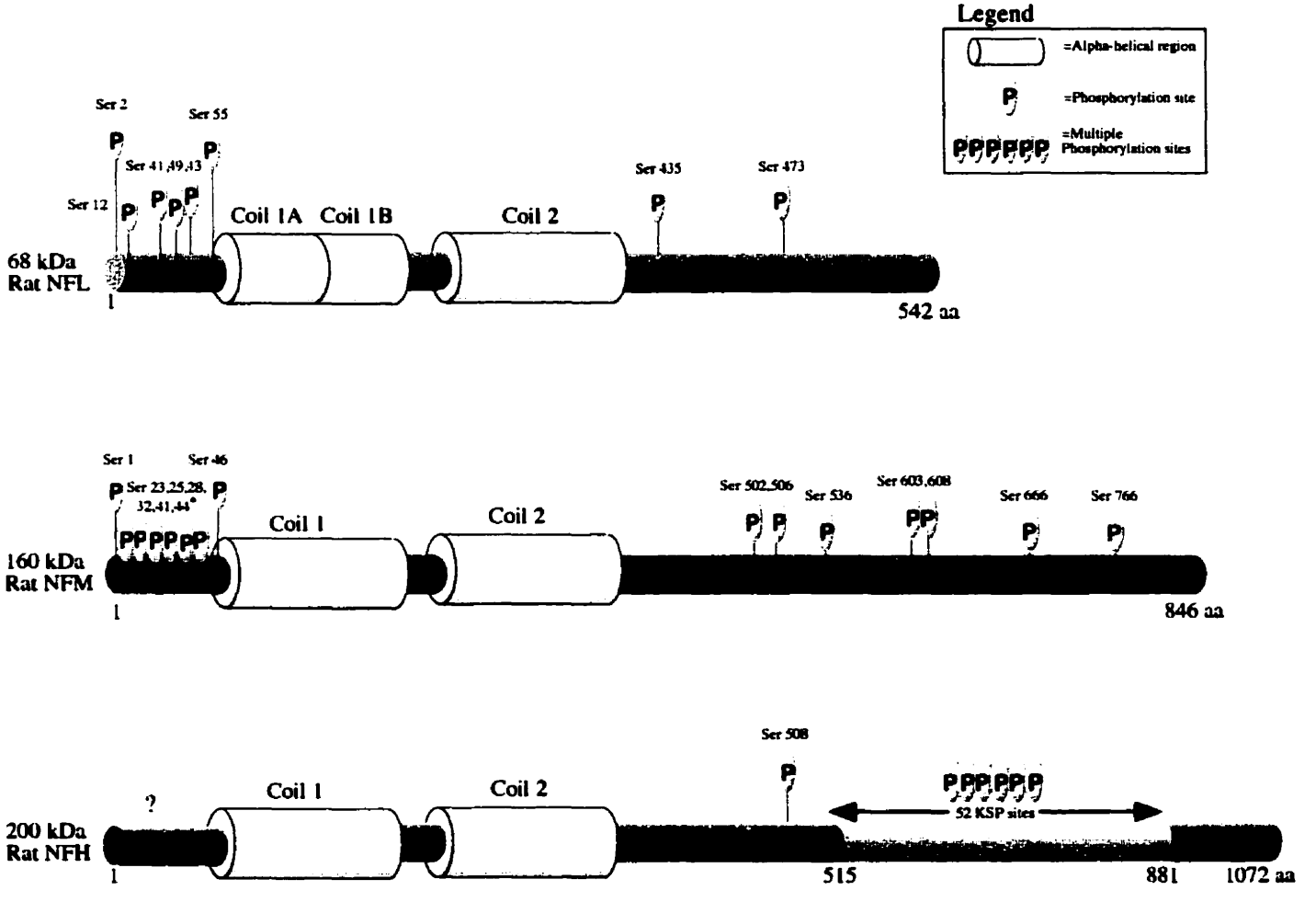
**Table 1-1: Phosphorylation sites in the N-terminal head domain of the neurofilament proteins.**

| Neurofilament Subunit: | Head domain phosphorylation site(s): | Neurofilament source: | Reference(s): |
|---|---|---|---|
| NFM | Ser-22,-23,-25, -28,-32 | mouse spinal cord and retinal ganglia | Sihag et al., 1999 |
| | Ser-1,-46 | rat brain and recombinant rat NFs | Cleverley et al., 1998 |
| NFL | Ser-12,-27,-33, -51 | swine brain | Gonda et al., 1990 |
| | Ser-55 | mouse retinal ganglia | Sihag and Nixon, 1991 |
| | Ser-2 | rat DRG cultures | Giasson et al., 1996 |
| | Ser-26,-57 | bovine brain | Hashimoto et al., 1998 |
| | Ser-12,-41,-43, -49, -55 | rat brain and recombinant rat NFs | Cleverley et al., 1998 |

**Figure 1-1: Domain organization and phosphorylation sites in rat neurofilament proteins.**

Neurofilaments are composed of three subunits of low- (NFL), middle- (NFM) and high- (NFH) molecular mass (details shown pertain to rat neurofilaments). The three neurofilament subunits are composed of non-helical head and tail domains which flank a central α-helical rod domain. The central rod domains of NFM and NFH are subdivided into coils 1 and 2 while the central rod domain of NFL can be subdivided into coils 1A, 1B and 2. Coils are separated by non-helical linker regions. The head domains of NFM and NFL are phosphorylated although phosphorylation sites for NFH have yet to be identified (see Table 1-1). All three neurofilament proteins are phosphorylated in their tail domains (see Table 1-2). The region of NFH encompassing the KSP repeats is extensively phosphorylated (see text for references).

**Figure 1-1: Domain organization and phosphorylation sites in rat neurofilament proteins.**



*Role of head domain phosphorylation*

Phosphorylation of the head domain of NF subunits may regulate NF assembly. Head domain phosphorylation of NFL is thought to inhibit assembly and promote disassembly of NFs (Sihag and Nixon, 1991; Giasson et al., 1996). Phosphorylated NFL is assembly incompetent but can be assembled into filaments once it is dephosphorylated by PP2A (Saito et al., 1995). Filament disassembly was also shown to be promoted when NFL was phosphorylated either by PKA (Giasson et al., 1996), by PKC (Gonda et al., 1990) or by rho-kinase (Hashimoto et al., 1998). Treatment of cultured neurons with low concentrations of the PP2A inhibitor, okadaic acid, has been shown to cause NF fragmentation and this effect is enhanced by concomitant activation of PKA (Sacher et al., 1992; 1994; Giasson et al., 1996).

Recent studies of transgenic mice expressing mutant NFL in which Ser-55 was replaced by an Asp demonstrated a defect in NF assembly and accumulations of perikaryal NFs despite unchanged NF expression levels (Gibb et al., 1998). This study supports the hypothesis that phosphorylation/dephosphorylation of NFL on Ser-2 and Ser-55 regulates NF assembly and is crucial for proper assembly and function of NFs (Nixon and Sihag, 1991; Giasson et al., 1996).

Other observations that led to the formulation of a hypothesis linking head domain phosphorylation of NFL with regulation of NF assembly included the observation that perikaryally-incorporated phosphate residues are removed or undergo turnover as NFs are transported into the axon (Nixon and Lewis, 1986; Nixon et al., 1987; 1989). In addition, studies with retinal ganglion cells indicated that a significant portion of NFL remained Triton soluble for up to 6 hours after its synthesis (Nixon et al., 1989). Based on these observations and previous evidence demonstrating that NFL head domain phosphorylation inhibits assembly, it was proposed that NFL is phosphorylated on its head domain immediately after synthesis in the perikaryon in order to prevent premature NF assembly (Nixon and Sihag, 1991). This would allow adequate time for newly synthesized NFM and NFH subunits to properly dock and be integrated into NFs. The high initial turnover rate of NFL head domain phosphate residues may permit reversible polymerization of NF subunits until the most stable stoichiometric configuration is achieved (Nixon and Sihag, 1991).

*Tail domain phosphorylation*

The three NF subunits differ markedly in the size and sequence of their C-terminal tail domains. NFL possesses a highly acidic tail domain with many Glu residues in a section referred to as the "E segment" (Shaw, 1991). NFM, which has a longer tail domain than NFL, contains two "E segments" and other segments rich in Glu and Lys residues. All three NF subunits were shown to contain multiple phosphorylation sites, although the number of phosphate moieties in NFM and NFH were particularly high (Julien and Mushynski, 1982). This is because the tail domains of both NFM and NFH contain multiple Lys-Ser-Pro (KSP) motifs which are targets for Ser-phosphorylation by Pro-directed kinases. NFM in different species has between 5 and 12 KSP motifs (Levy et al., 1987; Myers et al., 1987; Napolitano et al., 1987). There are 43-44 KSP motifs in the human NFH tail domain (Lees et al., 1988; Figlewicz et al., 1994) and over 50 in the tail domain of murine and rat NFH (Julien et al., 1988; Chin and Liem, 1990).

Many NF tail domain phosphorylation sites have been identified (see Fig. 1-1 and Table 1-2) (reviewed in Nixon and Sihag, 1991; Pant and Veeranna, 1995; Ku et al., 1996). The tail domain KSP sites in NFM and NFH are reported to be extensively phosphorylated in several species although only two phosphorylation sites have been identified in the NFL tail domain, Ser-473 (Xu et al., 1990; Nakamura et al., 1999) and Ser-435 (Cleverley et al., 1998).

The tail domain in chicken NFM was found to be phosphorylated at as many as 19 sites (Hollander et al., 1996; Bennett and Quintana, 1997; Shaw et al., 1997) while 7 sites have been identified in the tail domain of rat NFM (Xu et al., 1992; Betts et al., 1997). The NFH tail domain is even more extensively phosphorylated, with 25-50 phosphate residues in axonal NFH (Julien and Mushynski, 1982; Elhanany et al., 1994; Jaffe et al., 1998).

**Table 1-2: Phosphorylation sites in the C-terminal tail domain of the neurofilament proteins.**

| Neurofilament subunit: | Tail domain phosphorylation site(s): | Neurofilament source: | Reference(s): |
|---|---|---|---|
| NFH | Ser-508,-516,-522, -782,-788,-802, -808,-816,-827, -852,-860,-880 | rat spinal cord | Elhanany et al., 1994 |
| | (38 sites) | human recombinant protein | Jaffe et al., 1998 |
| NFM | Ser-502,-506,-536, -603,-608,-666 | rat spinal cord and DRG cultures | Xu et al., 1992 |
| | Ser-502,-506,-603, -666 | bovine brain | Yang et al., 1995 |
| | Ser-502,-528, -536 | chicken spinal cord, sensory neuron cultures and recombinant proteins | Hollander et al., 1996 |
| | Ser-464*,-471*, -502,-528,-534 | rat recombinant protein(*) and chicken brain | Shaw et al., 1997 |
| | Ser-603,-608,-666, -766 | rat brain | Betts et al., 1997 |
| | Ser-573,- 578,-582, -586,-623,-629, -634,-640,-646, -652,-658,-670, -676,-682,-688, -694,-712,-718,-787 | chicken sensory neuron cultures | Bennett and Quintana, 1997 |
| NFL | Ser-473 | rat spinal cord and DRG cultures | Xu et al., 1990; Nakamura et al., 1999 |
| | Ser-435 | rat brain | Cleverley et al., 1998 |

*Protein kinases and protein phosphatases implicated in tail domain phosphorylation*

NFH exhibits a distinct regional phosphorylation pattern in neurons (Sternberger and Sternberger, 1983; Lee et al., 1987). The tail domain of perikaryal NFH is hypophosphorylated and becomes hyperphosphorylated once NFH enters and is transported along the axon (Nixon et al., 1987). This regional phosphorylation pattern may be maintained by one or more of several mechanisms. For example, it may be that the KSP kinases responsible for phosphorylating sites in the tail domain of NFM and NFH are differentially localized in the neuron. Alternatively, the KSP kinases may be ubiquitously expressed throughout the neuron, while kinase inhibitors, activators or signaling pathways are differentially localized in the axonal and perikaryal compartments. It is also possible that KSP phosphatases are responsible for the topographically distinct phosphorylation pattern of NFH due to differential distribution. Evidence has been presented indicating that different multimeric complexes responsible for NF phosphorylation exist in the cell body and axonal compartments of the squid giant axon (Grant et al., 1999) and rat brain (Veeranna et al., 2000).

Pro-directed kinases that may be involved in phosphorylating the KSP repeats in the tail domain of NFM and NFH include: cyclin-dependent kinase-5 (cdk5) (Veeranna et al., 1995; Bajaj and Miller, 1997), glycogen synthase kinase-3 (GSK-3) (Guan et al., 1991; Yang et al., 1995; Bajaj and Miller, 1997), extracellular-signal regulated kinase1/2 (ERK1/2) (Roder and Ingram, 1991; Veeranna et al., 1998; Li et al., 1999) and stress-activated protein kinases (SAPKs)/ cJun N-terminal kinases (JNKs) (Giasson and Mushynski, 1996; 1997; Brownlees et al., 2000). Potential KSP phosphatases have been less extensively studied although cdk-5 sites have been shown to undergo dephosphorylation by PP2A (Veeranna et al., 1995).

Casein kinase (CK) I and II have also been implicated in tail domain phosphorylation of NFs. Ser-473 of NFL is apparently the preferred site for CK II although both CK I and CK II can phosphorylate this residue (Nakamura et al., 1999). In the NFM tail domain CKI has been shown to phosphorylate Ser-464, -471, -502, -528 and -534 (Shaw et al., 1997).

*Functions of tail domain phosphorylation*

As noted above, most NF tail domain phosphorylation occurs on the C-terminal domains of NFH and NFM. Functional roles for tail domain phosphorylation of NFs include protection from proteolysis (Goldstein et al., 1987; Pant, 1988; Elhanany et al., 1994) and regulation of NF axonal transport rate (for review, see Nixon and Sihag, 1991). Tail domain phosphorylation of NFH and NFM may also affect NF spacing and crossbridging to other axonal components although this is less well-established (Hirokawa and Takeda, 1998).

Goldstein et al. (1987) demonstrated that the phosphorylated form of NFH was protected from proteolysis compared to the dephosphorylated form. All three NF proteins were later shown to be less sensitive to calpain-mediated degradation in their phosphorylated forms as compared to their dephosphorylated forms (Pant, 1988). Another study linking the phosphorylation state of NF proteins with stability demonstrated that a highly phosphorylated fragment from the tail domain of NFH was resistant to proteolysis and that the latter resistance was abolished by dephosphorylation of the fragment (Elhanany et al., 1994). These results suggest that the phosphorylation state of NFs, and of the NFH tail-domain in particular, contribute to NF protein stability.

A second possible role for NF tail domain phosphorylation is in regulating the rate of NF axonal transport. Correlative evidence has indicated that the extent of NF phosphorylation is inversely proportional to the rate of NF axonal transport (Watson et al., 1991; Nixon et al., 1994a) and that phosphorylated NFs are preferentially associated with a stationary pool of NFs (Lewis and Nixon, 1988). This hypothesis was tested and confirmed by the use of phosphatase inhibitors (Jung and Shea, 1999). Specifically, increased C-terminal phosphorylation of NFH was directly linked to a decreased rate NF transport (Jung et al., 2000). One possible explanation for this phenomenon is that phosphorylation of NFH regulates its association with NF axonal transport machinery (Lee and Cleveland, 1996). Recent evidence indicates that NFs may associate with kinesin and that this association is dependent on NF phosphorylation state (Yabe, et al., 2000).

In addition to its phosphorylation state, the level of NFH can also effect the rate of NF axonal transport. A decrease in the rate of NF axonal transport during development

coincides with the appearance of NFH (Willard and Simon, 1983; Nixon et al., 1994a). Transgenic mice with excess or deficient NFH expression have reduced or accelerated NF transport rates, respectively (Eyer and Peterson, 1994; Collard et al., 1995a; Marszalek et al., 1996; Zhu et al., 1998). Since the reduced axonal transport of NFs exhibited by human NFH-expressing mice can be reversed by co-expression of high levels of NFL (Meier et al., 1999), the relative stoichiometry of the NF subunits (and not simply NFH levels) may be an important factor in regulating NF assembly and transport. In summary, both the level and phosphorylation state of NFH are thought to affect the rate of NF transport in axons.

It has been proposed that tail domain phosphorylation may affect axonal caliber. Support for this idea stems from a study of the *trembler* (dysmyelinating mutant) mouse, which shows decreased phosphorylation of NFH, increased NF density and decreased axonal diameter (de Waegh et al., 1992). In addition, comparisons of the interfilament distance in myelinated versus nonmyelinated axonal segments demonstrate increased NF spacing and axonal caliber in the myelinated segments most highly reactive for NF phosphoepitopes (Cole et al., 1994; Hsieh et al., 1994; Nixon et al., 1994b).

Additional studies have shown that adequate NF levels are also important for normal axonal caliber (Yamasaki et al., 1991; Zhu et al., 1997; Elder et al., 1998; 1999a; 1999b). A strain of mutant quail (Yamasaki et al., 1991) as well as NFL knockout mice (Zhu et al., 1997) lack axonal NFs and exhibit decreased axonal caliber. Transgenic mice expressing increased amounts of NFM display reduced axonal NFH levels and reduced axonal caliber (Wong et al., 1995). Mice engineered to express NFH-lacZ fusion protein or human NFH show a lack of axonal NFs which correlates with a reduction in axonal caliber (Cote et al., 1993; Eyer and Peterson, 1994). Increases in NFH have also been correlated with increased axonal caliber in transgenic mice (Marszalek et al., 1996).

On the other hand, studies of transgenic mice lacking NFH have not provided unequivocal support for the notion that NFH is involved in maintaining axonal caliber (Hirokawa and Takeda, 1998). NFH-deficient mice engineered by Zhu et al. (1998) and Rao et al. (1998) showed only a slight to moderate reduction in axonal caliber in large myelinated axons. A third group found that axonal caliber in NFH-deficient mice did

dramatically decrease and concluded that NFH is a major determinant of axonal caliber, at least for large diameter axons (Elder et al., 1998).

*Glycosylation of neurofilament proteins*

The head domains of all three NF subunits contain O-Linked N-acetylglucosamine residues (Dong et al., 1993) . Glycosylation also occurs on the NFM and NFH tail domains (Dong et al., 1996). This O-linked glycosylation of Ser or Thr residues is as dynamic and perhaps as widespread as phosphorylation, and may be involved in modulating NF assembly (Haltiwanger et al., 1992).

**The Mitogen Activated Protein Kinase Family**

As stated above, SAPKs and ERKs are Pro-directed kinases that have been implicated in phosphorylation of the NFH and NFM tail domains, which contain an abundance of KSP motifs. Both SAPKs and ERKs belong to the mitogen-activated protein kinase (MAPK) family and target consensus sequences that resemble sites in the NFH tail domain (Giasson et al., 1996; Veeranna et al., 1998; Brownlees et al., 2000).

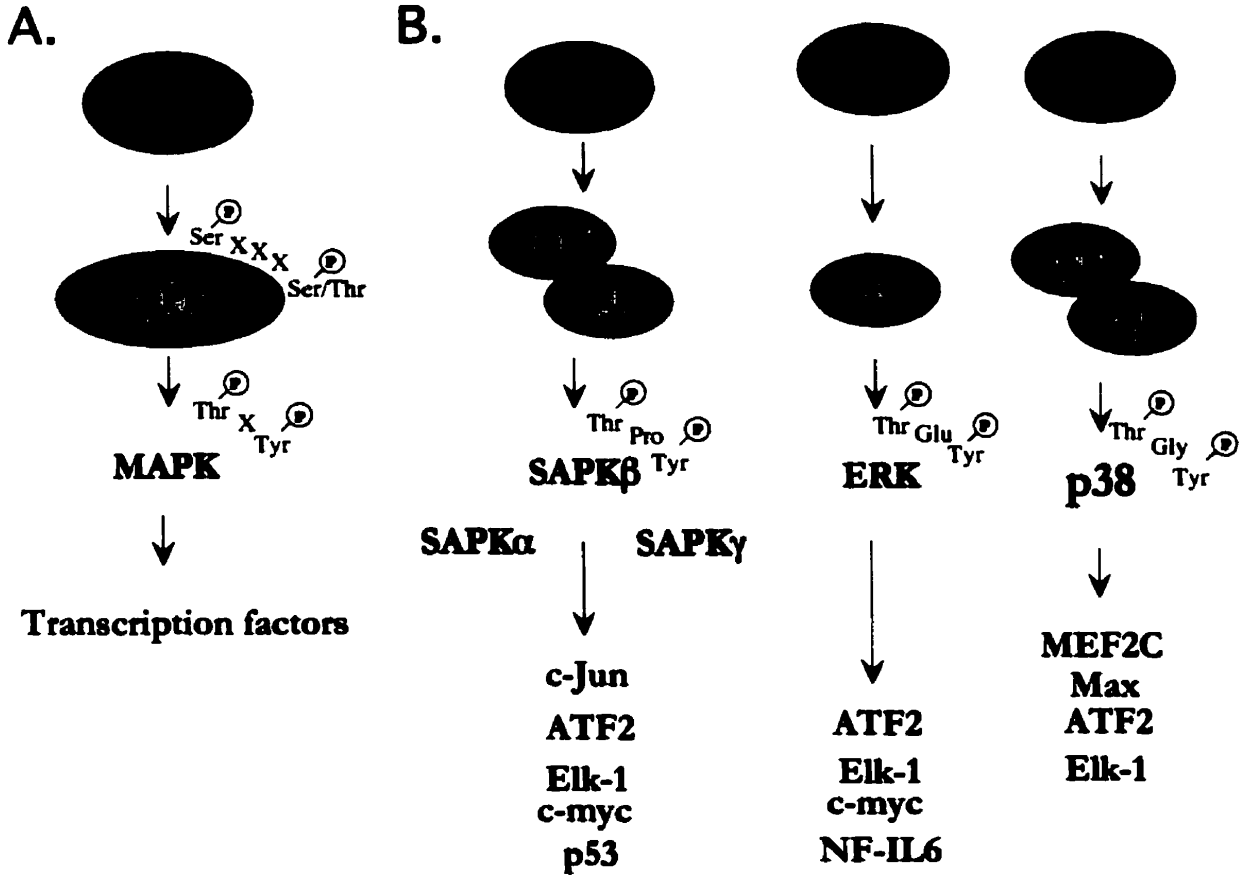
MAPKs play important roles in cellular responses to extracellular stimuli and are characteristically activated by signaling pathways involving a MAPK kinase kinase (MAPKKK) and a MAPK kinase (MAPKK) as immediate upstream elements (Fig. 1-2A) (reviewed in Minden and Karin, 1997). The three best characterized MAPK family members in mammalian cells are the p38 kinases, ERK1 and ERK2 (Cobb et al., 1991a; 1991b) and SAPKs (also referred to as JNKs) (Hibi et al., 1993; Derijard et al., 1994; Kyriakis et al., 1994). More recently discovered members of the MAPK family include ERK3 (Cheng et al., 1996) and ERK 5 (Zhou et al., 1995).

MAPKs mediate cellular responses to a wide range of extracellular stimuli through phosphorylation of substrates such as transcription factors. The SAPKs have many putative functions including regulation of cell survival, apoptosis, differentiation, proliferation, and certain developmental processes. In particular, SAPKs have been implicated in several models of neuronal apoptosis both during development (Kuan et al., 1999) and in response to stress (Yang et al., 1997). An understanding of SAPK regulation may therefore be important for the elucidation of pathogenic mechanisms underlying certain neurodegenerative disorders.

**Figure 1-2: Schematic representation of the three main MAPK pathways in mammals.**

There are three important groups of MAPKs in mammalian cells, the SAPKs, the ERKs and the p38 kinases. Each MAPK is activated through a signal transduction pathway resulting from the sequential phosphorylation of a MAPKKK, a MAPKK, and a MAPK (see **A**). Identified MAPKKKs for each of the three MAPK cascades are shown in **B**. Note that although MEKK1 and ASK1 are the MAPKKKs shown for the pathways leading to SAPK and p38 kinase activation, respectively, there are multiple MAPKKKs for these two pathways (see Fig. 1-3). The MAPKKs are activated by dual phosphorylation at a Ser-X-X-X-(Ser/Thr) motif by an upstream MAPKKK. Once activated, MAPKKs phosphorylate their respective MAPK substrates on Thr and Tyr residues contained in a Thr-X-Tyr motif where X is Pro, Glu or Gly for the SAPKs, ERKs or p38 kinases, respectively. The best characterized substrates for MAPKs are transcription factors (shown in **B**) but cytoplasmic substrates have been identified (not shown). See text for references.

**Figure 1-2: Schematic representation of the three main MAPK pathways in mammals.**



*The extracellular signal-regulated kinases*

ERK1 and ERK2 are members of the first group of MAPKs to be discovered in mammalian cells (reviewed in Adler et al., 1997). The ERKs are activated by mitogenic stimuli such as growth factors, which bind to receptor tyrosine kinases. This initiates a signaling cascade resulting in the sequential activation of Ras (Dent et al., 1992; Kyriakis et al., 1992; Avruch et al., 1994), Raf (a MAPKKK) (Marais et al., 1995), MEK1 or MEK2 (MAPKKs) (Seger et al., 1992a; 1992b), and finally, ERK1 or ERK2 (Fig. 1-2) (Cobb et al., 1991a; 1991b). MEK1 and MEK2 are dual specificity MAPK kinases which activate ERK1/2 by phosphorylation of Thr and Tyr in a Thr-Glu-Tyr motif (Seger et al., 1992a). ERK substrates include the transcription factors Elk-1 (Gille et al., 1992; Hill et al., 1993), ATF2 (Abdel-Hafiz et al., 1992), NF-IL6 (Nakajima et al., 1993), and c-myc (Seth et al., 1992; Davis, 1993; Gupta and Davis, 1994). ERK has also been shown to activate CREB kinase which in turn activates the transcription factor CREB (Xing et al., 1996).

In addition to growth factor stimuli, ERKs can also be weakly activated in certain cell types by UVC irradiation (Sachsenmaier et al., 1994), by arsenite (Liu et al., 1996), and by hydrogen peroxide (Guyton et al., 1996). These cellular stressors also activate p38 and SAPKs (see below).

*The p38 kinases*

Unlike the mitogen-activated ERK signaling pathway, p38 and SAPKs are not preferentially activated by mitogens. Instead, p38 kinases and SAPKs are activated by pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), and by cellular stressing agents such as UV light, X-rays, hydrogen peroxide, heat shock, osmotic shock, and growth factor withdrawal (reviewed in Ichijo, 1999). The p38 group of MAPKs are derived from four genes: *p38α*, *p38β*, *p38γ*, and *p38δ* (Han et al., 1994; Lee et al., 1994; Goedert et al., 1997a; Jiang et al., 1997; Stein et al., 1997; Wang et al., 1997b). MAPKKs that activate p38 kinases, by dual phosphorylation at a Thr-Gly-Tyr motif, include MKK3 and MKK6 (see Fig. 1-2) (Derijard et al., 1995; Moriguchi et al., 1996a; 1996b; Stein et al., 1996). MKK4, which activates SAPKs, may also phosphorylate p38 kinases (Sanchez et al., 1994; Derijard et al., 1995; Lin et al.,

1995). MAPKKKs involved in p38 kinase activation pathways include TAK (Yamaguchi et al., 1995). ASK1 (Wang et al., 1996; Ichijo et al., 1997) and MTK1 (Gerwins et al., 1997; Takekawa et al., 1997) (also see Tables 1-3 and 1-3). Since these signaling components also activate SAPKs, there are at present no known MAPKKKs that activate p38 kinases exclusively (Ichijo, 1999). Small G-proteins of the Rho family, rac (rac-1 and rac-2) and cdc42, have also been shown to be upstream activators of both the p38 kinases and the SAPKs (Bagrodia et al., 1995; Coso et al., 1995; Minden et al., 1995; Brown et al., 1996).

Downstream targets of the p38 kinases include the transcription factors ATF2 (Price et al., 1996; Raingeaud et al., 1996), myocyte-enhancer factor-2C (MEF2C) (Han et al., 1997), and Elk-1 (Price et al., 1996). These transcription factors are also phosphorylated by SAPKs (see below). The p38 kinases also activate another kinase, MAP kinase-activated protein kinase-2 (MAPKAP2) (Tan et al., 1996), which phosphorylates the transcription factor CREB and the heat shock protein Hsp25/Hsp27 (Stokoe et al., 1992; Rouse et al., 1994). p38 also phosphorylates Max, the heterodimeric partner of c-myc (Zervos et al., 1995).

*The stress-activated protein kinases*

SAPKs are the products of three genes: *SAPKα* (*JNK2*), *SAPKβ* (*JNK3*) and *SAPKγ* (*JNK1*) (Hibi et al., 1993; Derijard et al., 1994; Kyriakis et al., 1994). Each SAPK can be expressed as a 45 kDa or a 55 kDa species although as many as 10 different SAPK isoforms can arise due to alternative splicing of primary mRNA transcripts (Gupta et al., 1996; Kumagae et al., 1999). The different SAPK isoforms exhibit differing affinities for the transcription factors that normally serve as SAPK substrates (Gupta et al., 1996).

SAPKs are activated by a number of cellular stressors such as osmotic shock (Galcheva-Gargova et al., 1994; Moriguchi et al., 1995), ceramide (Verheij et al., 1996) heat shock (Adler et al., 1995) and UV irradiation (Hibi et al., 1993; Derijard et al., 1994; Adler et al., 1995) as well as by the pro-inflammatory cytokines, TNF-α (Sluss et al., 1994) and IL-1 (Gupta et al., 1996). Activation of SAPKs is effected by dual phosphorylation at a Thr-Pro-Tyr motif by the MAPKKs, MKK4/SEK1 and MKK7 (see Fig. 1-2) (Sanchez et al., 1994; Derijard et al., 1995; Moriguchi et al., 1997; Yao et al.,

1997). There is a large number of MAPKKKs and MAPKKKKs upstream of the MAPKKs (see Tables 1-3 and 1-4, and Fig. 1-3). Unlike MAPKKKs, the MAPKKKK group is homologous in function but not in amino acid sequence.

SAPKs are also termed c-Jun N-terminal kinases (JNKs) due to their ability to phosphorylate the transcription factor c-Jun on serines 63 and 73 (Hibi et al., 1993; Derijard et al., 1994). The Jun family of transcription factors include cJun, JunB and JunD (Angel and Karin, 1991). Jun proteins form dimers with each other, or with Fos or ATF transcription factor family members, to form the AP-1 transcriptional activator. Fos family members, c-Fos, FosB, Fra-1, Fra-2, as well as ATF proteins, ATF-2 and ATF-a, are also components of AP-1 dimers (Angel and Karin, 1991; Leppa and Bohmann, 1999). AP-1 components are all bZIP (basic region leucine zipper) proteins which form homo- or heterodimers that recognize the DNA sequences TGACTCA, TGACGTCA or variants thereof.

Additional transcription factors such as c-myc (Noguchi et al., 1999), Elk-1 (Cavigelli et al., 1995), ATF2 (Gupta et al., 1995) and p53 (Adler et al., 1997) are also phosphorylated by SAPKs.

*Regulation of transcription factor activity by SAPKs*

Phosphorylation of the transcription factors ATF2, Elk-1 and members of the Jun family by SAPK increases their ability to stimulate transcription (Hibi et al., 1993; Derijard et al., 1994; Cavigelli et al., 1995; Gupta et al., 1995; 1996). In contrast, SAPK phosphorylation of another transcription factor, NFAT (Nuclear Factor of Activated T-cells), inhibits nuclear translocation thus blocking its ability to stimulate transcription (Chow et al., 1997).

Another mechanism by which SAPKs can influence transcription factors is by regulating their turnover rate. For example, SAPK has been shown to target the ubiquitination of cJun (Fuchs et al., 1996), p53 (Adler et al., 1997), JunB and ATF2 (Fuchs et al., 1997). Inactive SAPK promotes ubiquitination by binding to these transcription factors while activated-SAPK inhibits their ubiquitination by phosphorylating them. In contrast, SAPK neither associates with nor targets Elk-1 for ubiquitination (Fuchs et al., 1997).

**Table 1-3: MAPK kinases and MAPKK kinases implicated in SAPK activation.**

| | Activation of other MAPKs? | | |
|---|---|---|---|
| | p38 kinases | ERK1/2 kinases | Reference(s): |
| **MAPKKs:** | | | |
| MKK4/ SEK1 | - | - | Sanchez et al., 1994; Derijard et al., 1995 |
| MKK7 | - | - | Moriguchi et al., 1997; Yao et al., 1997 |
| **MAPKKKs:** | | | |
| MEKK1 | - | + | Lange-Carter et al., 1993 |
| MEKK2 | - | + | Blank et al., 1996 |
| MEKK3 | - | + | Blank et al., 1996 |
| MEKK4/ MTK1 | + | - | Gerwins et al., 1997; Takekawa et al., 1997 |
| Tpl-2/Cot | - | + | Aoki et al., 1991; Salmeron et al., 1996 |
| MUK/DLK/ ZPK | - | - | Holzman et al., 1994; Reddy and Pleasure, 1994; Hirai et al., 1996 |
| MLK2/ MST | - | - | Dorow et al., 1995; Hirai et al., 1997 |
| MLK-3/ SPRK/ PTK-1 | - | - | Gallo et al., 1994; Ing et al., 1994; Rana et al., 1996 |
| TAK1 | + | - | Yamaguchi et al., 1995 |
| MAPKKK5 /ASK-1 | + | - | Wang et al., 1996; Ichijo et al., 1997 |
| MAPKKK6 /ASK-2 | - | - | Wang et al., 1998 |

**Figure 1-3: Multiple upstream activators of the SAPKs.**

Multiple upstream MAPKKKKs phosphorylate and activate one or more of the MAPKKKs. MAPKKK activation results in phosphorylation and activation of one or both MAPKKs. MKK7/SKK4/JNKK2 and/or MKK4/SEK1/JNKK1. References for the implicated MAPKKKKs, MAPKKKs and MAPKKs are provided in Tables 1-3 and 1-4.

**Figure 1-3: Multiple upstream activators of the SAPKs.**

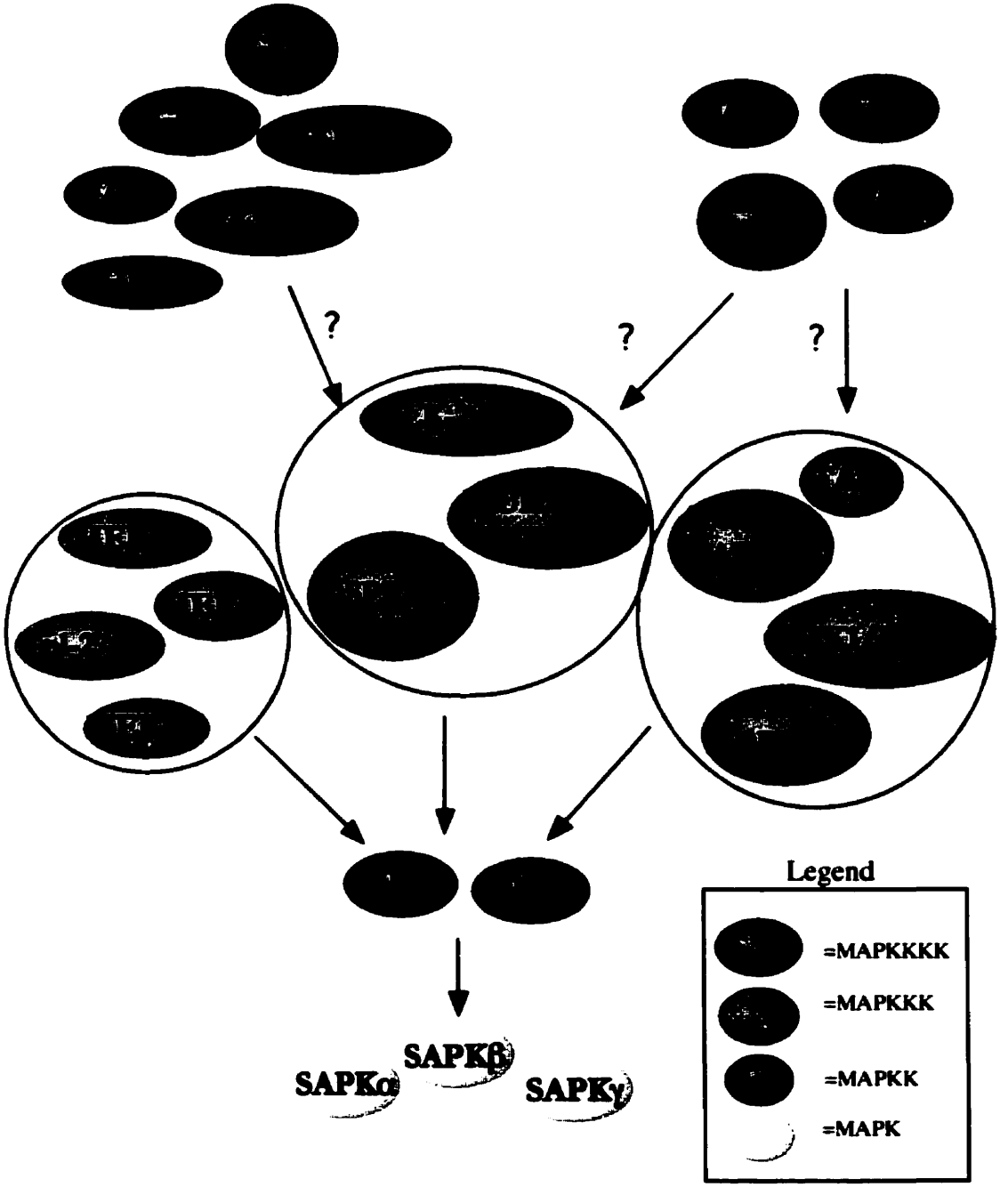


**Table 1-4: MAPKKK kinases implicated in SAPK activation.**

| **Putative MAPKKKKs:** | Reference(s): |
|---|---|
| PAK1,2,3,4 | Manser et al., 1994; Knaus et al., 1995; Abo et al., 1998 |
| GCK | Katz et al., 1994 |
| GCKR/KHS | Shi and Kehrl, 1997; Tung and Blenis, 1997 |
| GLK | Diener et al., 1997 |
| HPK1 | Hu et al., 1996; Kiefer et al., 1996; Wang et al., 1997a |
| NIK/HGK | Su et al., 1997; Yao et al., 1999 |
| Krs-1 | Taylor et al., 1996 |
| MST1/Krs-2 | Creasy and Chernoff, 1995; Taylor et al., 1996 |
| MST3 | Schinkmann and Blenis, 1997 |
| SOK1 | Pombo et al., 1996 |

*Other putative SAPK substrates*

In addition to NFs and the above-mentioned transcription factors, SAPKs may phosphorylate additional cellular substrates. A 46 kDa SAPK is reported to be, at least *in vitro*, the volume-sensitive kinase responsible for phosphorylating the Na-K-2Cl cotransporter in response to osmotic stress (Klein et al., 1999). SAPK has also been shown to phosphorylate Bcl-2 family members (Basu et al., 2000; Kharbanda et al., 2000). In addition, tau protein may be a SAPK substrate although this remains to be shown *in vivo* (Goedert et al., 1997b; Reynolds et al., 2000).

*MAPK signaling modules*

Recent evidence has indicated that signaling elements of the MAPK cascades may be organized into separate signaling modules which respond to distinct stimuli (for review, see Schaeffer and Weber, 1999). Currently four putative MAPK modules have been characterized in mammalian cells (see Fig. 1-4). The first two MAPK modules employ scaffolding molecules which are not themselves signal transduction elements (i.e. they have no intrinsic kinase activity) while a MEKK family member serves as a scaffold in the other two modules.

The first MAPK module is stimulated by certain mitogens and is composed of Ras, Raf, MEK1, ERK and MEK partner-1 (MP-1) (Schaeffer and Weber, 1999). MP-1 is considered to be a scaffolding molecule and lacks kinase activity. MEK1 and ERK1, but not MEK2 or ERK2, interact directly with MP-1 (Fig. 1-4A) (Schaeffer et al., 1998).

The second MAPK module is involved in SAPK activation and, like the first complex, employs a scaffolding molecule devoid of kinase activity, termed JNK/SAPK interacting protein (JIP) (Fig. 1-4B) (Schaeffer and Weber, 1999). Recent studies have indicated that multiple JIP isoforms, resulting from alternative splicing of three gene transcripts (*jip-1, jip-2, jip-3*), exist in mouse and rat brain suggesting that JIP scaffolding may be a widespread phenomenon (Kim et al., 1999; Yasuda et al., 1999; Kelkar et al., 2000). JIP-1 was first characterized as an inhibitor of SAPK activation and was shown to bind to SAPK with high affinity, but not to ERK or p38 kinases (Dickens et al., 1997). Overexpression of JIP-1 was demonstrated to inhibit SAPK translocation to the nucleus and cJun phosphorylation (Dickens et al., 1997; Whitmarsh et al., 1998). Further studies

demonstrated that JIP-1 also interacts directly with MKK7, hematopoetic progenitor kinase-1 (HPK1), and mixed lineage kinases (MLKs), MLK3 and DLK (Whitmarsh et al., 1998). Co-transfection studies where JIP-1 was co-expressed in cells with MLK3 and MKK7 demonstrated enhanced activation of SAPK (Whitmarsh et al., 1998).

The third MAPK module (Fig. 1-4C) consists of MEKK2 interacting directly with MKK7 and SAPK (Cheng et al., 2000). The C-terminal domain of MEKK2 is responsible for interaction with the downstream MAPK elements. The function of the large N-terminal domain remains unknown (Cheng et al., 2000) although it may interact with upstream MAPKKKKs and/or other signaling elements.

The fourth putative MAPK module consists of MEKK1, MKK4 and SAPK (Fig. 1-4D) (Schaeffer and Weber, 1999). It remains to be demonstrated whether this MAPK module functions through sequential interactions between MEKK1-MKK4 and MKK4-SAPK (Xia et al., 1998; Schaeffer and Weber, 1999; Cheng et al., 2000) or whether it consists of a ternary complex containing MEKK1 associated with both MKK4 and SAPK (Xu and Cobb, 1997). Nck interacting kinase (NIK), an upstream MAPKKKK, may also associate directly with MEKK1 (Su et al., 1997).

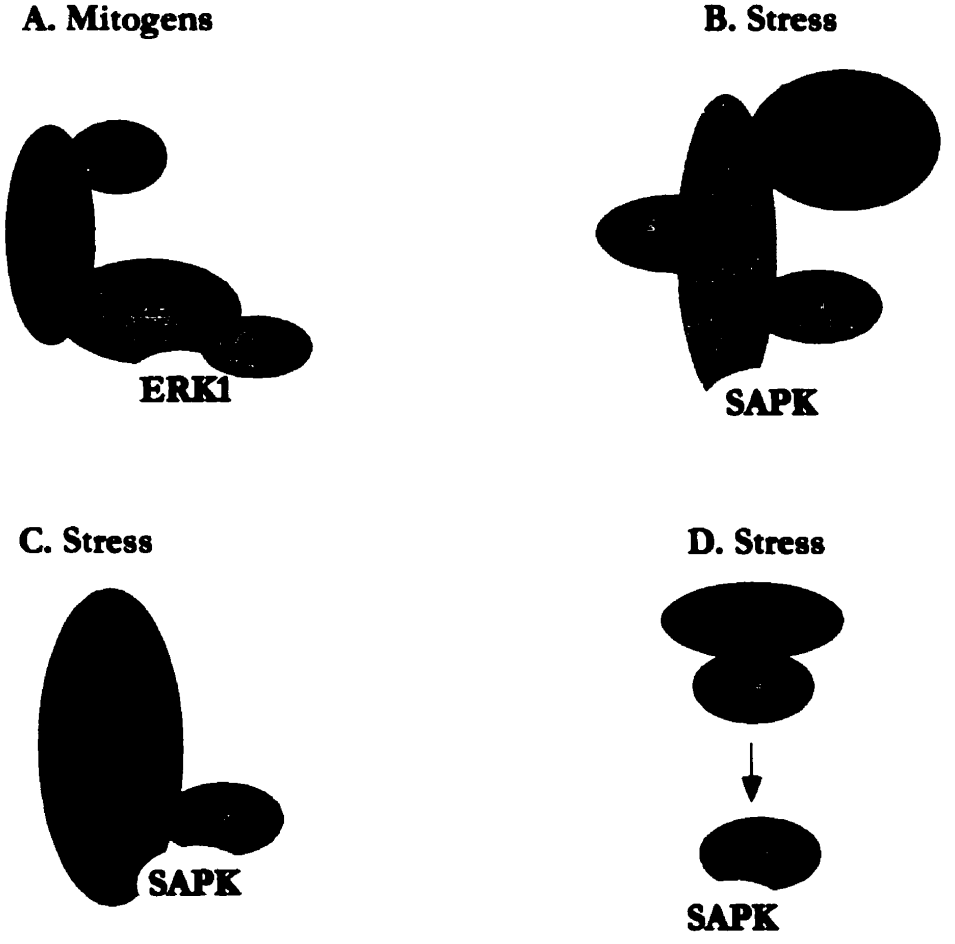
*Role of SAPKs in neuronal apoptosis*

Studies have indicated that SAPKs may play a role in both survival and apoptosis (for review, see Leppa and Bohmann, 1999). In neurons, SAPK activation and/or cJun phosphorylation have frequently been correlated with induction of apoptosis (Estus et al., 1994; Ham et al., 1995; Xia et al., 1995). There is evidence that SAPKs are activated in Huntingtin protein- and amyloid-β-induced models of neuronal apoptosis (Liu, 1998; Kihiko et al., 1999). In addition, one report found that SAPKs were selectively activated in astrocytes, but not motor neurons, in ALS (Migheli et al., 1997).

**Figure 1-4: Four putative mammalian MAPK signaling modules activated by mitogens or by stress.**

Certain mitogenic stimuli activate the Ras-->Raf-->MEK1-->ERK1 pathway (**A**). In this MAPK signaling module MEK partner-1 (MP-1) acts as a scaffold protein by binding to both MEK1 and ERK1 but not MEK2 or ERK2. The following three MAPK modules (**B-D**) lead to SAPK activation and are initiated by various stressors. The JNK/SAPK interacting protein (JIP) binds multiple SAPK signal transduction elements including hematopoetic progenitor kinase (HPK), mixed-lineage kinase-3 (MLK3), MKK7 and SAPK (**B**). MEKK2 forms a signaling complex with MKK7 and SAPK (**C**). MEKK1 can activate MKK4 which may then dissociate from MEKK1 and activate SAPK, although these three proteins may exist in a ternary complex (**D**). See text for references.

**Figure 1-4: Four putative mammalian MAPK signaling modules activated by mitogens or by stress.**



Specific SAPK inhibitors, such as CEP-1347, are the subject of intense study due to their putative neuroprotective properties. CEP-1347 (KT7515) is a 3,9-disubstituted-[(alkylthio)methyl]-K252a derivative (Fig. 1-5) that has been shown to prevent neuronal death both *in vivo* (DiCamillo et al., 1998; Glicksman et al., 1998; Saporito et al., 1998) and *in vitro* (Borasio et al., 1998; Maroney et al., 1998; 1999). CEP-1347 is a specific inhibitor of SAPK activation which does not affect ERKs (Glicksman et al., 1998; Maroney et al., 1998) or p38 kinases (Maroney et al., 1998). In several cell culture models of neuronal death, including trophic factor withdrawal from motorneurons and sympathetic neurons (Maroney et al., 1998; 1999), motorneuron axotomy (Glicksman et al., 1998) and stress-induced neuronal dying in response to oxidative stress and UV light (Maroney et al., 1999). CEP-1347 has been shown to reduce neuronal death. In animals treated with ibotenic acid, an *in vivo* model of excitotoxic damage, CEP-1347 was shown to both prevent neuronal death and, perhaps more importantly, to prevent loss of behavioral function (DiCamillo et al., 1998; Saporito et al., 1998).

Although the neuroprotective effects of CEP-1347 have been demonstrated, it is not likely to be a universal inhibitor of apoptosis. For example, CEP-1347 does not protect cultured neurons against apoptosis induced by Fas Ligand activation or serum withdrawal (Maroney et al., 1999). In fact, studies of gene knockout mice lacking both SAPKα and SAPKγ or the upstream elements, MKK4/SEK1 or MEKK1, have shown that SAPKs may actually be required for survival and/or proliferation in certain cell types and contexts (Nishina et al., 1997; Yujiri et al., 1998; Kuan et al., 1999; Nishina et al., 1999).

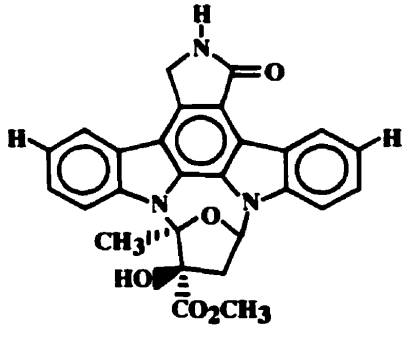
**Objectives and Rationale**

The objective of my thesis project was to extend earlier correlative studies (Giasson and Mushynski, 1996; 1997) by providing direct evidence implicating SAPKs in NFH phosphorylation. This was made possible through the availability of a specific inhibitor of SAPK activation, CEP-1347, from Cephalon, Inc. It has previously been shown that CEP-1347 does not affect ERKs or p38 kinases (Glicksman et al., 1998; Maroney et al., 1998). By testing the effects of CEP-1347 on stress-induced as well as constitutive phosphorylation of NFH, I was able to demonstrate that SAPKs are NF kinases in PC12 cells and cultured DRG neurons. In addition, localized DRG cultures

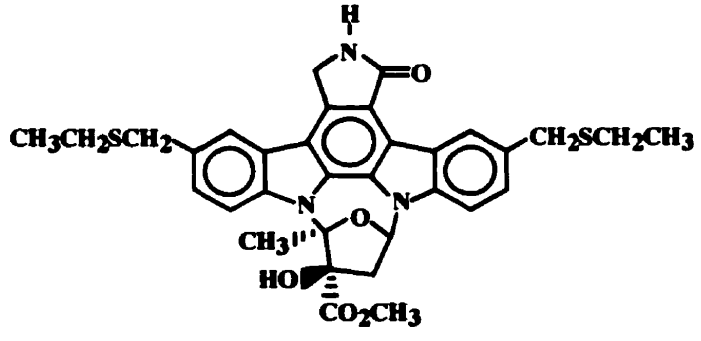
prepared according to established protocols (Giasson and Mushynski, 1997) allowed for the separate analysis of cell body- and axon-enriched fractions. This enabled me to study the response of SAPKs to inhibition by CEP-1347 in order to determine whether SAPK activation pathways in cell bodies and axons are different.

**Figure 1-5: Chemical structure of K252a and its derivative, CEP-1347(KT7515).**

**K252a**




**CEP-1347**



## Chapter 2: Materials and Methods

Neurofilament Antibodies:

Monoclonal antibodies for NFH (N52), NFM (NN18) and NFL (NR4) were purchased from Sigma (Oakville, ON, Canada). SMI-34, from Sternberger Monoclonals (Baltimore, MD), was used to detect highly phosphorylated epitopes in NFH. Monoclonal anti-vimentin antibody was from Roche Diagnostics (Laval, QC, Canada).

Signal Transduction Antibodies:

SAPKβ (JNK3) and MKK4/SEK1 polyclonal antibodies were purchased from StressGen Biotechnologies Corp. (Victoria, BC, Canada). SAPKα (JNK2) (N18) and SAPKγ (JNK1) (C17) were from Santa Cruz Biotechnology Corp. (Santa Cruz, CA). SAPKγ (JNK1) monoclonal antibody was obtained from Pharmingen (Mississauga, ON, Canada). Phospho-specific anti-SAPK/JNK (Thr 183/Tyr 185) polyclonal antibody was purchased from New England Biolabs (Mississauga, ON, Canada).

Secondary Antibodies:

For Western blots analyzed by enhanced chemiluminescence, horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG (Santa Cruz Biotechnologies) were used to label blots probed with monoclonal and polyclonal primary antibodies, respectively. [$^{125}$I]-labelled goat anti-mouse IgG was provided by New England Nuclear (Guelph, ON, Canada). FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was used for immunofluorescence microscopy.

Cell Culture Materials

Nerve Growth Factor (NGF, 2.5 S) was purchased from Prince Laboratories (Toronto, ON, Canada) while extracellular matrix (ECM) was from Sigma and bovine apotransferrin from ICN (Montreal, QC, Canada). Basal media (L-15, Dulbecco's Modified Eagle's medium (DMEM), DMEM/F12), horse serum, fetal bovine serum, bovine serum albumin (BSA) for cell culture, antibiotics, and trypsin were obtained from Life Technologies, Inc. (Burlington, ON, Canada).

Other Materials

N-acetyl-Leu-Leu-norleucinal (CI) was from ICN, MG-132 and PD098059 from Calbiochem (San Diego, CA) and [γ-$^{32}$P]ATP from New England Nuclear. CEP-1347

(KT 7515), an inhibitor of SAPK activation, was kindly provided by Cephalon (West Chester, PA). The inhibitor was stored as a 4 mM stock solution in dimethylsulfoxide (DMSO) and was diluted in DMEM/F12 containing 1% BSA immediately before addition at the desired concentration to the cell cultures. For all cell culture experiments, CEP-1347 was added 1 hr prior to the addition of stressing agents (CI, arsenite, osmotic shock).

**Preparation of Dorsal Root Ganglion Primary Cultures**

The protocol described here for preparation of dorsal root ganglion (DRG) cultures is adapted from Giasson and Mushynski (1996). Untimed pregnant (embryonic day 15-16) Sprague Dawley rats were obtained from Charles River Laboratories (St. Constant, QC, Canada). Under sterile conditions, embryos were removed from their sacs in Leibovitz's (L-15) medium and transferred to a petri dish containing fresh L-15. The spinal column was dissected from each embryo and the spinal cords with associated DRGs were individually removed and transferred to a 60 mm petri dish containing L-15 medium. DRGs were counted as they were plucked from the spinal cords and were collected by centrifugation. Dissociation of DRGs was achieved by 15 min. of trypsinization (0.025% in Hank's BSS) at 37°C, followed by treatment with soybean trypsin inhibitor (5 mg/ml in L-15) and trituration in the latter until clumps were no longer visible. Finally, the dissociated cells were suspended for plating in defined medium consisting of DMEM/F12 containing N1 additive (Giasson and Mushynski, 1996), 0.09% BSA, 10 ng/ml 2.5 S NGF and antibiotics (penicillin/streptomycin). Between 40-50 DRGs were obtained from each embryo and each DRG contains about 5000 sensory neurons.

Localized cultures were prepared to allow for the manual separation of neuronal cell bodies from neurites. To prepare the localized cultures, a small volume (20 µl) containing 15 dissociated DRGs was added to the center of each well in a 6-well culture dish and cells were allowed to attach for 30 min at 37°C before flooding the wells with medium. In these localized cultures, neurites extend radially to form a halo surrounding the cell body mass. The antimitotic agents, 5-fluoro-2'-deoxyuridine and cytosine-β-D-arabinofuranoside, were used at concentrations of $10^{-5}$ M and $10^{-6}$ M, respectively, to rid

the cultures of Schwann cells and most fibroblasts. The protocol involved two 3-day antimitotic treatments, beginning the day after cell plating and separated by a 3-day period in normal medium. The cultures were then maintained in N1 medium (as described above) until their use at 21-25 days in culture. Similarly treated DRG cultures maintained in N1 medium lacking in NGF were used as a source of fibroblasts for determination of fibroblast contamination of the neuronal cultures (Giasson and Mushynski, 1997).

Culture dishes for plating DRGs were coated with rat-tail collagen (50 μg/ml) overnight, washed with water, and then the collagen was cross-linked for 2 hrs with 0.3 mM 1-cyclo-3(2-morpholino-ethly)carbodiimide-metho-p-toluenesulfonate (Sigma) (Macklis et al., 1985). In addition to cross-linked collagen, culture dishes for localized DRG cultures were subsequently coated with ECM (diluted one part in fifty in DMEM/F12) which was added to strengthen neuronal attachment in cultures devoid of non-neuronal cells. The latter were washed with sterile water and allowed to dry completely before cells were plated.

The experimental protocol for preparation of primary cell cultures was approved by the McGill University Animal Care Committee and meets the guidelines of the Canadian Council on Animal Care.

**PC12 Cell Culture**

PC12 cells were obtained from American Type Culture Collection (Rockville, MD) and maintained according to published procedures (Greene and Tischler, 1976; Lindenbaum et al., 1988). The PC12 cells were maintained in DMEM (high glucose) containing 10% heat-inactivated horse serum, 5% fetal bovine serum and antibiotics. PC12 cells slated for NGF treatment were plated in the serum-containing medium for 1 day, washed 3 times with basal DMEM and maintained for 5 days in DMEM containing 0.1% BSA and 50 ng $ml^{-1}$ 2.5 S NGF before experiments were performed.

**Gel Electrophoresis and Western blot analysis**

Cells were washed three times with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$) (PBS) at 37°C then harvested in cold PBS. DRG cell bodies and neurites in localized cultures were manually separated using a

circular punch with a diameter slightly larger than that of the cell body mass. Cell fractions were subsequently solubilized in equal volumes of cell lysis buffer (73.7 mM Tris, 11.7% glycerol, 2.37% sodium dodecyl sulfate) and boiled for 7 min. Aliquots (5-10 µl) from each sample were analyzed for protein content using the Bicinchoninic acid assay according to the Sigma protocol. The Optical Density (OD) at 562.5 nm was recorded for each sample and protein content was determined by comparison with BSA standards. The linear relationship between $OD_{562.5nm}$ and the amount of protein in each BSA standard sample was confirmed by plotting a curve. Samples containing equal amounts of protein were adjusted to contain 5% (v/v) β-mercaptoethanol and resolved on 10% and 5% polyacrylamide gels for SDS-PAGE analysis (Laemmli, 1970) of lower molecular weight (30 kDa to 70 kDa) and higher molecular weight (>100 kDa) proteins, respectively.

Proteins were subsequently transferred electrophoretically to Immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore Corp.) in transfer buffer (48 mM Tris, 39 mM glycine). Membranes were pretreated with 3% non-fat milk powder in Tris buffered saline (TBS)/Tween (20 mM Tris pH 7.7, 137 mM NaCl, 0.1% Tween-20) for 30 min. to block non-specific protein binding sites and then incubated with primary antibodies for 1 hr. The membrane was then washed in TBS/Tween 3-4 times over a period of 30 min. and incubated with secondary antibody for 1 hr. Following 3-4 washes in TBS/Tween, the membrane was developed with the Renaissance Western Blotting Detection Kit (NEN). For analysis of phospho-SAPK, Western blots were blocked with 5% BSA in TBS/Tween instead of the non-fat milk powder.

**Preparation of GST-cJun**

The cDNA encoding glutathione-S-transferase (GST)-cJun (amino acids 1-89) fusion protein was incorporated into a pGEX-2T construct (provided by Dr. J. Woodgett). *Escherichia coli* expressing the GST-cJun recombinant protein was incubated at 37°C with vigorous shaking in 1L of Terrific Broth (12 g Bacto Tryptone, 24 g Bacto Yeast (DIFCO) per liter, plus 0.4% gylcerol, 34 mM $KH_2PO_4$ and 144 mM $K_2HPO_4$) (TB) in the presence of 100 µg/ml carbencillin. When the culture reached late log phase, as indicated by an $OD_{600nm}$ between 0.7 and 0.8, expression of the GST-cJun protein was

induced by adding 1 mM isopropylthiogalactoside. The culture was then incubated at 37°C with shaking for 3 hr.

The bacteria were collected by centrifugation and extracted with bacterial protein extraction reagent from Pierce (Rockford, Illinois) containing 1mM phenylmethyl-sulfonylfluoride. The solution was shaken at room temperature for 20 min. then centrifuged for 15 min. at 4°C (15 000 rpm). GST-cJun was purified from the supernatant by affinity chromatography using glutathione-agarose beads (Sigma). The supernatant was extracted with glutathione-agarose beads three times to maximize GST-cJun recovery. The beads were washed three times with a buffer composed of one-tenth volume bacterial extraction reagent, 20 mM Tris pH 7.5, 100 mM NaCl and GST-cJun was eluted from the beads with 10 mM glutathione in 20 mM Tris, 100 mM NaCl and 0.1% β-mercaptoethanol. The eluted GST-cJun was then concentrated using a Centriplus column (Millipore Corp.).

**Immunoprecipitation kinase assay**

SAPKγ activity was analyzed following essentially the immunoprecipitation kinase assay protocol described by Giasson and Mushynski (1996). Cells were rinsed three times with PBS at 37°C then harvested into cold PBS. Each cell pellet was suspended in an equal volume of SAPK lysis buffer (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1mM sodium orthovanadate, 2 mM pyrophosphate, 1% Triton X100, 10% glycerol and 25 μl $ml^{-1}$ protease inhibitor cocktail (Sigma)). After centrifugation for 5 min. at 4°C, the supernatant and pellet fractions were processed separately. Each pellet fraction was solubilized in SDS sample buffer (see recipe above) and used for analysis of cytoskeletal proteins (NFH and NFM). Aliquots (5 μl each) of the soluble fractions were analyzed for protein content by the Bradford assay according to the protocol provided by Sigma. Immunoprecipitation was carried out with the following components: 75 μg of total protein, 50 μl of a 1:1 Protein A Sepharose (Pharmacia) suspension in SAPK lysis buffer, 1.0 μg of anti-SAPKγ (C17) antibody and additional SAPK lysis buffer to give identical total volumes of 500 μl for each sample. Samples were rotated at 4°C for 3 hr to allow binding of SAPKγ to the Protein A

Sepharose-linked antibody. The beads were then washed 4 times with SAPK lysis buffer followed by a final wash in SAPK kinase buffer (25 mM HEPES at pH 7.4, 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 2 mM DTT, and 0.1 mM sodium vanadate). The SAPK reaction was carried out in the latter buffer containing 0.5 μg GST-cJun, 2.5 μCi [$\gamma$-$^{32}$P]-ATP and a final concentration of 5.0 nmol ATP in a total volume of 30 μl for 20 min. at 30°C. The [$^{32}$P]-labelled GST-cJun was released from the beads by adding 10 μl of 3X SDS sample buffer (containing 5% (v/v) β-mercaptoethanol) and boiling for 5 min. The supernatants were collected for SDS-PAGE and Western blot analysis (as described above). Gel bands containing [$^{32}$P]-labelled GST-cJun were visualized by autoradiography of dried gels.

**Determining Changes in NFH Phosphorylation**

Changes in NFH phosphorylation were evaluated by two different approaches, using either spread DRG cultures or localized DRG cultures (see rationale below). Both approaches involved quantification of NFH phosphorylation by Western blot analysis carried out as described above except for the following procedural modifications. Instead of 3% milk powder, 0.5% Casein (Hammerstein) in TBS/Tween was used for membrane blocking. The primary antibodies used were N52, SMI-34, and NN18 (see below). [$^{125}$I]-goat anti-mouse IgG was used as the secondary antibody. The [$^{125}$I]-IgG labelled Western blots were quantified using a BAS-III Imaging Plate and the Fujix BAS2000 Bio-Imager (Fuji Bio-Imaging).

*Quantification in neuronal cell bodies from localized DRG cultures-*

N52, a phosphorylation independent anti-NFH antibody (Harris et al., 1991), was used to determine phosphorylation changes in the cell body fraction of localized DRG cultures. In these localized cultures some neurites extend across the cell body mass and thus cell body fractions contain small amounts of hyperphosphorylated NFH. As a result, NFH hyperphosphorylation in the cell body fractions cannot be quantified with antibodies, such as SMI-34, which recognize highly phosphorylated epitopes. Instead changes in the level of the dephosphorylated form of NFH, which is the only form present in the perikaryon of DRG neurons (Giasson and Mushynski, 1997), were quantified. A window encompassing the dephosphorylated NFH (dpNFH) band (as seen in the control with no treatment in

Fig. 3-6D) was used to obtain data for quantification by Bio-Imager analysis. Background readings were measured in each lane and subtracted from the NFH values. The Western blots were concomitantly probed with both N52 and NFM antibody NN18, which is also phosphorylation-independent (Harris et al., 1991). Quantitative values obtained for the NFM band were used to correct NFH levels for small differences in protein loading in individual lanes.

*Quantification in spread DRG cultures-*

In experiments involving long-term CEP-1347 treatment, changes in the level of highly phosphorylated NFH were monitored using the phosphoepitope-specific monoclonal antibody, SMI-34 (Shea and Beermann, 1993). The phosphorylation level of NFH was monitored by measuring depletion of the phosphorylated NFH band (pNFH) from a window encompassing the band representing the highly phosphorylated NFH (pNFH) in neurites (see control lane in Fig. 3-7A). Since this highly phosphorylated NFH is found only in neurites, we did not have to separate the neurites from the cell bodies for analysis and spread DRG cultures were therefore used for these experiments. The Western blots were probed with both anti-NFM (NN18) and SMI-34. Quantification values obtained from the NFM band were used to correct for small differences in protein loading in individual lanes.

## Chapter 3: Results

### CEP-1347 inhibits SAPK activation and NFH phosphorylation in differentiated PC12 cells.

The PC12 cell line is derived from a rat adrenal pheochromocytoma and has been previously shown to differentiate into sympathetic neuron-like cells when treated with nerve growth factor (NGF) (Greene and Tischler, 1976). PC12 cells maintained with or without NGF are referred to as differentiated or naive, respectively. Giasson and Mushynski (1996) showed that activation of SAPKγ in naive PC12 cells by agents such as the proteasome inhibitor, CI, was accompanied by an increase in NFH phosphorylation. A related proteasome inhibitor, MG-132, has also been demonstrated to activate SAPKγ in PC12 cells (Giasson et al., 1999). The gift from Cephalon of a specific inhibitor of SAPK activation, CEP-1347 (KT7515) (Maroney et al., 1998; 1999), enabled us to test directly whether SAPKs were involved in phosphorylation of NFH.

The results in Fig. 3-1 are representative of 5 separate experiments. Fig. 3-1A demonstrates that MG-132-induced activation of SAPKγ in differentiated PC12 cells was inhibited by CEP-1347 as determined by immunoprecipitation kinase assay. A similar inhibition profile was revealed by Western blot analysis of the same samples with phospho-SAPK antibody (Fig. 3-1B). This antibody recognizes a phospho-epitope encompassing Thr 183/ Tyr 185, whose phosphorylation is required for SAPK activation (Derijard et al., 1994). Since the results of Western blot analysis with phospho-SAPK antibody paralleled the results obtained by immunoprecipitation kinase assay and had the added advantages of greater sensitivity and of allowing detection of both the 45 kDa and 55 kDa SAPK isoforms, it was the preferred method used to monitor SAPK activation in subsequent experiments.

In-gel kinase assays with GST-cJun as the substrate were also carried out in order to determine the kinase activity of SAPK isoforms (not shown) but this procedure had several limitations. First, much larger amounts of sample protein were required for the in-gel kinase assay whereas the amount of protein in isolated cell body- and neurite-enriched fractions was limited. Secondly, although the 45 kDa SAPK species strongly labelled the in-gel GST-cJun substrate, the 55 kDa SAPK showed only weak activity. SAPK isoforms

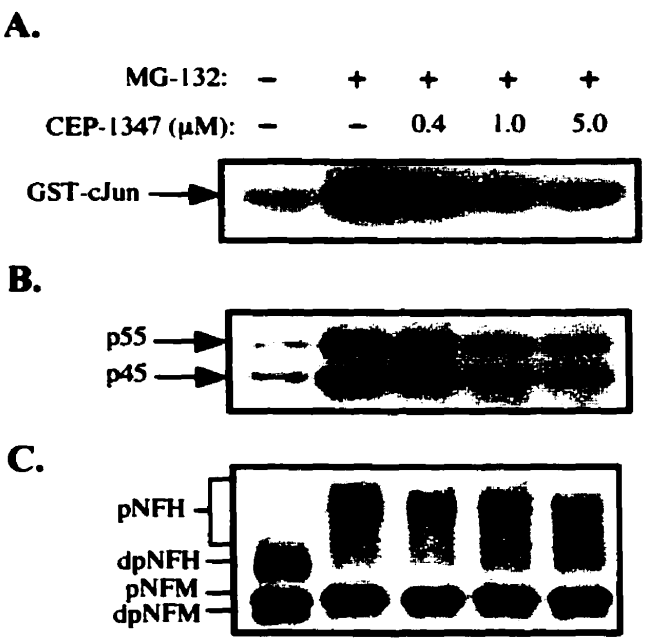
have previously been shown to exhibit varying affinities for their transcription factor substrates (Gupta et al., 1996) and the 55 kDa SAPK isoform in DRG neurons may not phosphorylate cJun as efficiently as does the 45 kDa SAPK. Thirdly, recent evidence has shown that certain SAPK species are inhibited by GST (Adler et al., 1999) and the GST portion of GST-cJun may similarly inhibit the 55 kDa SAPK. This raises questions regarding the validity of in-gel kinase assays using GST-cJun as the substrate. For these reasons, phospho-SAPK analysis by Western blotting was considered to provide a better index of SAPK activation than in-gel kinase assay.

Fig. 3-1C shows samples from these differentiated PC12 cells analyzed by Western blotting with anti-NFH (N52) and anti-NFM (NN18) antibodies. MG-132 treatment caused a reduction in the gel electrophoretic mobility of NFH indicating the conversion of hypophosphorylated NFH (dpNFH) to more highly phosphorylated forms (pNFH) (Julien and Mushynski, 1982). A lesser reduction in electrophoretic mobility was seen for a minor band representing hypophosphorylated NFM (dpNFM). The binding of NFH monoclonal antibody, N52, is essentially independent of the phosphorylation state of NFH (Shaw et al., 1986); but see (Guidato et al., 1996a) as indicated by the wide diffuse band comprising NFH in a broad range of phosphorylation states in samples from MG-132-treated PC12 cells (Fig. 3-1C). The NFM antibody (NN18) is also reported to be phosphorylation-independent (Harris et al., 1991) and is therefore suitable for monitoring phosphorylation-dependent changes in the gel electrophoretic mobility of NFM. CEP-1347 inhibited the MG-132-induced increase in NFH and NFM phosphorylation although the gel electrophoretic mobility of the NFH band was still markedly retarded even at 5.0 μM CEP-1347. This probably reflects the high residual SAPK activity seen at 5.0 μM CEP-1347 compared to the control (no treatment) (Figs. 3-1A and 3-1B).

**Figure 3-1: CEP-1347 inhibits SAPK activation and NFH phosphorylation in differentiated PC12 cells.**

PC12 cells maintained in NGF-containing minimal medium for 5 days were pretreated with the indicated concentrations of CEP-1347 for 1 hr prior to the addition of the proteasome inhibitor, MG-132, to a final concentration of 5 μM for 10 hr. An autoradiograph of [$^{32}$P]-labelled GST-cJun from an immunoprecipitation SAPKγ assay is shown in (**A**). The content of phospho-SAPK (activated SAPK) in the same cultures is shown in the Western blot in (**B**). SAPK isoforms with apparent molecular masses of 45 kDa (p45) and 55 kDa (p55) are indicated. NFH and NFM in the same cultures were analyzed by Western blotting using the NFH and NFM monoclonal antibodies, N52 and NN18, respectively (**C**). dpNFH/dpNFM and pNFH/pNFM refer to hypo- and hyperphosphorylated NFH and NFM, respectively.

**Figure 3-1:**


A.
MG-132: – + + + +
CEP-1347 (μM): – – 0.4 1.0 5.0
GST-cJun
B.
p55
p45
C.
pNFH
dpNFH
pNFM
dpNFM

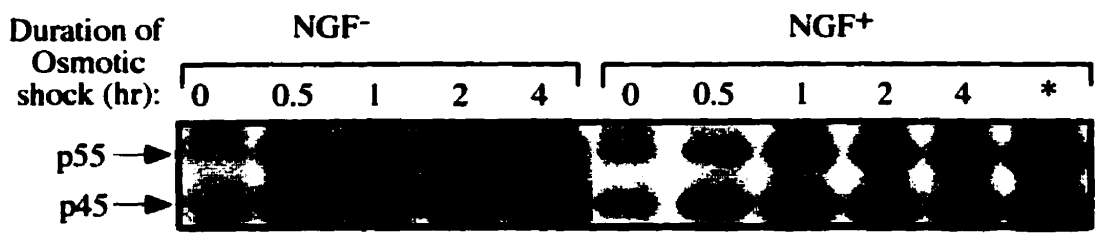
**Naive PC12 cells are more sensitive to osmotic stress than differentiated PC12 cells.**

SAPKs were previously shown to be activated by hyperosmotic shock (Galcheva-Gargova et al., 1994; Moriguchi et al., 1995). Giasson and Mushynski (1996) have previously shown by immunoprecipitation kinase assay with SAPKγ antibody, that SAPK is activated in response to hyperosmotic shock in naive PC12 cells. In order to determine which SAPK isoforms are activated in response to osmotic stress and to learn whether the SAPK activation pattern differs in naive versus differentiated PC12 cells, Western blot analysis was carried out using antibody against activated, phospho-SAPK. Neuronally differentiated ($NGF^+$) and naive PC12 ($NGF^-$) cells were treated with 0.4 M NaCl for up to 4 hr. Fig. 3-2 shows that slightly higher basal levels of the 45 kDa (p45) and 55 kDa (p55) phospho-SAPK species were present in differentiated as compared to naive PC12 cells. In naive PC12 cells both the p45 and p55 SAPK species were fully activated after only 30 min. of hyperosmotic shock and phospho-SAPK levels remained high for the duration of treatment. In differentiated PC12 cells, phospho-SAPK levels increased more slowly in response to hyperosmotic shock, reaching a maximum at 2-4 hr. At all time points phospho-SAPK levels were lower in differentiated than in naive PC12 cells. The latter difference was not due to a limitation in available SAPK in differentiated PC12 cells since treating these cells with arsenite produced higher levels of phospho-SAPK than did hyperosmotic shock (Fig. 3-2).

**Figure 3-2: Naive PC12 cells ($NGF^-$) are more sensitive to osmotic stress than differentiated PC12 cells ($NGF^+$).**

PC12 cells maintained in either NGF-free medium ($NGF^-$) or NGF-containing medium ($NGF^+$) were treated for the indicated periods with 0.4 M NaCl. Cells were harvested, lysed and equal amounts of protein were resolved by SDS-PAGE. Western blot analysis was carried out using phospho-SAPK polyclonal antibody and bands were visualized by ECL. Two phospho-SAPK isoforms were visible with apparent molecular weights of 45 kDa (p45) and 55 kDa (p55). For comparison, NGF-treated PC12 cells were exposed to 0.5 mM Arsenite treatment for 4 hr (see *).

**Figure 3-2:**



**CEP-1347 inhibits SAPK activation in differentiated PC12 cells in response to hyperosmotic shock and arsenite treatment.**

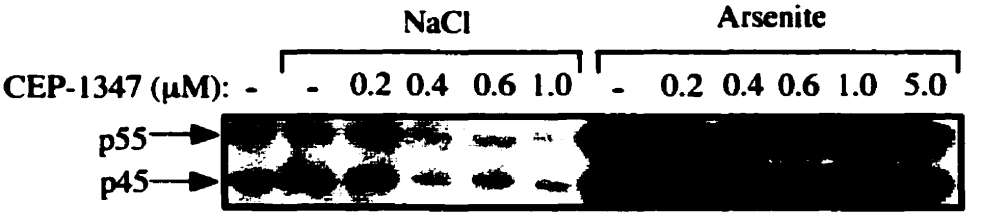
A comparison of the inhibitory effect of CEP-1347 on SAPK activation by other cellular stressors in addition to MG-132, was carried out by treating differentiated PC12 cells with either 0.4 M NaCl or 0.5 mM sodium arsenite for 4 hr. Samples were then harvested and analyzed by Western blotting for phospho-SAPK immunoreactivity. The results in Fig. 3-3 are representative of 3 separate experiments.

Arsenite was a more potent activator of both p45 and p55 SAPK isoforms than was hyperosmotic shock (Fig. 3-3). In cells subjected to osmotic stress, 0.4 μM CEP-1347 reduced activated SAPK levels to below those seen in the control (no treatment). On the other hand, arsenite-induced SAPK activation was not inhibited to levels seen in control even at 5.0 μM CEP-1347. In effect, CEP-1347 completely inhibited hyperosmotic shock induced SAPK activation but had a lesser effect on arsenite-induced SAPK activation, indicating that different SAPK signal transduction pathways were being activated by the two stressing agents.

**Figure 3-3: CEP-1347 inhibits SAPK activation in differentiated PC12 cells in response to osmotic shock and arsenite treatment.**

PC12 cells cultured in the presence of NGF for 6d were pretreated with the indicated concentrations of CEP-1347 for 1 hr. Either NaCl or arsenite was then added to a final concentration of 0.4 M or 0.5 mM, respectively, for an additional 4 hr. Cells were harvested and analyzed by Western blot with phospho-SAPK antibody. SAPK isoforms of 45 kDa (p45) and 55 kDa (p55) are shown.

**Figure 3-3:**



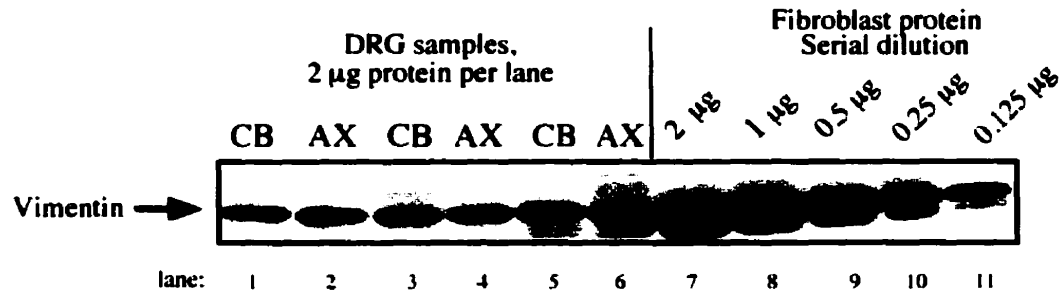
**Localized DRG cultures treated with antimitotic agents are minimally contaminated with fibroblasts.**

We next studied the effects of CEP-1347 on stress-induced phosphorylation of perikaryal NFH in primary neuronal cultures treated with antimitotic agents to reduce nonneuronal cell content. DRG cultures treated with antimitotic agents do not contain Schwann cells but a population of quiescent fibroblasts remains that is resistant to the antimitotic treatment (Giasson and Mushynski, 1997). Parallel cultures of anitmitotic-treated DRG neurons and DRG fibroblasts (obtained from cultures maintained without NGF and therefore devoid of neurons and Schwann cells) were prepared and analyzed by Western blot analysis with vimentin antibody (Fig. 3-4). Vimentin is a marker of fibroblast contamination since it is expressed in mesenchymal cells but not neurons. A comparison of vimentin levels in the cell body- and neurite-enriched fractions of three different DRG neuronal preparations with a dilution series of DRG fibroblast protein indicated a contamination level of less than 10% fibroblast protein (Fig. 3-4). This finding, coupled with evidence that higher levels of SAPK protein are found in DRG neurons than in fibroblasts (Giasson and Mushynski, 1996), indicates that the SAPKs we study in antimitotic-treated neurons are by and large neuronal in origin.

**Figure 3-4: Determining fibroblast contamination in localized DRG cultures treated with antimitotic agents.**

Cell extracts from localized DRG cultures treated with antimitotic agents were assayed for fibroblast contamination. Cells were harvested and analyzed by Western blot analysis using vimentin antibody. Lanes 1 through 6 contain 2 ug each of protein from the cell body (CB)- and neurite (AX)-enriched fractions of three different DRG neuronal cultures. Lanes 7 through 11 contain a dilution series of fibroblast cell protein. Cell body- and neurite-enriched samples contain, on average, approximately 0.125 ug of fibroblast protein per 2 ug protein.

**Figure 3-4:**


DRG samples,
2 µg protein per lane
Fibroblast protein
Serial dilution
CB
AX
CB
AX
CB
AX
2 µg
1 µg
0.5 µg
0.25 µg
0.125 µg
Vimentin
lane:
1
2
3
4
5
6
7
8
9
10
11

**CEP-1347 inhibits arsenite-induced SAPK activation and hyperphosphorylation of perikaryal NFH in DRG neurons.**

To assess CEP-1347 inhibition of SAPK activation in DRG neurons, cultures were incubated with various concentrations of CEP-1347 for 1 hr and then arsenite was added to a final concentration of 0.5 mM for an additional 4 hr. This was the longest exposure to arsenite that DRG neurons could tolerate without detachment from the substratum and was also the interval required to detect significant changes in phosphorylation of perikaryal NFH. The results in Fig. 3-5 are representative of 4 separate experiments. Neurite- and cell body-enriched fractions were harvested and analyzed by Western blotting with phospho-SAPK antibody (Fig. 3-5A). The blot was then stripped and reprobed with anti-SAPK$\gamma$ (Fig. 3-5B) to verify that similar amounts of protein were loaded in each lane and that changes in phospho-SAPK levels were not due to increases in SAPK protein. Note that the latter antibody labels a single, 45 kDa band in both the cell body and neurite fractions and that the SAPK level in neurite samples is slightly higher than that in cell body despite equivalent amounts of total protein.

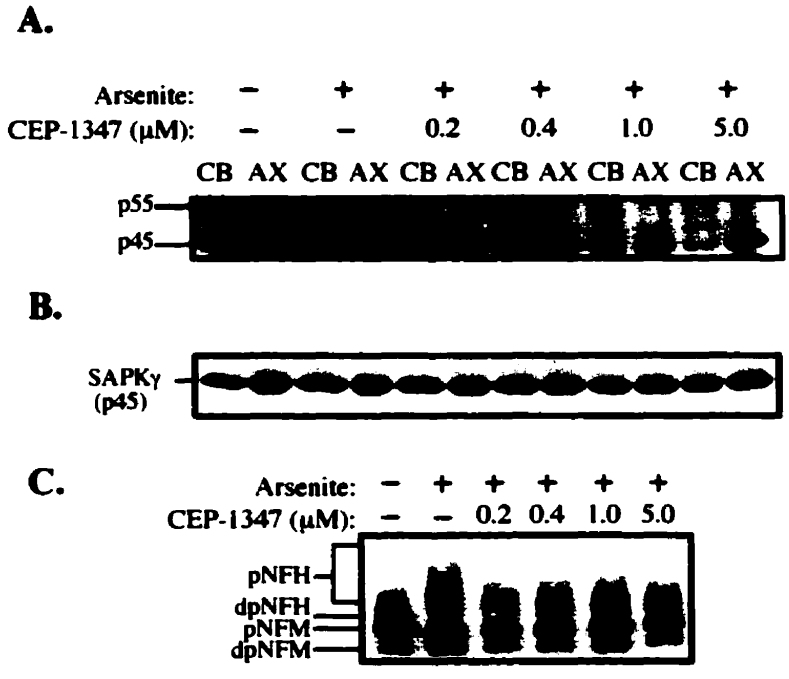
The neurite-enriched fraction contained high basal levels of activated SAPK, as indicated in Fig. 3-5A by intense staining of the 45 kDa and 55 kDa phospho-SAPK bands in the control sample (no arsenite treatment) and the apparent lack of a further increase in staining in the neurite sample from arsenite-treated neurons. The unstressed cell body fraction (control) also contained significant levels of phospho-SAPK, although arsenite treatment caused a further increase relative to the control (Fig. 3-5A).

Formation of the phospho-SAPK isoforms was differentially inhibited by CEP-1347 and, interestingly, all isoforms could be reduced to levels below those in untreated control samples by the inhibitor (Fig. 3-5A). The 55 kDa phospho-SAPK in the neurite-enriched fraction was particularly sensitive to CEP-1347 treatment and its formation was completely inhibited by 0.2 μM CEP-1347. Formation of the 45 kDa and 55 kDa phospho-SAPK species in the cell body-enriched fraction was less sensitive to CEP-1347 inhibition and was not reduced below control levels until a CEP-1347 concentration of 1.0 μM was reached. However, a 45 kDa phospho-SAPK species in the neurite fraction persisted even at 5.0 μM CEP-1347.

**Figure 3-5: CEP-1347 inhibits arsenite-induced SAPK activation and hyperphosphorylation of perikaryal NFH in DRG neurons.**

DRG cultures were pretreated for 1 hr with the indicated concentrations of CEP-1347 and then arsenite was added to a final concentration of 0.5 mM for an additional 4 hr. Equal amounts of protein from cell body (CB)- and neurite (AX)-enriched fractions were resolved by SDS-PAGE and analyzed by Western blotting. The Western blot was probed with phospho-SAPK antibody, which labelled 45 kDa (p45) and 55 kDa (p55) bands corresponding to the two major forms of immunoreactive phospho-SAPK (**A**). The blot was then erased and reprobed with anti-SAPKγ to show the amount of SAPKγ protein in these samples (**B**). Cell body-enriched fractions were analyzed by Western blotting with NFH (N52) and NFM (NN18) antibodies (**C**). dpNFM/dpNFH and pNFM/pNFH refer to the hypo- and hyperphosphorylated forms of NFM/NFH, respectively.

**Figure 3-5:**


A.
Arsenite: – + + + + +
CEP-1347 (μM): – – 0.2 0.4 1.0 5.0
CB AX CB AX CB AX CB AX CB AX CB AX
p55
p45
B.
SAPKγ
(p45)
C.
Arsenite: – + + + + +
CEP-1347 (μM): – – 0.2 0.4 1.0 5.0
pNFH
dpNFH
pNFM
dpNFM

The NFH in cultured DRG neurons shows the typical pattern, with neuritic (axonal) NFH being hyperphosphorylated and NFH in the cell body hypophosphorylated (Giasson and Mushynski, 1997). Cell body-enriched fractions from the same cell samples analyzed in Fig. 3-5A were analyzed by Western blotting with the NFH (N52) and NFM (NN18) antibodies (Fig. 3-5C). Fig. 3-5C shows that arsenite treatment induced hyperphosphorylation of perikaryal NFH, as indicated by the reduction in its gel electrophoretic mobility (Julien and Mushynski, 1982). Since the NFH antibody, N52, is essentially phosphorylation-independent (Harris et al., 1991), the broad band formed by perikaryal NFH in arsenite-treated samples (Fig. 3-5C) reflects a pool of heterogeneously phosphorylated NFH polypeptides. This heterogeneous pool represents NFH polypeptides that have variable numbers of phospho-serine residues out of a possible total of 52 representing the KSP-repeats in the rat NFH tail domain (Chin and Liem, 1990). CEP-1347 treatment inhibited arsenite-induced hyperphosphorylation of perikaryal NFH (Fig. 3-5C). A reduced shift in the gel electrophoretic mobility of perikaryal NFH was evident beginning at 0.2 μM CEP-1347 and inhibition of perikaryal NFH phosphorylation was essentially complete at 1.0 μM CEP-1347.

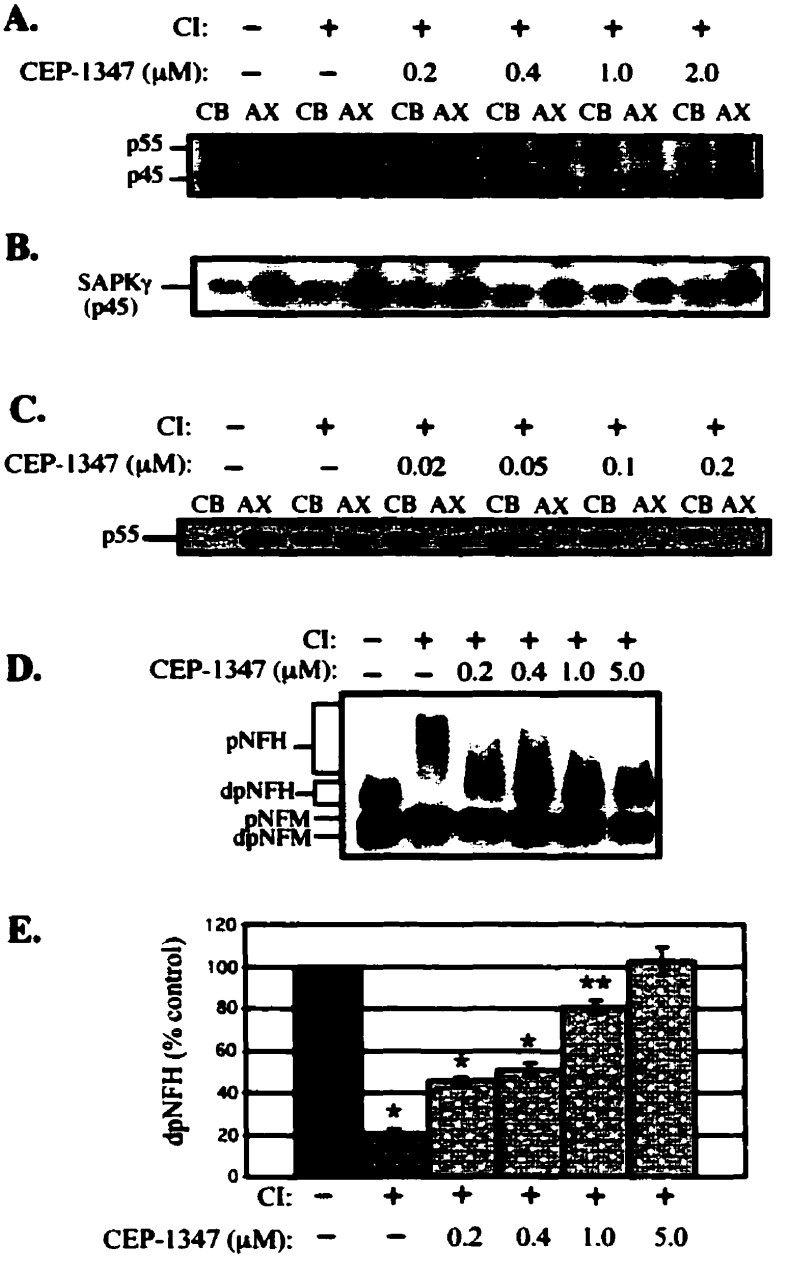
**CEP-1347 inhibits CI-induced SAPK activation and hyperphosphorylation of perikaryal NFH in DRG neurons.**

To assess the effect of CEP-1347 on CI-induced SAPK activation in DRG neurons, cultures were pretreated with indicated concentrations of CEP-1347 for 1 hr and then CI was added to a concentration of 30 μM for an additional 10 hr. Neurite- and cell body-enriched fractions were harvested and analyzed by Western blotting with phospho-SAPK antibody (Fig. 3-6A). Twice as much neurite protein (AX) as cell body protein (CB) was loaded in the designated lanes in Fig. 3-6A. This was done to increase the visibility of the 55 kDa phospho-SAPK band from neurites, as formation of this activated species was highly sensitive to CEP-1347 inhibition. The difference in protein loading is apparent in Fig. 3-6B which shows the SAPKγ content of the same samples following erasure of the blot in Fig. 3-6A and reprobing with SAPKγ antibody. A separate experiment using CEP-1347 concentrations in the 0.02 -0.2 μM range was carried out to estimate the $IC_{50}$ for inhibition of p55 phospho-SAPK formation (Fig. 3-6C).

**Figure 3-6: CEP-1347 inhibits both CI-induced SAPK activation and hyperphosphorylation of perikaryal NFH in DRG neurons.**

Localized DRG cultures were pretreated for 1 hr with the indicated concentrations of CEP-1347 and then CI was added to a final concentration of 30 μM for an additional 10 hr. Equal amounts of protein from cell body (CB)-enriched fractions and equal amounts of twice as much protein from neurite (AX)-enriched fractions were resolved by SDS-PAGE and the Western blot was probed with phospho-SAPK antibody, which labelled the 45 kDa (p45) and 55 kDa (p55) bands corresponding to the two major forms of immunoreactive phospho-SAPK (**A**). The blot was then erased and reprobed with anti-SAPKγ to show the amount of SAPKγ protein in these samples (**B**). The effect of CEP-1347 concentrations in the 0.02-0.2 μM range on 55 kDa phospho-SAPK levels (p55) is shown in (**C**). Samples from the same cell body-enriched fractions were subjected to Western blot analysis with anti-NFH (N52) and anti-NFM (NN18) (**D**). dpNFM/dpNFH and pNFM/pNFH refer to the hypo- and hyperphosphorylated forms of NFM/NFH, respectively. Inhibition by CEP-1347 of CI-induced hyperphosphorylation of perikaryal NFH was quantified by Western blot analysis using [$^{125}$I]-labelled goat anti-mouse IgG as the secondary antibody (**E**). A window encompassing the lower, dephosphorylated NFH band (as indicated for dpNFH in the control (no treatment) in **D**) was quantified. The NFH values were adjusted for variations in protein content between separate lanes by relating them to the amount of NFM in each lane and are expressed as percent of control (no treatment). The data are presented as the mean ± SEM from three separate experiments. Columns marked with an asterisk are significantly different from the control as assessed by the Student's *t* test (*$p$ <0.001 or **$p$ <0.05).

**Figure 3-6:**


A.
CI: – + + + + +
CEP-1347 (μM): – – 0.2 0.4 1.0 2.0
CB AX CB AX CB AX CB AX CB AX CB AX
p55
p45
B.
SAPKγ
(p45)
C.
CI: – + + + + +
CEP-1347 (μM): – – 0.02 0.05 0.1 0.2
CB AX CB AX CB AX CB AX CB AX CB AX
p55
D.
CI: – + + + + +
CEP-1347 (μM): – – 0.2 0.4 1.0 5.0
pNFH
dpNFH
pNFM
dpNFM
E.
dpNFH (% control)
120
100
80
60
40
20
0
CI: – + + + + +
CEP-1347 (μM): – – 0.2 0.4 1.0 5.0

As demonstrated for arsenite-treated DRG neurons (Fig. 3-5A), basal levels of phospho-SAPK were high in the control DRG samples, especially in the neurite-enriched fraction and activation of SAPKs in CI-treated neurons was differentially inhibited by CEP-1347 (Fig. 3-6A). Inhibition profiles for the 45 kDa and 55 kDa phospho-SAPK species in the cell body-enriched fraction were similar to those for arsenite-activated samples (cf. Fig. 3-5A and 3-6A). Again, a 45 kDa SAPK species in the neurite-enriched fraction was resistant to CEP-1347 inhibition (Fig. 3-6A). From Fig. 3-6C, the $IC_{50}$ for inhibition of 55 kDa SAPK activation in the neurite-enriched fraction was estimated to be approximately 0.02 μM CEP-1347.

Cell body-enriched fractions from these CI-treated DRG neurons were analyzed by Western blotting with NFH (N52) and NFM (NN18) antibodies. As demonstrated for arsenite-treated DRG neurons (Fig. 3-5C), CI treatment also induced hyperphosphorylation of perikaryal NFH. Fig. 3-6D shows the characteristic reduction in the gel electrophoretic mobility of NFH signifying increased phosphorylation (Julien and Mushynski, 1982). CI treatment caused a more extensive shift in the gel electrophoretic mobility of NFH than did arsenite treatment (cf. Fig. 3-5C and 3-6D). This is most likely due to the longer duration of CI treatment (10 hr) compared to arsenite-treatment (4 hr), since it was previously shown that the extent of aberrant phosphorylation of perikaryal NFH correlated with both the degree and duration of SAPK activation (Giasson and Mushynski, 1996).

CEP-1347 treatment inhibited the CI-induced hyperphosphorylation of perikaryal NFH (Fig. 3-6D). A reduced shift in the gel electrophoretic mobility of perikaryal NFH was evident beginning at 0.2 μM CEP-1347 and was essentially complete at 5 μM. Interestingly, CI treatment also caused a reduction in the mobilities of both phosphorylated (pNFM) and hypophosphorylated (dpNFM) perikaryal NFM (Fig 6D). CEP-1347 treatment inhibited the CI-induced mobility shift of the perikaryal pNFM and dpNFM bands similar to the situation for NFH.

Several possible approaches were considered to quantify changes in the phosphorylation state of perikaryal NFH in response to CI and CEP-1347 treatment. Enhanced chemiluminescence (ECL) staining (e.g. Fig. 3-6D) was unsuitable due to the rather limited linear relationship between signal intensity and epitope concentration. A

phosphoepitope-specific antibody such as SMI-34 could not be used because the cell body fraction contains small amounts of highly phosphorylated NFH from neurites that run through the cell body mass. This axonal NFH is strongly labelled by SMI-34 on Western blots, thereby obscuring changes in perikaryal NFH. In addition, quantifying the broad smear formed by heterogenously phosphorylated perikaryal NFH (Fig. 3-6D) presented difficulties relating to background subtraction. Changes in phosphorylation of perikaryal NFH were therefore quantified by Western blotting with N52 primary antibody followed by [$^{125}$I]-labelled anti-mouse IgG secondary antibody. A defined zone, that occupied by hypophosphorylated NFH (dpNFH) (see bracket for dpNFH in Fig. 3-6D), was then scanned for quantification. Hypophosphorylated NFH is found exclusively in the perikaryal compartment (Giasson and Mushynski, 1997).

The results in Fig. 3-6E demonstrate a ~80% reduction in dpNFH due to the mobility shift that accompanies CI-induced phosphorylation. Inhibition of SAPK activation by CEP-1347 was accompanied by increased dpNFH levels beginning at 0.2 μM CEP-1347, and attaining control (no treatment) values at 5 μM CEP-1347.

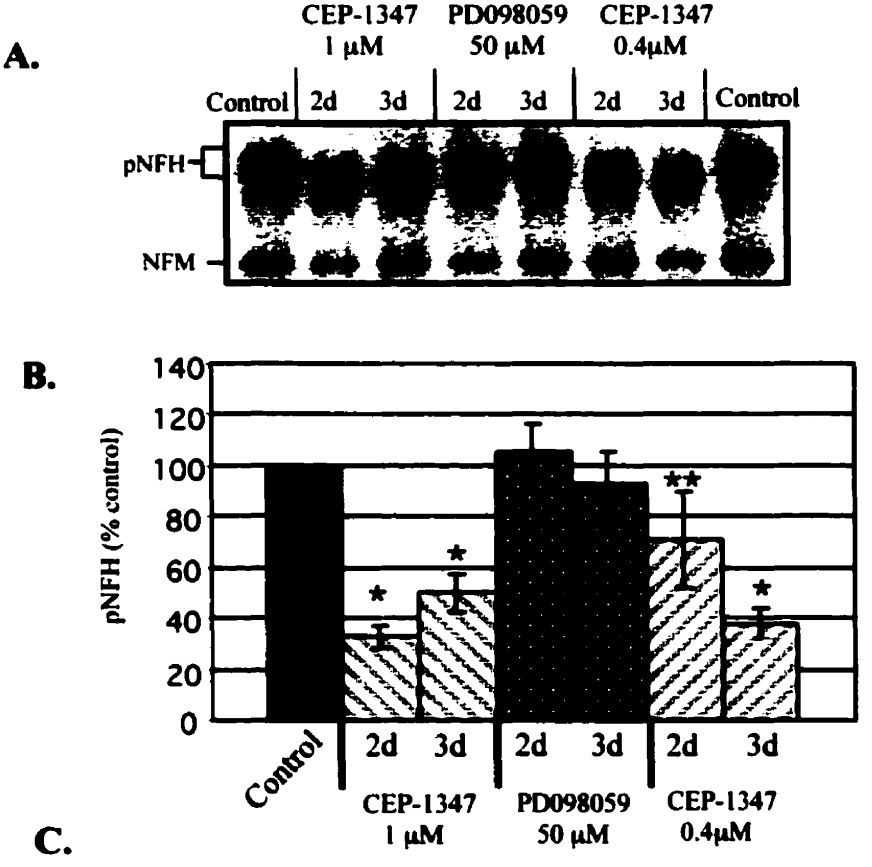
**Long-term treatment of DRG cultures with CEP-1347 decreased the steady-state phosphorylation level of axonal NFH.**

In neurons, perikaryal NFH is hypophosphorylated and becomes progressively hyperphosphorylated after it enters the initial axon segment. Hence, highly phosphorylated NFH normally exists exclusively in the axon (Sternberger and Sternberger, 1983; Lee et al., 1987; Nixon et al., 1989). This phosphorylation pattern is maintained in cultured DRG neurons (Giasson and Mushynski, 1997) and may in part reflect the high basal levels of activated SAPK in the neurite (axonal) compartment (Figs. 3-5A and 3-6A). Various concentrations of CEP-1347 were added to spread DRG cultures for a duration of up to 3 d to test whether the SAPKs in DRG neurites were involved in constitutive phosphorylation of NFH in this compartment. There was no need to separate cell body and neurite fractions in this experiment because, as already pointed out, hyperphosphorylated NFH is found only in neurites in unstressed DRG cultures.

**Figure 3-7: Long-term CEP-1347 treatment lowers the phosphorylation state of axonal NFH in DRG neurons.**

Spread DRG cultures not treated with antimitotic agents were maintained with CEP-1347 or PD098059 at the indicated concentrations beginning at day 21 in culture for the 3 day treatment. Fresh medium or medium and inhibitor were added every 24 hr and all cultures were harvested at day 24 in culture. Samples were analyzed by Western blot and probed with SMI-34 antibody, which recognizes highly phosphorylated forms of NFH, and anti-NFM (NN18) (**A**). For quantification (**B**), Western blots were probed with SMI-34 and NN18 primary antibodies and [$^{125}$I]-labelled goat anti-mouse IgG was the secondary antibody. Levels of highly phosphorylated NFH were determined by quantifying the SMI-34 signal in a window encompassing the most highly phosphorylated NFH band seen in the control (no treatment) in (**A**). These values were adjusted for small variations in protein content between separate lanes by relating them to the amount of NFM in each lane and are expressed as percent of control. The data are presented as means ± SEM from four separate experiments. Columns marked with an asterisk are significantly different from the control as assessed by the Student's *t* test (*$p$ <0.001 or **$p$ <0.05). Values for PD098059-treated samples did not differ significantly from control. In (**C**), DRG cultures were treated for 2d with CEP-1347 in the 0.02-0.4 μM range. The samples were analyzed by Western blot with SMI-34 antibody. Designations for the various forms of NFH and NFM are the same as in the legend to Fig. 3-6.

**Figure 3-7:**


A.
CEP-1347 1 μM
PD098059 50 μM
CEP-1347 0.4μM
Control
2d
3d
2d
3d
2d
3d
Control
pNFH
NFM
B.
pNFH (% control)
140
120
100
80
60
40
20
0
Control
2d
3d
2d
3d
2d
3d
CEP-1347 1 μM
PD098059 50 μM
CEP-1347 0.4μM
C.
CEP-1347 (μM): - 0.02 0.05 0.1 0.2 0.4 -
pNFH

Spread DRG cultures that had not been treated with antimitotic agents were subjected to long-term treatment (2-3 d) with CEP-1347 or with the MEK inhibitor, PD098059, which blocks ERK activation (Alessi et al., 1995). We chose to focus on the SAPK and ERK proline-directed kinases because in the DRG neuronal culture model used here, p38 is not expressed in neurites and cdk-5 is not active (Giasson and Mushynski, 1997).

Western blot analysis with phosphoepitope-specific antibody, SMI-34, demonstrated that treatment with 1 μM or 0.4 μM CEP-1347, but not 50 μM PD098059, caused a small increase in the gel electrophoretic mobility of the slowly migrating NFH band (pNFH), signifying a reduction in phosphorylation state (Fig. 3-7A). Fig. 3-7A shows an autoradiograph of samples analyzed by Western blotting with SMI-34 primary antibody followed by [$^{125}$I]-labelled anti-mouse IgG secondary antibody. A 2-3 day exposure to either 1 μM or 0.4 μM CEP-1347 had two effects on pNFH as detected with SMI-34 antibody. First, CEP-1347 treatment caused an increase in the gel electrophoretic mobility of the slowly migrating pNFH band, signifying a reduction in phosphorylation state. This effect was also seen with a phosphorylation-independent antibody (N52) (not shown). Secondly, CEP-1347 treatment caused a reduction in SMI-34 staining intensity of the highly phosphorylated pNFH band, indicating a loss of phosphoepitopes recognized by the antibody. The MEK inhibitor, PD 098059, had little or no effect on either the mobility or the SMI-34 staining intensity of the pNFH band.

To quantify these changes, the amount of highly phosphorylated NFH (pNFH) in the SMI-34-labelled band bracketed in the control lane in Fig. 3-7A was determined by probing Western blots of the DRG samples with SMI-34 antibody followed by [$^{125}$I]-labelled anti-mouse IgG secondary antibody. Quantification of the [$^{125}$I]-signal was performed for four separate experiments and the means ± SEM are shown in Fig. 3-7B. The results in Fig. 3-7B show that treatment with 1 μM CEP-1347 for 2 or 3 d or 0.4 μM CEP-1347 for 3 d caused a ~50-70% reduction in highly phosphorylated NFH (pNFH) in neurites whereas PD098059 had no significant effect. The effect of 0.4 μM CEP-1347 appeared to be maximal after 3 d of treatment since similar results were seen for 1 μM CEP-1347 at 2 d and 3 d.

To test the effect of lower CEP-1347 concentrations, in the 0.02-0.05 μM range, DRG cultures were treated with CEP-1347 as described above and samples were analyzed by Western blotting with SMI-34 antibody. Fig. 3-7C shows that an effect of long-term CEP-1347 treatment on the mobility and staining intensity of pNFH with SMI-34 could be seen beginning at 0.02-0.05 μM CEP-1347, which is similar to the $IC_{50}$ value for CEP-1347 inhibition of 55kDa phospho-SAPK formation in the neurite fraction (Fig. 3-6C). A continuous decline in gel electrophoretic mobility of pNFH occurred throughout the CEP-1347 concentration range tested in Fig. 3-7C.

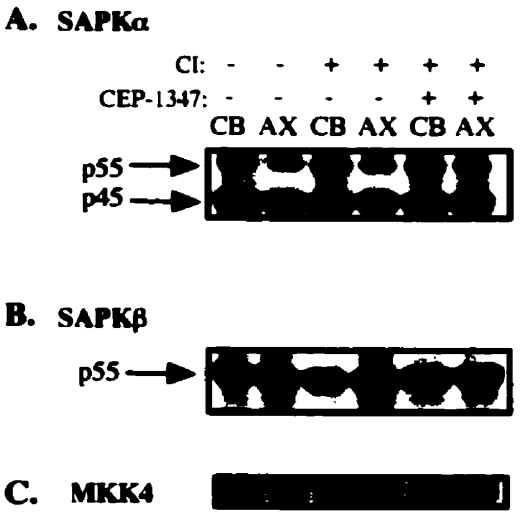
**SAPKα and SAPKβ levels in DRG neurons are not affected by stressing agents or CEP-1347 treatment.**

Each of the three SAPK genes can give rise, by alternative splicing, to at least two isoforms with molecular weights of 45 kDa and 55 kDa. Previous studies have indicated that as many as 10 isoforms can be generated by alternative splicing of mRNA transcripts from the 3 genes (Gupta et al., 1996; Kumagae et al., 1999). Samples from cell body- and neurite-enriched fractions of DRG neurons were analyzed by Western blotting to determine the SAPKα and SAPKβ forms present (Fig. 3-8). Only a 45 kDa SAPKγ isoform was shown to exist in both neurites and cell bodies (see Figs. 3-5B and 3-6B). SAPKα antibody reacted with 45 kDa and 55 kDa SAPK isoforms with the 45 kDa SAPK isoform being the major band (Fig. 3-8A). SAPKβ antibody detected only a 55 kDa species (Fig. 3-8B). Fig. 3-8C shows that MKK4/SEK1 is present in both cell body and axonal fractions. The similar SAPK levels in control samples and in samples from DRG neurons treated with 30 μM CI (10 hr) with or without 1μM CEP-1347 demonstrated that these treatments do not alter levels of SAPK protein.

**Figure 3-8: SAPKα– and SAPKβ–immunoreactivities in DRG neurons are not affected by CI or CEP-1347 treatments.**

Localized DRG neuronal cultures treated with antimitotic agents were maintained until day 21 in culture. Cells were pre-incubated with 1 μM CEP-1347 (where indicated) for 1 hr and then CI was added to a final concentration of 30 μM for a duration of 10 hr. Cell body (CB)- and neurite (AX)-enriched fractions were harvested separately and equal amounts of protein were analyzed by SDS-PAGE and Western blot analysis. Western blots were probed with SAPKα (**A**), SAPKβ (**B**) and MKK4/SEK1 (**C**) primary antibodies. p45 and p55 refer to the 45 kDa and 55 kDa SAPK isoforms.

**Figure 3-8:**



**Immunocytochemical staining of DRG neuronal cultures**

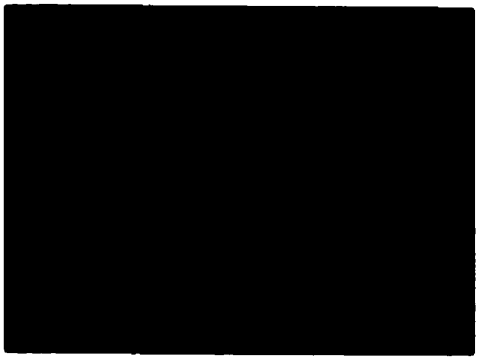
Immunocytochemical staining was carried out to determine the subcellular locations of SAPKα, SAPKβ and SAPKγ in DRG neurons. The immunofluorescence micrographs in Fig. 3-9 were obtained using the three different anti-SAPK antibodies followed by FITC-conjugated secondary antibody. Immunofluorescence staining for all three SAPKs was seen in both the cell body and neurites which confirmed results obtained by Western blot analysis of the DRG cultures (Figs. 3-7 and 3-8). In addition, immunofluorescence microscopy showed that SAPKα immunoreactivity was most prominent in the neuronal nucleus (Fig. 3-9B). It is interesting to note that a fraction of neuronal nuclei in cells treated with SAPKβ antibody exhibited strong nuclear staining indicating that localization of SAPKβ at a given time can vary within the neuronal population (Fig. 3-9C).

**Figure 3-9: Immunocytochemical staining of SAPKα, SAPKβ and SAPKγ in DRG neurons.**

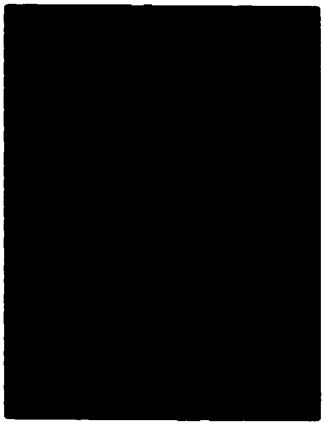
DRG neuronal cultures treated with antimitotic agents were maintained until day 21 in culture. Immunofluorescence micrographs show cells treated with SAPKγ (**A**). SAPKα (**B**) or SAPKβ (**C**) primary antibodies followed by FITC-conjugated secondary antibody.

**Figure 3-9:**

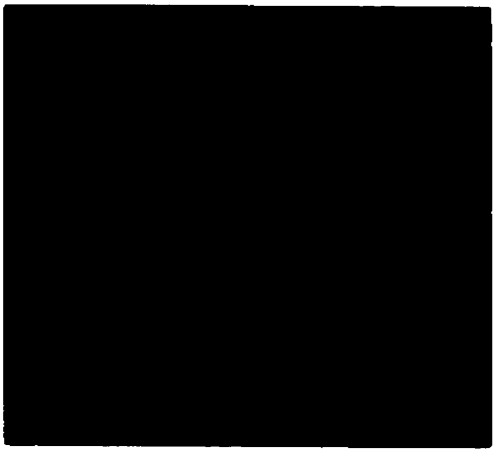
**A. SAPKγ**



**B. SAPKα**



**C. SAPKβ**



## Chapter 4: Discussion

*CEP-1347 inhibits stress-induced and constitutive phosphorylation of NFH*

Previous correlative studies have implicated SAPKs in phosphorylation of NFH (Giasson and Mushynski, 1996; 1997; Brownlees et al., 2000). The present study involved the use of CEP-1347, a specific inhibitor of SAPK activation, to provide more direct evidence that SAPKs are involved in stress-induced as well as constitutive phosphorylation of NFH. Results presented here demonstrate that CEP-1347 inhibited stress-induced hyperphosphorylation of NFH and NFM in both differentiated PC12 cells and sensory neurons and that the decrease in phosphorylation paralleled the reduction in SAPK activation. In addition, long-term treatment of unstressed DRG neurons with CEP-1347 caused a reduction in the phosphorylation state of NFH in neurites, signifying the involvement of SAPKs in constitutive phosphorylation of axonal NFH.

Other Pro-directed kinases in addition to SAPKs have been implicated in NFH tail domain phosphorylation. They include: cdk-5 (Lew et al., 1992; Hisanaga et al., 1993; Shetty et al., 1993; Guidato et al., 1996a; Sharma et al., 1999), GSK3α/β (Guan et al., 1991; Guidato et al., 1996b) and ERK1/2 (Roder and Ingram, 1991; Veeranna et al., 1998). However these various kinases were studied in other cell systems, often in transfected non-neuronal cells, therefore their role in NFH phosphorylation in sensory neurons remains to be confirmed.

Immunoprecipitation kinase assays have previously demonstrated that cdk-5 is not active in cultured DRG neurons (Giasson and Mushynski, 1997), likely due the absence of its activator ligand, p35/p25 (Tsai et al., 1994). The latter may explain the selective sparing of DRG neurons in cdk-5 gene knockout mice, although deletion has a deleterious effect on many types of CNS neurons (Ohshima et al., 1996). GSK3 has only been demonstrated to phosphorylate NFH *in vitro* and in transfected cells (Guan et al., 1991; Guidato et al., 1996b) and its role in primary neurons remains to be established. ERK1/2 can be ruled out as an NFH kinase in DRG neurons since long-term treatment with PD098059, a specific inhibitor of the ERK activator, MEK-1 (Alessi et al., 1995), caused neither a reduction in the gel electrophoretic mobility of NFH nor a decrease in the SMI-34 phosphoepitopes (Fig. 3-7). This indicates that ERK1/2 may not be universally

involved in NFH phosphorylation in all types of neurons although it does appear to play a role in cultured hippocampal neurons and neuroblastoma (Veeranna et al., 1998; Sharma et al., 1999). Another potential NFH kinase, p38 MAPK, was previously shown to be expressed only the cell bodies of DRG neurons and treatment of these neurons with SB203580, a specific inhibitor of p38, had no effect on the stress-induced phosphorylation of NFH (Giasson and Mushynski, 1997). These various considerations, together with unequivocal evidence provided through use of the specific inhibitor of SAPK activation, CEP-1347, indicate that in DRG neurons SAPK is the Pro-directed kinase responsible for stress-induced phosphorylation of NFH and also contributes to the constitutive phosphorylation of axonal NFH.

Evidence presented here implicates SAPKs in the stress-induced phosphorylation of perikaryal NFM in addition to NFH. CI-induced SAPK activation caused a reduction in the gel electrophoretic mobility of perikaryal NFM and this effect was blocked by CEP-1347 (Fig. 3-6D). Inhibition of CI-induced phosphorylation of NFM by CEP-1347 paralleled the inhibition profile for CI-induced phosphorylation of NFH. Arsenite treatment did not cause a detectable shift in the gel electrophoretic mobility of NFM, presumably due to its shorter treatment duration (4 hr) compared to CI treatment (10 hr). It was not possible to treat DRG cultures with arsenite for longer periods since it caused the cells to detach from the substratum. Since previous studies have shown that both the degree and duration of SAPK activation are required for NFH hyperphosphorylation (Giasson and Mushynski, 1996), it is likely that arsenite-treatment was of too short duration to obtain a shift in the gel electrophoretic mobility of perikaryal NFM.

*SAPK isoforms are differentially inhibited by CEP-1347*

In cultured sensory neurons, CEP-1347 differentially inhibited the stress-induced activation of various SAPK isoforms as determined by Western blot analysis of phospho-SAPK levels in the neuronal cell body and neurite fractions (Figs. 3-5 and 3-6). Specifically, activation of a 55 kDa SAPK isoform in the neurite fraction was highly sensitive to CEP-1347 inhibition with an $IC_{50}$ value of approximately 0.02 μM. The corresponding $IC_{50}$ value for the 45 kDa and 55 kDa SAPKs in neuronal cell bodies was approximately 0.2 μM. In contrast, activation of a 45 kDa SAPK in neurites was not

completely inhibited even at 5 μM CEP-1347. These results suggest that SAPKs in neurites and cell bodies are activated by different signaling pathways or modules (see Fig. 1-5) characterized by their varying susceptibilities to CEP-1347 inhibition.

In PC12 cells, SAPKs activated by two different stressing agents also exhibited different susceptibilities to CEP-1347 inhibition indicating that distinct signaling pathways were involved. Results presented here show that CEP-1347 is a more potent inhibitor of SAPK activation induced by hyperosmotic shock than of arsenite-induced SAPK activation since only the former was completely inhibited by the drug (Fig. 3-3). The more robust SAPK activation seen in arsenite-treated PC12 cells may be due to stimulation of multiple signaling pathways, including the pathway activated by hyperosmotic shock. This possibility is supported by recent evidence showing that arsenite-mediated SAPK activation involves several MAPKKKs including MEKK2, MEKK3 and MEKK4 (Porter et al., 1999) although the precise activation mechanism remains controversial. Other studies have indicated that arsenite activates SAPKs by inhibiting a SAPK phosphatase (Cavigelli et al., 1996). Less is known about the signal transduction pathway leading to SAPK activation by hyperosmotic shock. Further studies of this pathway in differentiated PC12 cells may help identify the signaling element(s) upstream of the SAPKs that is(are) targeted by CEP-1347.

CEP-1347 inhibition profiles of SAPK activation shown here indicate that the SAPK isoforms can be linked to different signaling pathways and that the latter can be differentially localized in neurites and the cell body. The signal transduction pathway in differentiated PC12 cells that is activated by hyperosmotic shock may be similar to that which activates the 55 kDa SAPK species found in DRG neurites since both are potently inhibited by CEP-1347. The profile for inhibition of 45 kDa SAPK activation in neurites resembled that for arsenite activation of SAPKs in PC12 cells since the two were highly insensitive to CEP-1347 treatment. This indicates that the axonal 45 kDa and 55 kDa SAPK isoforms are regulated differently despite being in the same subcellular compartment. Scaffolding proteins such as MEKKs, JIPs or some as yet unidentified species, may be involved in targeting the CEP-1347-sensitive signal transduction pathway to the axon. In fact, recent evidence indicates that cytoskeletal protein phosphorylation in

the squid giant axon and rat brain is topographically regulated by multimeric complexes (Grant et al., 1999; Veeranna et al., 2000).

*DRG neurons and differentiated PC12 cells contain high basal levels of phospho-SAPKs*

It is interesting to note that DRG neurites contain high basal levels of activated SAPKs (Figs. 3-5 and 3-6). It is unlikely that this is due to stresses associated with cell culture since the basal level of activated SAPKs in cell bodies is not as high and SAPK activation in this compartment responds more markedly to stress. High basal levels of activated SAPK have previously been observed in other cell types (Dhar et al., 1996) as well as in the brain (Xu et al., 1997; Gillardon et al., 1999). Furthermore, immunocytochemical analysis has shown that both the cell bodies and axons of DRG neurons in tissue sections contain phospho-SAPK (Fernyhough et al., 1999).

The high basal levels of activated SAPK in unstressed DRG neurons suggest that SAPKs function not only to mediate the response of a cell to stress but are also involved in maintenance or regulation of the constitutive phosphorylation of cellular proteins such as NFH (see discussion below). One can speculate that the high basal level of activated SAPK in neurites reflects the participation of SAPKs in maintaining the highly phosphorylated state of axonal NFH in cultured DRG neurons. As noted previously, this high level of NFH phosphorylation is attained in the absence of Schwann cells in antimitotically treated cultures (Giasson and Mushynski, 1997). The cues responsible for the activation of SAPKs in DRG neurites remain to be demonstrated.

A comparison of naive and differentiated PC12 cells showed that basal SAPK activity was increased in the latter (Fig. 3-2). This is consistent with a previous report that basal SAPK levels are on average five-fold higher in differentiated, than in naive, PC12 cells (Goodman et al., 1998). It was also evident that the differentiated PC12 cells were relatively insensitive to osmotic stress, which is also consistent with a previous study (Goodman et al., 1998). The changes that accompany NGF treatment are likely due to an effect of the growth factor on upstream signaling elements and/or SAPK phosphatases.

Results presented here demonstrate that phospho-SAPK levels can be reduced below basal (control) levels by CEP-1347 treatment of both differentiated PC12 cells subjected to hyperosmotic shock and CI-treated DRG neurons (Figs. 3-3, 3-5 and 3-6).

This may be due to stress-induced activation of a SAPK phosphatase or to CEP-1347 inhibition of a kinase whose constitutive activity is required to maintain basal phospho-SAPK levels. Evidence for the former possibility stems from studies of the MAPK phosphatases, a group currently composed of nine phosphatases in mammals that dephosphorylate Thr and Tyr residues within the MAPK activation loop (for review, see Camps et al., 1999; Haneda et al., 1999). Cellular stressors and growth factors that activate MAPKs can also induce MAPK phosphatase transcription and activation (Camps et al., 1999). Phosphatase activation may function as an "off switch" to attenuate the MAPK signal by dephosphorylating the kinase once the desired signal has been relayed. MAPK phosphatase-1 (MKP-1) has been previously implicated in a feedback loop that inactivates MAPKs during the cellular response to stress or after mitogenic stimuli (Misra-Press et al., 1995; Lai et al., 1996; Begum et al., 1998a; 1998b) and SAPK is a candidate substrate for MKP-1 (Sun et al., 1994; Liu et al., 1995; Misra-Press et al., 1995). Since hyperosmotic shock has been shown to increase transcription of the MKP-1 gene, this may explain why phospho-SAPK levels descend below basal levels in CEP-1347-treated PC12 cells subjected to hyperosmotic shock (Fig. 3-3) (Schliess et al., 1998). The effects, if any, of CI or arsenite on MKP-1 (or other phosphatases) transcription remain to be demonstrated.

A second mechanism which may explain the reduction of activated SAPK to below basal levels in CEP-1347-treated cells is that the signaling element targeted by the drug is also responsible for basal SAPK activation. The high basal level of activated SAPK in DRG neurons would thus reflect a high basal activity of one or more of its upstream activator(s).

*SAPKs are involved in constitutive phosphorylation of NFH*

Long-term treatment of unstressed sensory neurons with CEP-1347, but not PD098059 (an inhibitor of the ERK activator, MEK, Alessi et al., 1995), decreased the steady-state phosphorylation level of NFH in neurites as detected by a decrease in phosphoepitopes recognized by SMI-34 antibody and an increase in gel electrophoretic mobility (Fig. 3-7). The observed decrease in NFH phosphorylation began at around 0.02-0.05 μM CEP-1347 which correlates well with the $IC_{50}$ value of ~0.02 μM for inhibition

by CEP-1347 of the activation of a 55 kDa SAPK found in neurites (Figs. 3-5 and 3-6). These results suggest that the 55 kDa SAPK may contribute to the elevated phosphorylation state of NFH in the axon. According to the results of Western blot analysis with currently available commercial antibodies against the various SAPKs, the 55 kDa SAPK may be an isoform of SAPKβ or SAPKα (Fig. 3-8). Recent studies in cortical neurons indicate that SAPKβ may, at least in part, be responsible for NFH tail domain phosphorylation (Brownlees et al., 2000), although this conclusion was based on the assumed specificity of an anti-SAPKβ antibody.

Immunocytochemical analysis of DRG cultures using commercially available antibodies showed that SAPKα, SAPKβ and SAPKγ were present in cell bodies and neurites although there appeared to be an increased localization of SAPKα to the nuclei (Fig. 3-9). In addition, SAPKβ was heterogeneously distributed within the neuronal population with some neurons displaying prominent nuclear immunoreactivity (Fig. 3-9B). Nuclear localization of SAPKs may represent a novel mechanism for their regulation. Mizukami et al. (1997) previously found that inactive SAPK is translocated to the nucleus during ischemic insult but is only activated upon reperfusion due to nuclear entry of MKK4/SEK1.

*Functional significance of NFH phosphorylation*

The study of stress-induced hyperphosphorylation of perikaryal NFH is medically relevant since aberrant NF phosphorylation has been observed in toxic neuropathies induced by acrylamide (Gold et al., 1988) and β,β'-iminodipropionitrile (IDPN) (Gold et al., 1991). In addition, abnormal patterns of NF phosphorylation are seen in neurodegenerative disorders such as ALS, Parkinson's disease and Alzheimer's disease (for reviews, see Lee and Cleveland, 1994; Williamson et al., 1996; Julien and Mushynski, 1998). Whether phosphorylation of perikaryal NFs serves a protective function, contributes to pathology or is physiologically irrelevant remains to be determined.

One hypothesis suggests that hyperphosphorylation of perikaryal NFH may lead to the accumulation of NFs due to retardation of axonal transport. Previous work has shown that phosphorylation of the C-terminal domain of NFH correlated with a decrease

in the velocity of NF axonal transport (see Chapter 1 and Lewis and Nixon, 1988; Archer et al., 1994; Jung et al., 2000) and in animal models of ALS, transgenic mice expressing mutant forms of superoxide dismutase-1 (SOD1) exhibited a reduction in axonal transport velocity which may represent an early feature of toxicity mediated by the mutant protein (Williamson and Cleveland, 1999). Although it is clear that NF phosphorylation can slow NF axonal transport, it remains to be shown whether or not aberrant hyperphosphorylation of perikaryal NFH (and NFM) leads to NF accumulation. The physiological relevance of perikaryal NF accumulations is also unclear. Initial correlative evidence suggested that perikaryal NF accumulations had deleterious effects since they were correlated with neurodegenerative pathologies (see above and Williamson et al., 1996). Additional correlative evidence comes from a transgenic mouse model of ALS in which expression of human NFH causes ALS-like pathology associated with blockage of axonal transport and perikaryal NF accumulations (Cote et al., 1993; Collard et al., 1995b). Alleviation of this condition by crossbreeding with transgenic mice overexpressing human NFL highlights the importance of subunit stoichiometry in NF assembly and transport (Julien and Mushynski, 1998; Meier et al., 1999).

Other studies with transgenic mice have shown that perikaryal NF accumulations may not in themselves be sufficient to induce pathology. Transgenic mice expressing increased levels of NFM (Wong et al., 1995) and NFH (Marszalek et al., 1996) have no overt phenotype despite perikaryal NF accumulations and defects in axonal NF transport. Mice expressing an NFH-LacZ fusion protein also have perikaryal swellings and defective axonal transport but no ALS-type pathology (Eyer and Peterson, 1994). The reasons for these discrepancies are unclear at the moment although they may in part reflect differences in the type of NF subunit overexpressed or in the degree to which axonal transport is compromised.

Recent studies have indicated that under certain circumstances increased expression of perikaryal NF subunits confers protection against ALS pathology (Julien, 1999). Crossing transgenic mice expressing human NFH with mice carrying the SOD1 G37R mutation produced mice which developed ALS pathology later and survived significantly longer than mice expressing the SOD1 mutation alone (Couillard-Despres et al., 1998). A similar protective effect was reported when transgenic mice overexpressing

murine NFL or NFH were crossed with mice expressing SOD1 with the G93A mutation (Kong and Xu, 2000). Enigmatically, NFL-deficient mice crossed with mice expressing the SOD1 G85R mutation exhibited a slower onset and progression of ALS pathology (Williamson et al., 1998). However, features common to all of these mutant SOD1 mouse models in which life-span is increased by NF perturbation are the accumulation of NFs in the perikarya of motorneurons and decreased levels of axonal NFs.

Here we have shown that several cellular stressors induce hyperphosphorylation of perikaryal NFH and/or NFM by activation of SAPKs. Although the physiological relevance of stress-induced hyperphosphorylation of perikaryal NF proteins is unknown, it could conceivably serve a protective role by buffering the effects of SAPK activation brought on by neuronal insult. Another IF protein, keratin, is also hyperphosphorylated in response to stress and correlative evidence had led to the proposal that hyperphosphorylation has a protective role (Ku et al., 1996). Like keratins, which make up as much as 5% of total cellular protein in epithelial cells, NFs can similarly make up a significant proportion of the total cell protein particularly in large neurons. Hyperphosphorylation of such abundant proteins has been proposed to protect the cell by sequestering its ATP stores and slowing normal cell functions until the initiating stressor has subsided (Ku et al., 1996). Perhaps this is a component of the protective mechanism exerted by accumulations of perikaryal NFs in slowing the progression of ALS-like pathology in transgenic mice expressing mutant SOD1 although additional mechanisms have been proposed. For example, it has been suggested (Couillard-Despres et al., 1998) that perikaryal NFs are protective due to their ability to chelate calcium (Lefebvre and Mushynski, 1987; 1988).

Additional insights into the role of NFs in neuronal homeostasis and pathology will be forthcoming as our knowledge of NF structure and function increases. It is likely that SAPKs will emerge as key players in light of the multiplicity of compatible phosphorylation sites in the tail domains of NFM and NFH.

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