Glutamate Slows Axonal Transport of Neurofilaments in Transfected Neurons

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Abstract. Neurofilaments are transported through axons by slow axonal transport. Abnormal accumulations of neurofilaments are seen in several neurodegenerative diseases, and this suggests that neurofilament transport is defective. Excitotoxic mechanisms involving glutamate are believed to be part of the pathogenic process in some neurodegenerative diseases, but there is currently little evidence to link glutamate with neurofilament transport. We have used a novel technique involving transfection of the green fluorescent protein-tagged neurofilament middle chain to measure neurofilament transport in cultured neurons. Treatment of the cells with glutamate induces a slowing of neurofilament transport. Phosphorylation of the side-arm domains of neurofilaments has been associated with a slowing of neurofilament transport, and we show that glutamate causes increased phosphorylation of these domains in cell bodies. We also show that glutamate activates members of the mitogen-activated protein kinase family, and that these kinases will phosphorylate neurofilament side-arm domains. These results provide a molecular framework to link glutamate excitotoxicity with neurofilament accumulation seen in some neurodegenerative diseases.

Key words: neurofilament proteins • phosphorylation • amyotrophic lateral sclerosis • Alzheimer’s disease

Introduction

Neurofilaments are the major intermediate filaments of neurons and, in mature neurons, comprise three subunit proteins: neurofilament light chain (NF-L), neurofilament middle chain (NF-M), and neurofilament heavy chain (NF-H; for review see Lee and Cleveland, 1996). Neurofilament proteins are synthesized in cell bodies and transported into and through axons, with other components of the cytoskeleton, by slow axonal transport. Neurofilament and microtubule proteins travel in slow component a, whereas actin and a number of other proteins travel in slow component b (for reviews see Baas and Brown, 1997; Hirokawa et al., 1993; Schmidt et al., 1996). The speed of transport of neurofilaments varies between ~0.25 and 3 mm/d, and is dependent on a number of factors, including the type of neuron, location of neurofilament protein within the axon, and the age of the animal (Baas and Brown, 1997; Hirokawa et al., 1993; Nixon, 1998).

Accumulations of neurofilament proteins are seen in a number of neurodegenerative diseases. These include amyotrophic lateral sclerosis (ALS), Parkinson’s disease, dementia with Lewy bodies, and Alzheimer’s disease (Hirano, 1991; Trojanowski et al., 1993; Schmidt et al., 1996). Although it is not clear how neurofilament accumulations contribute to the neurodegenerative process in these diseases, their presence suggests that neurofilament transport is somehow disrupted in affected neurons. Indeed, direct measurements of axonal transport in several transgenic mouse models of ALS have revealed that slowing of neurofilament transport is a common and early pathological feature (Collard et al., 1995; Zhang et al., 1997; Williams and Cleveland, 1999).

The events that might lead to the slowing of neurofilament transport are not known, but a body of evidence implicates excitotoxic mechanisms involving glutamate in several neurodegenerative diseases (Rothstein, 1996; Guo et al., 1999). However, there is currently no direct evidence to link excessive extracellular glutamate with the
Materials and Methods

Cell Culture

Intermediate filament negative SW 13 cells were grown in DMEM containing 10% (vol/vol) FBS supplemented with 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (GIBCO BRL). Primary cortical neurons were obtained from E18 rat embryos and cultured on glass coverslips coated with poly-o-lysine and laminin in 12-well plates (Falcon) in a neurobasal medium and B27 supplement (GIBCO BRL) containing 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. Cells were collected for 5 d and, under these conditions, were almost exclusively neurons (staining with antibodies to neurofilaments and glial fibrillary acidic protein) (Dako) revealed that glial cells comprised <0.1% of the population.

Plasmids and Cell Transfection

For expression of NF-L, NF-M, and NF-H, the rat cDNA (Chin and Liem, 1989, 1990) were cloned into EcoRI fragments into pCIneo (Promega). NH2-terminal EGFP-tagged rat NF-M was expressed by cloning a rat NF-M cDNA into the EcoRI site of pEGFP-c3 (CLONTECH) and β-galactosidase by cloning into pQBIA PCM V (Quantum) following end neared both insert and vector.

7-d-old primary cortical neurons were transfected by calcium phosphate methods using a calcium phosphate Profection kit (Promega) essentially as previously described (Nikolic et al., 1996; Xia et al., 1996). In brief, neurons grown on coverslips in 12-well plates were transfected with 6 μg of pEGFP-NF-M plasmid DNA prepared using a ENDOfree plasmid kit (Qiagen). As described previously (Xia et al., 1996), the duration of incubation with the DNA/calcium phosphate precipitate was dictated by how fast the precipitate formed on the plate that was routinely between 10 and 20 min. The incubation was stopped 20 min later by removal of the media and shaking with 2% DM SO/5% glycerol in Hepes-buffered saline. The cells were washed three times in culture media and returned to the incubator. The efficiency of transfection was routinely 1–3%, and since contaminating glial cells represent <0.1% of the cells in the population, the majority of transfected cells are neurons which is in agreement with earlier studies on cortical neurons using this transfection method (Nikolic et al., 1996; Xia et al., 1996). However, some coverslips were additionally immunostained for neurofilament proteins (see below) or glial fibrillary acidic protein to further demonstrate that transfected cells were neurons. SW13 cells were transfected as previously described using a calcium phosphate Profection kit (Gibb et al., 1996).

Immunofluorescence Studies and Detection of EGFP-NF-M

For immunofluorescence studies of both SW13 cells and neurons, cells were fixed in 4% (wt/vol) paraformaldehyde in PBS, processed as above, and mounted in Vectashield. Transfected neurons were routinely stained with Hoechst 33258 (Molecular Probes) for analyses of nuclear morphology and fragmentation so as to assess any potential toxicity. Additionally, lactate dehydrogenase cell viability assays (CytoTox 96; Promega) were used to determine toxicity including that induced by glutamate. AII cells were examined using a Zeiss A xioskop microscope, and images were collected via a CCD camera (Princeton Instruments) and analyzed using Metamorph image analysis software. Statistical analyses of neurofilament transport rates were performed using One-way ANOVA tests. The rate of transport was calculated using linear regression analysis.

For studies of transfected EGFP-NF-M in primary cortical neurons, cells were fixed in 4% (wt/vol) paraformaldehyde in PBS, processed as above, and mounted in Vectashield. Transfected neurons were routinely stained with Hoechst 33258 (Molecular Probes) for analyses of nuclear morphology and fragmentation so as to assess any potential toxicity. Additionally, lactate dehydrogenase cell viability assays (CytoTox 96; Promega) were used to determine toxicity including that induced by glutamate. AII cells were examined using a Zeiss A xioskop microscope, and images were collected via a CCD camera (Princeton Instruments) and analyzed using Metamorph image analysis software. Statistical analyses of neurofilament transport rates were performed using One-way ANOVA tests. The rate of transport was calculated using linear regression analysis.

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dent neurons (Julien et al., 1986; Carden et al., 1987). Therefore, NF-M is probably a constituent of most cellular neurofilaments. Additionally, exogenous-tagged NF-M has been successfully used to measure neurofilament transport in previous studies (Terada et al., 1996; Wang et al., 2000). We tagged rat NF-M at its NH\textsubscript{2} terminus with EGFP. To demonstrate that this NH\textsubscript{2}-terminal addition of EGFP does not influence NF-M assembly properties, we studied EGFP-NF-M assembly in transfected SW13- cells that do not express intermediate filaments and in transfected primary cortical neurons.

Transfection of EGFP-NF-M alone into SW13- cells resulted in the formation of NF-M-containing aggregates but not NF-M intermediate filament networks (data not shown). This is consistent with previous observations on rodent neurofilament assembly properties that demonstrate that the formation of NF-M-containing neurofilaments requires coexpression with NF-L (Ching and Lien, 1993; Lee et al., 1993). However, cotransfection of EGFP-NF-M with NF-L led to filament formation (Fig. 1, a and b) that was not noticeably different from filaments formed by cotransfection of NF-L and untagged-NF-M (Fig. 1, c and d). Additionally, experiments involving cotransfection of EGFP-NF-M with NF-L and NF-H, with NF-L and NF-M, and with NF-L, NF-M, and NF-H, all produced intermediate filament networks of normal appearance (all data not shown but see Fig. 1, e and f, for networks in cells cotransfected with EGFP-NF-M and all three untagged neurofilament subunits).

To determine whether EGFP-NF-M was also capable of incorporation into neurofilaments in neurons, we analyzed neurofilament networks in EGFP-NF-M-transfected rat cortical neurons. Here, EGFP-NF-M colocalized with NF-L in typical neurofilament networks (Fig. 1, g and h). Thus, NH\textsubscript{2}-terminal tagging of NF-M with EGFP has no noticeable effect on its ability to coassemble into neurofilaments in both SW13- cells and neurons. These observations are consistent with similar assembly studies of vimentin and NF-M, which also involved NH\textsubscript{2}-terminal GFP-tagging of these proteins (Ho et al., 1998; Yoon et al., 1998; Wang et al., 2000).

**Transfected EGFP-NF-M Is Transported through Neurites at Rates Consistent with that of Slow Axonal Transport in Cultured Neurons, and Transport Requires Metabolic Energy**

To study anterograde transport of EGFP-NF-M through neurites, we transfected rat cortical neurons with the EGFP-NF-M plasmid, and 140 min after transfection, analyzed cells by fluorescence microscopy after fixation at 20-min intervals for periods of up to 280 min following transfection. Images of transfected cells were captured, and the distance traveled by EGFP-NF-M at each time point was measured. In these studies, measurements of the distance traveled by EGFP-NF-M were taken from the cell body to the front of the fluorescent signal and were of the longest distances in each neuron. The fluorescent front was taken as the most distal point at which fluorescence above background was detected. For neurites that exhibited branching, measurements were of the major neurite as determined by the length and brightness of fluorescence. To verify the objectivity of the measurements, two independent observers determined the distance to the fluorescent front in the same selected transfected neurons, and the results were not significantly different. Cells were routinely counterstained with Hoechst 33258 to assess nuclear morphology and apoptosis. These studies revealed no nuclear abnormalities or increased cell death associated with the transfected cells, which is in agreement with previous studies of cortical neurons using this transfection method (Nikolic et al., 1996).

Images of at least 20 transfected neurons for each time point were used to calculate the average rate of transport simultaneously transfected cells with antibodies to either NF-L and NF-M, NF-L or NF-H, and EGFP-NF-M and NF-H revealed that ~90% of cells express both plasmids. Thus, most cells appear to take up and express all three neurofilament subunits in these experiments. Bars, 25 μm.
of EGF-P-NF-M in each experiment (Fig. 2, representative images of transfected cells at 140–280 min after transfection). Since we measured the distance traveled by EGF-P-NF-M for only up to 300 μm from the cell body, and since the average length of the major neurites in the transfected cortical neurons exceeded 700 μm, the assays are of transport within neurites and are not a reflection of EGF-P-NF-M in neurite terminals and neurite growth rates. The average length of neurites in the cultures and also their rate of growth was determined by transfection of β-galactosidase and staining for this enzyme 24 h later. β-Galactosidase has been used on many occasions to determine the shape of neurons, and is known to be present in growth cones (Nikolic et al., 1996). These studies demonstrated that the average length of neurites 24 and 28 h after transfection was 777.5 ± 25.1 μm and 780.3 ± 21.8 μm, respectively (not significantly different using One-way ANOVA test; P = 0.932).

From these analyses, we calculated that the average rate of transport of EGF-P-NF-M was 80 ± 2 μm/h (Fig. 3). Although this rate of transport of EGF-P-NF-M showed no noticeable differences between experiments (compare, for example, the rate of transport of EGF-P-NF-M in Figs. 3 and 4, which are from two different experiments), the average distance traveled at the first time point (140 min) differed by ~15 μm between experiments. We attribute this to differences between transfections for each experiment that result in small variations in the precise time at which EGF-P-NF-M is first expressed. Indeed, the period of incubation with the DNA/calcium phosphate precipitate during transfection varies by ~10 min depending on the time in which a particular precipitate takes to form on the cells (see Materials and Methods). This 10-min variation is in keeping with the differences observed between experiments in the average distance traveled by EGF-P-NF-M at each time point. Other groups have also reported that the time taken for DNA/calcium phosphate precipitates to form on neurons using this transfection method is variable and can lead to small differences in the incubation period (Xia et al., 1996). However, to facilitate comparisons between different experiments, we have adjusted the displayed figures (Figs. 3 and 4) so that the distance traveled by EGF-P-NF-M is calculated as the distance from the first measurement (i.e., 140 min after transfection); the distance traveled at this first data point is, therefore, recorded as zero.

To determine whether transport of EGF-P-NF-M requires metabolic energy, measurements were taken from cells treated for 30 min with 50 mM 2-deoxy-D-glucose/
Glutamate Inhibits Transport of Transfected EGFP-NF-M

To study how the treatment of neurons with glutamate influenced EGFP-NF-M transport rates, we treated cells with 500, 50, and 30 μM glutamate. Glutamate treatment was applied 140 min after transfection (i.e., at the same time as the first recording of distance traveled was made) and maintained over the course of the experiment. 7-d-old cortical neurons are known to be susceptible to glutamate treatment and to express glutamate receptors (Cheng et al., 1994; Davis et al., 1995). Although glutamate had no significant effect on EGFP-NF-M movement over the first 20 min, at later time points, a significant inhibition of transport was observed. The extent of this inhibition of EGFP-NF-M transport was related to the concentration of glutamate applied to the cultures (Fig. 4).

Previous studies have shown that treatment of cultured cortical neurons with concentrations of up to 1 mM glutamate, for periods in excess of the times used here, does not influence cell viability (Davis et al., 1995). Indeed, the Hoechst 33258 staining that we routinely performed to monitor viability of the cells revealed no evidence of increased cell death over the period of treatment. However, to confirm that the slowing of EGFP-NF-M transport was not due to a simple loss of viability of the cells, we performed lactate dehydrogenase cell viability assays on glutamate-treated cells for periods of up to 180 min (i.e., longer than the 120-min period in which EGFP-NF-M transport was studied in the presence of glutamate). These studies revealed that there was no significant loss of viability over the time course of the experiment (data not shown), which is in agreement with previous reports of glutamate-treated cortical neurons (Davis et al., 1995).

To further confirm that this slowing of EGFP-NF-M transport was induced by the glutamate treatment, and to determine which glutamate receptor subtype(s) might mediate this effect, we studied EGFP-NF-M transport in either untreated or 50 μM glutamate-treated neurons, in the presence or absence of ionic glutamate receptor antagonists. Treatment with 5 μM CNQX (AMPA/kainate receptor blocker) had no significant effect on glutamate-induced retardation of the distance traveled; however, 1 μM MK-801 (NMDA receptor blocker) completely inhibited the effect of glutamate (Fig. 5). We also treated cells with 1 μM nifedipine, an inhibitor of L-type voltage-gated calcium channels, which are activated after depolarization of the postsynaptic membrane (Ghosh and Greenberg, 1995). However, nifedipine had no effect on glutamate-induced slowing of EGFP-NF-M transport (Fig. 5).

We also studied whether glutamate induced changes in the levels of NF-L, NF-M, and NF-H or tubulin proteins in the neurons by immunoblotting (Fig. 6). However, no alterations to the steady state levels of any of these proteins were detected. Finally, we assessed the mitochondrial membrane potential after glutamate treatment by staining with the probe MitoTracker red. These studies did not reveal any changes to mitochondria in response to gluta-
Glutamate activates MAP kinases in neurons treated with glutamate in the presence of MK801. A significant difference was observed between untreated and glutamate-treated neurons as analyzed by One-way ANOVA tests. No change in the total levels of either p42 or p44 MAPK (arrowheads), but a noticeable increase in the active forms of both these kinases was observed after glutamate treatment. Glutamate did not alter the levels of NF-L, NF-M, NF-H, or tubulin. An identical sample is shown at the bottom to demonstrate equal protein loading of the samples.

We have recently demonstrated that, in rat cortical neurons (as used here), glutamate activates SA PK1b but has no effect on cdk5 activity and causes a decrease in glycogen synthase kinase-3 activity (Brownlees et al., 2000). Others have also shown that glutamate activates SA PK1s in neurons (Bading and Greenberg, 1991; Kurino et al., 1995; Xia et al., 1996; Kawasaki et al., 1997; Schwarzschild et al., 1999). However, we did not investigate the effect of glutamate that has on p42/p44 MAPK activities in our earlier studies, and rectified this omission by the use of antibodies that detect total and active p42/p44 MAPK. These studies demonstrated that glutamate induced a marked activation of both MAPK isoforms, and that this activation extended for at least 120 min (i.e., the time course over which EGF-NF-M transport was studied; Fig. 6). Others have also shown that glutamate can activate p42/p44 MAPK in neurons (Bading and Greenberg, 1991; Kurino et al., 1995; Xia et al., 1996; Schwarzschild et al., 1999). Thus, p42/p44 MAPK and SA PK1s are good candidates for altering NF-M/NF-H side-arm domain phosphorylation in response to glutamate.

We also studied the subcellular localization of active p42/p44 MAPK and SA PK1s in the neurons using antibodies that detect the active forms of these kinases. In untreated cells, active p42/p44 MAPK was only weakly detected where it localized to cell bodies and some neurites (Fig. 7 a); active SA PK1 was present mainly in neurites (Fig. 7 c). Treatment with glutamate induced a marked increase in active p42/p44 MAPK labeling in both cell bodies (including some nuclei) and neurites (Fig. 7 b). Similarly, glutamate also increased labeling for active SA PK1s, particularly in cell bodies and nuclei (Fig. 7 d).

NF-M side-arms are phosphorylated by p42/p44 MAPK (Veeranna et al., 1998; Li et al., 1999) and SA PK1b, which is a major SA PK in neurons (Oh et al., 1995; Martin et al., 1996; Carboni et al., 1997, 1998; Lee et al., 1999). However, there is currently little data to demonstrate that SA PK1b will phosphorylate NF-M side-arms. Therefore, we prepared the side-arm domain of NF-M as a GST fusion protein and tested its ability to be phosphory-

**Figure 5.** Effect of glutamate receptor antagonists on glutamate-induced inhibition of EGF-NF-M transport. Histogram shows distance traveled by EGF-NF-M 240 min after transfection in either untreated or 50-μM glutamate-treated neurons in the presence or absence of 1 μM MK-801, 5 μM CNQX, or 1 μM nifedipine. Glutamate was applied at the 140-min time point and inhibitors 10 min before this. Data from one representative experiment are shown. Asterisks indicate treatments that displace a significant fraction (P < 0.001) compared with 50-μM glutamate treatment as analyzed by one-way ANOVA tests. No significant difference was observed between untreated neurons and neurons treated with glutamate in the presence of MK-801. Error bars are SEM.

**Figure 6.** Glutamate activates p42 and p44 MAPKs and does not alter the levels of neurofilament or tubulin proteins in cortical neurons. 7-d-old cortical neurons were either untreated (un), or treated with 100 μM glutamate for 30, 60, or 120 min, and then analyzed by 12% SDS-PAGE and immunoblotting. No change in the total levels of either p42 or p44 MAPK (arrowheads), but a noticeable increase in the active forms of both these kinases was observed after glutamate treatment. Glutamate did not alter the levels of NF-L, NF-M, NF-H, or tubulin. An identically loaded Coomassie-stained gel is shown at the bottom to demonstrate equal protein loading of the samples.
lated by recombinant SA PK1b. For comparisons, we also used as a substrate the MPR domain of NF-H side-arms (Brownlees et al., 2000) and recombinant p42MAPK. Both NF-M and NF-H substrates, but not GST, were phosphorylated by p42MAPK and SAPK1b (Fig. 8). Not all Ser-Thr-Pro motifs are phosphorylated under these conditions, since we have shown that Thr668 in the Alzheimer’s disease amyloid precursor protein, which is a known in vivo phosphorylation site, is not phosphorylated by MAPK (Aplin et al., 1996).

We next examined if glutamate induced alterations to the phosphorylation of NF-M/NF-H side-arms in vivo in cortical neurons. SMI36, an antibody that reacts with phosphorylated but not nonphosphorylated NF-M/NF-H side-arms (Sternberger and Sternberger, 1983), labeled neurites but only weakly labeled cell bodies in untreated cells (Fig. 7e). This is consistent with many studies, which have shown that NF-M and NF-H side-arms are heavily phosphorylated in axons but are much less phosphorylated in cell bodies and proximal axons (Julien and Mushynski, 1982; Sternberger and Sternberger, 1983; Carden et al., 1985; Lee et al., 1987, 1988; Nixon et al., 1994b). However, glutamate induced a marked increase in SMI36 labeling of cell bodies that was evident as early as 30 min after glutamate treatment (Fig. 7f). We also confirmed that glutamate induced an increased cell body phosphorylation of NF-M/NF-H side-arms in the EGFP-NF-M–transfected neurons by the staining of transfected cultures with SM136 (Fig. 9, a–d). Thus, glutamate activates members of the MAP kinase family including p42/p44MAPK and SAPK1b, neurofilament side-arm domains are substrates for these kinases, and glutamate induces increased phosphorylation of neurofilament side-arms in cell bodies of cortical neurons.

Finally, we analyzed whether the glutamate-induced slowing of EGFP-NF-M transport might lead to the formation of neurofilament accumulations such as are seen in some neurodegenerative diseases. Accumulations typical of these disorders were rarely detected over the time course in which transport rates were studied (140–260 min after transfection and 120-min glutamate treatment). However, after 180 min of glutamate treatment (i.e., 320 min after transfection), increased EGFP-NF-M labeling and swelling of proximal neurites become more noticeable in a proportion of the neurons. To quantify this more carefully, we randomly captured images of EGFP-NF-M–transfected cells either untreated or treated with 100 μM glutamate at this time point and scored them blind for the presence of accumulations. In two different experiments, accumulations typical of the ones shown in Fig. 10 (see also accumulation in Fig. 9 double labeled with SMI36) were found in greater numbers in the glutamate-treated cells (experiment 1, 29.6% treated [n = 81] versus 7.6% untreated [n = 92]; experiment 2, 33.3% treated [n = 69] versus 9.9% [n = 111]). The accumulations seen in untreated neurons may be a consequence of increased ex-
after transfection, were fixed and immunostained with SMI36. a neurons. Cells were transfected with EGFP-NF-M and, 320 min M/NF-H side-arms in cell bodies of EGFP-NF-M–transfected Figure 9. Glutamate induces increased phosphorylation of NF-M glutamate-treated cell. Bar, 25 μm.

expression of EGFP-NF-M since overexpression of NF-M in transgenic mice can also lead to neurofilament accumulations (Vickers et al., 1994; Wong et al., 1995).

Discussion

Discussion

A nterograde axonal transport of the cytoskeleton has been studied using a variety of experimental systems both in vivo and in cultured cells (for reviews see Baas and Brown, 1997; Hirokawa et al., 1996; Hirokawa et al., 1997; Nixon, 1998). These include monitoring the movement of radiolabeled proteins (Reinsch et al., 1991; de Waegh et al., 1992; Lasek et al., 1992; Archer et al., 1994; Nixon et al., 1994b; Collard et al., 1995; Campenot et al., 1996; Zhang et al., 1997; Williamson and Cleveland, 1999), the use of fluorescently labeled cytoskeletal proteins, and photoactivation and photo-bleaching techniques (Keith, 1987; Okabe and Hirokawa, 1992, 1995). Recent studies have shown that neurofilaments accumulate in fast transport rates (up to 2.3 μm/s), but that this movement is interrupted by prolonged pauses (Wang et al., 2000). Therefore, only a proportion of neurofilaments are moving at any one time (estimated at 1–15%), such that an overall slow transport rate is observed. Since we analyzed EGFP-NF-M movement in cells fixed at 20-min intervals rather than in living cells, our assays calculate this overall slow transport rate. In the cortical cells used, this is 80 ± 2 μm/h, which is consistent with the known rates of transport as assayed in vivo by other methods.

Abnormal accumulations of neurofilament proteins are seen in ALS, A lzheimer’s disease, and Lewy body in Parkin-son’s disease and Lewy body dementia (H irano, 1991; Trojanowski et al., 1993; Schmidt et al., 1996). Such accumulations suggest that the transport of neurofilament proteins through axons is disrupted in these disease states. Indeed, measurements of axonal transport in several transgenic mouse models of ALS reveal that the slowing of cytoskeletal transport is an early pathological feature (Collard et al., 1995; Zhang et al., 1997; Williamson and Cleveland, 1999).

The events that lead to cell death in these neurodegenerative diseases are not known, but excitotoxic mechanisms involving glutamate have been suggested to be part of the disease process for both ALS (for reviews see Shaw, 1994; Rothstein, 1996) and Alzheimer’s disease (Guo et al., 1999). Indeed, a loss of the glial glutamate transporter (EAAT2) has been described in sporadic forms of ALS (Lin et al., 1998) in a transgenic mouse model of ALS (Brujin et al., 1997). Moreover, mutant SOD1s, which cause familial forms of ALS, induce selective damage to EAAT2 by oxidative mechanisms (Trotti et al., 1999); uptake of glutamate via sodium-dependent glutamate transporters is a primary mechanism for inactivation of extracellular glutamate. Thus, glutamate-induced excitotoxicity might be part of the pathogenic process in several neuro-
degenerative diseases. Our finding that glutamate inhibits anterograde axonal transport of NF-M, therefore, provides a mechanism to link excitotoxicity with neurofilament accumulation in these disorders.

The series of molecular events by which glutamate exposure might lead to a slowing of neurofilament transport are not clear. However, increased phosphorylation of NF-M and NF-H side-arm domains has been associated with slower neurofilament transport rates in many studies (Watson et al., 1989a, b, 1991; A rcher et al., 1994; Nixon et al., 1994a, b; Jung and She a, 1999). Therefore, it is notable that we observe an increase in neurofilament side-arm phosphorylation in cell bodies after glutamate treatment. Other studies have shown that glutamate can cause increased neurofilament side-arm phosphorylation in neurons (A sahara et al., 1999; Brownlee et al., 2000). NF-M and NF-H side-arms are more heavily phosphorylated in axons than cell bodies (J ulien and M ushynski, 1982; S ternberger and S ternberger, 1983; C arden et al., 1985; L ee et al., 1987, 1988; Nixon et al., 1994b), and so it is also possible that changes to neurofilament phosphorylation occurred in neurites but were not so easily detectable.

Glutamate is known to activate members of the M A PK family including p42/p44 MAPK and SA PKs (B ading and G reenberg, 1991; K urino et al., 1995; X ia et al., 1996; S chwarzchild et al., 1997, 1999; Brownlee et al., 2000), and we demonstrate that this is also the case in the cortical neurons used here. p42/p44 MAPK and SA PK 1b/c will all phosphor ylate NF-M/NF-H side-arms (G iasson and M ushynski, 1996, 1997; V eran na et al., 1998; L i et al., 1999; Brownlee et al., 2000; data shown here), and we show that the active forms of these kinases are present in cell bodies in the glutamate-treated neurons. Together, these observations suggest that these particular kinases are the link between glutamate and increased neurofilament phosphorylation.

Thus, activation of p42/p44 MAPK and SA PKs, and phosphorylation of NF-M/NF-H side-arms might be at least part of the process by which glutamate causes a slowing of neurofilament transport in the cortical neurons used here. Whether the same processes occur in other neuronal cell types including lower motor neurons remains to be established. However, other possible mechanisms including glutamate-induced alterations to the neurofilament transport motor, which has to date not been identified, or the way in which neurofilaments attach to the motor, which is again not known (B rady, 2000), cannot be excluded.

Indeed, one recent suggestion is that NF-M/NF-H side-arm phosphorylation regulates association of neurofilaments with kinesin, a fast motor (Y abe et al., 2000). Whatever the precise mechanism, the results presented here are the first to show a link between glutamate excito toxicity, a proposed pathogenic process for several human neurodegenerative diseases, and slowing of neurofilament transport, which is a pathological feature in at least some of these disorders.

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