

## Increased $M_r$ 60,000 Protein Phosphorylation is Correlated with Neocortical Neurofibrillary Tangles in Alzheimer's Disease

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**Abstract.** Increased  $M_r$  60,000 protein phosphorylation has been found in the cytosol fraction of brain tissue from Alzheimer's disease patients. A correlation between this biochemical change and the morphologic abnormalities found in Alzheimer's disease was sought. Three neuropathologic features were studied: neurofibrillary tangles and neuritic plaques, findings characteristic of Alzheimer's disease, and gliosis, a non-specific change. The number of tangles correlated well with the extent of  $M_r$  60,000 protein phosphorylation ( $p < 0.001$ ); but the number of plaques did not. To investigate the possibility that gliosis causes the increased  $M_r$  60,000 protein phosphorylation, cases of Pick's disease and multi-infarct dementia were also studied. The levels of  $M_r$  60,000 protein phosphorylation in these cases were comparable to those seen in normal controls. These findings suggest that the increased  $M_r$  60,000 protein phosphorylation is closely related to diseased, tangle-bearing neurons and is not directly related to neuritic plaque formation or secondary gliosis.

**Key Words:** Alzheimer's disease; Gliosis; Neuritic plaque; Neurofibrillary tangles; Phosphorylation.

### INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disease, affecting more than 5% of the population beyond age 65. This disease is characterized pathologically by two types of lesions, neuritic plaques (NP) and neurofibrillary tangles (NFT). Although the pathogenic mechanisms are not known, increasing immunohistochemical evidence suggests that NFT are composed of normal cytoskeletal components, although their state of phosphorylation differs from that of their normal counterparts (1, 2). Aberrant phosphorylation reactions have also been found biochemically (3, 4). *In vitro* phosphorylation of a  $M_r$  60,000 protein is increased in homogenates prepared from Alzheimer brain tissue compared with control (3). This altered biochemistry may be causally related to the neuronal loss occurring in Alzheimer's disease, or it may reflect secondary changes. Is the increased  $M_r$  60,000 protein phosphorylation intrinsic to the pathogenesis of the disease? If significant correlations can be shown to exist between the increased  $M_r$  60,000 protein phosphorylation and either of the histopathologic hallmarks of Alzheimer's disease, i.e. NP and NFT, this possibility becomes more likely. Alternatively, the biochemical abnormality may be secondary to the astrocytic gliosis encountered in AD as a reaction to neuronal loss. In this study we tried to correlate  $M_r$  60,000 protein phosphorylation with the number of NFT and NP in well-documented cases of AD.

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We also studied the relationship between  $M_r$  60,000 protein phosphorylation and gliosis per se. For this purpose, we examined brain tissue from patients with Pick's disease and multi-infarct dementia. In these instances, significant neocortical gliosis and neuronal loss are present but NP and NFT are lacking. In this report, we present evidence that  $M_r$  60,000 phosphorylation correlates well with NFT counts but not with NP or gliosis. We hypothesize, therefore, that the  $M_r$  60,000 protein, or its kinase, may be involved in the pathogenesis of the NFT.

## MATERIALS AND METHODS

A total of 22 brains comprising four diagnostic categories were studied. There were 12 cases of AD, three of Pick's disease (PD), one of multi-infarct dementia (MID), and six normal controls. All patients from the first three categories were clinically demented, whereas those in the control group were not. Postmortem times varied but did not exceed 24 hours (h), and the bodies were kept at 4°C from death until autopsy. Following removal, the brains were divided sagittally and the left hemibrain was fixed in 10% formalin while the right hemibrain was frozen at -70°C. Before dissection, the right hemibrains were kept for four h at 4°C and sections approximately 1 cm thick were sliced while the brains were still frozen. Midfrontal cortex was dissected from these sections on a glass plate cooled from below by a bed of powdered dry ice. Dissected cortex was placed in 10 volumes of the homogenization buffer (0.32 M sucrose; 5 mM HEPES, pH 8.0; 5 mM benzamidine; 2 mM  $\beta$ -mercaptoethanol; 3 mM EGTA; 0.5 mM  $MgSO_4$ ; 0.5 mM  $ZnSO_4$ ; 0.1 mM PMSF; 10  $\mu$ g/ml leupeptin; 5  $\mu$ g/ml pepstatin and 10  $\mu$ g/ml aprotinin) and homogenized by two five-second strokes of a Polytron homogenizer (Brinkmann, Westbury, NY). This homogenate was centrifuged one h at 100,000  $\times$  g at 2°C to prepare the cytosol. Cytosol fractions were divided into aliquots, frozen in an ethanol/dry-ice bath, and stored at -70°C until used for the phosphorylation reaction.

After seven to ten days of fixation, the left hemibrains were examined grossly, dissected, and blocks were taken from all cerebral lobes, hippocampus, basal ganglia, substantia innominata, hypothalamus, amygdala, mesencephalon, pons, and cerebellum. Hematoxylin and eosin (H&E) and Nissl preparations from these areas were examined for overall neuropathologic evaluation. Thioflavin S-stained sections (10  $\mu$ m thickness, 1% aqueous thioflavin S) were viewed with ultraviolet illumination and fluorescein filters for the identification of neocortical NFT and NP. These lesions were quantified in sections of midfrontal cortex, superior temporal gyrus, inferior parietal cortex, and hippocampus. Neocortical NP and NFT quantification first involved evaluation of the entire thioflavin S-stained slide. A representative area of cortex along the side of the gyrus where the pial surface was parallel to the gray-white junction was then chosen for lesion quantification. Plaques were counted in three  $\times$ 125 microscopic fields of neocortex. The fields in which NP were enumerated were not chosen at random, but rather represented regions of high NP density within the examined cortex. This area typically involved the upper cortical layers, where NP are found in largest numbers (5). The resulting three figures from the microscopic fields were then averaged to provide single NP counts for each case. In a similar manner, the entire slide was first evaluated at low power for NFT counts. Since NFT, unlike NP, tend to occur in definite clusters (5), some fields with no NFT could be found adjacent to those with several. In order to increase the sensitivity of NFT detection, we counted these lesions at higher magnification ( $\times$ 500) only in areas of high NFT density. Three such fields were counted, and the resulting figures averaged to obtain single NFT counts for each case. Counts of zero, then, actually represent an absence of NFT on the entire slide rather than in just three  $\times$ 500 fields.

Diagnoses of AD were made when large numbers of NP and NFT were seen in the neocortex. In some cases, only NP were present, but they occurred in sufficient numbers to assure the diagnosis. A significant percentage of elderly AD cases lack tangles in the neocortex (6). Pick's disease (PD) was diagnosed when many of the pathognomonic intracytoplasmic argyrophilic neuronal inclusions (i.e. Pick bodies) were seen within a milieu of severe neuron loss and extensive gliosis in the absence of plaques and tangles. The one case of clinical dementia

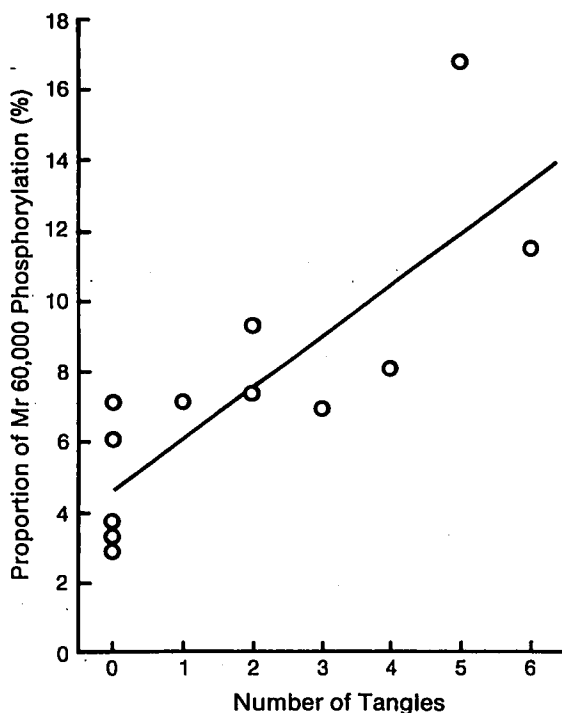


Fig. 1. The proportion of  $M_r$  60,000 protein phosphorylation determined by SDS-gel electrophoresis and liquid scintillation counting in the midfrontal cortex is plotted against the average number of NFT found in a  $\times 500$  field.  $r = 0.85$ ;  $p < 0.001$ .

without either AD or PD displayed numerous, widespread but predominantly small, subacute to old infarcts in the cerebral hemisphere and brain stem. The brains of some of the six control patients without clinical dementia contained small foci of old or recent infarction, but without sufficiently extensive parenchymal loss to result in clinical dementia.

#### Phosphorylation and Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis

The conditions of the phosphorylation reaction have been described previously (3). Ten microliters of cytosol fraction in the homogenization buffer containing  $6.5 \mu\text{g}$  total protein was mixed with  $15 \mu\text{l}$  of reaction mixture containing 50 mM Tris-HCl, pH 7.6 (all concentrations are for final 25- $\mu\text{l}$  volume), 10 mM  $\text{MgSO}_4$ , 100  $\mu\text{g/ml}$  protein kinase A inhibitor (Sigma, St. Louis, MO), 5 mM  $\beta$ -mercaptoethanol, 10  $\mu\text{M}$  ATP, 1  $\mu\text{Ci}$  per tube [ $\gamma$ - $^{32}\text{P}$ ]ATP (ICN, Irvine, CA), and 0.2 mM EDTA. The mixture was incubated for 12 minutes at  $30^\circ\text{C}$ . The reaction was stopped by addition of  $6.3 \mu\text{l}$  of a buffer containing 40% glycerol, 25%  $\beta$ -mercaptoethanol, 12% SDS, 0.31 M Tris-HCl (pH 6.8), 25 mM EDTA, and 0.1% bromophenol blue. The samples were then subjected to 6.5–12.5% polyacrylamide gel (1 mm thickness) electrophoresis with 2-cm stacking gel (7) at a current of 20 mA per gel. The electrophoresis was stopped when the dye front reached 1 cm from the gel edge. Gels were fixed one h in 10% acetic acid containing 15% isopropyl alcohol, stained one h with 0.2% Coomassie brilliant blue in 10% acetic acid and 40% isopropyl alcohol, and destained in 10% acetic acid and 15% isopropyl alcohol. The destained gel was rinsed one h with water, dried, and exposed about ten h to X-Omat RP film (Kodak) with a Hi Plus intensifying screen (Du Pont) at  $-70^\circ\text{C}$ . Dried gels were dissected and radioactivity recovered in the  $M_r$  60,000 band and that of the entire lane (total proteins) were determined by liquid scintillation counting (Tm Analytic, Elk Grove Village, IL), and phosphorylation of the  $M_r$  60,000 protein was

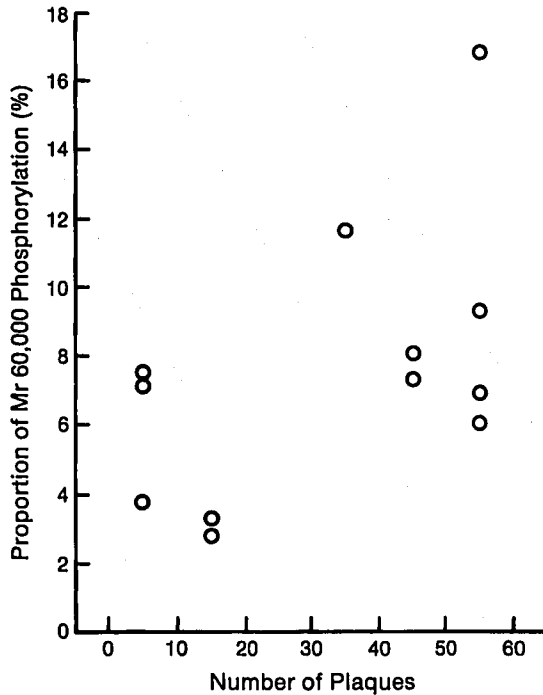


Fig. 2. Same as Figure 1 except the abscissa is the average number of neuritic plaques found in a  $\times 125$  field.  $r = 0.54$ . Not significant.

expressed as the percentage of radioactivity recovered in the gel lane. Protein concentrations were determined using the method of Lowry et al (8) with plasma globulin (Bio-Rad, Richmond, CA) as the standard.

## RESULTS

### *M<sub>r</sub>* 60,000 Protein Phosphorylation Correlates Positively with Neurofibrillary Tangle Counts

Neocortical NFT are highly characteristic of AD, although they are found in other conditions and some cases of AD among the elderly do not exhibit them (6). Tangle

TABLE 1  
*M<sub>r</sub>* 60,000 Protein Phosphorylation in Different Neurological Diseases

	Neurofibrillary tangles in midfrontal neocortex	Gliosis	<i>M<sub>r</sub></i> 60,000 phosphorylation (%)
Control	—	—	4.0 $\pm$ 1.0 (N = 6)
Alzheimer's disease	+	+	10.5 $\pm$ 3.5 (N = 7)*
Alzheimer's disease	—	+	4.6 $\pm$ 1.8 (N = 5)
Pick's disease	—	+	4.5 $\pm$ 0.9 (N = 3)
Multi-infarct dementia	—	+	5.3 (N = 1)

The *M<sub>r</sub>* 60,000 protein phosphorylation was determined by SDS-gel electrophoresis and scintillation counting of the *M<sub>r</sub>* 60,000 bands and total lanes from dried gels. Alzheimer cases in which NFT were not found in the midfrontal cortex section are tabulated separately from those which had both neocortical NP and NFT. Figures are means  $\pm$  standard deviations.

\* Different from control ( $p < 0.005$ ).

counts correlate well with other pathologic changes in AD, and increasing numbers of NFT reflect a greater disease severity in young patients. When the degree of  $M_r$  60,000 protein phosphorylation is plotted against NFT counts using linear regression analysis, there is a significant positive correlation ( $r = 0.85$ ,  $p < 0.001$ , Fig. 1). This result is consistent with the previous finding (3) of greater  $M_r$  60,000 protein phosphorylation in younger Alzheimer brains than in older ones, as young cases of AD typically have higher NFT counts. Therefore, the increased  $M_r$  60,000 protein phosphorylation may be a direct measurement of tangle-bearing neurons in AD. As tabulated in Table 1, AD with detectable neocortical NFT showed increased  $M_r$  60,000 protein phosphorylation, although Alzheimer cases with only NP and without NFT in the cortex do not show significant differences from the control in  $M_r$  60,000 protein phosphorylation.

#### **$M_r$ 60,000 Protein Phosphorylation Does Not Correlate with Neuritic Plaque Counts**

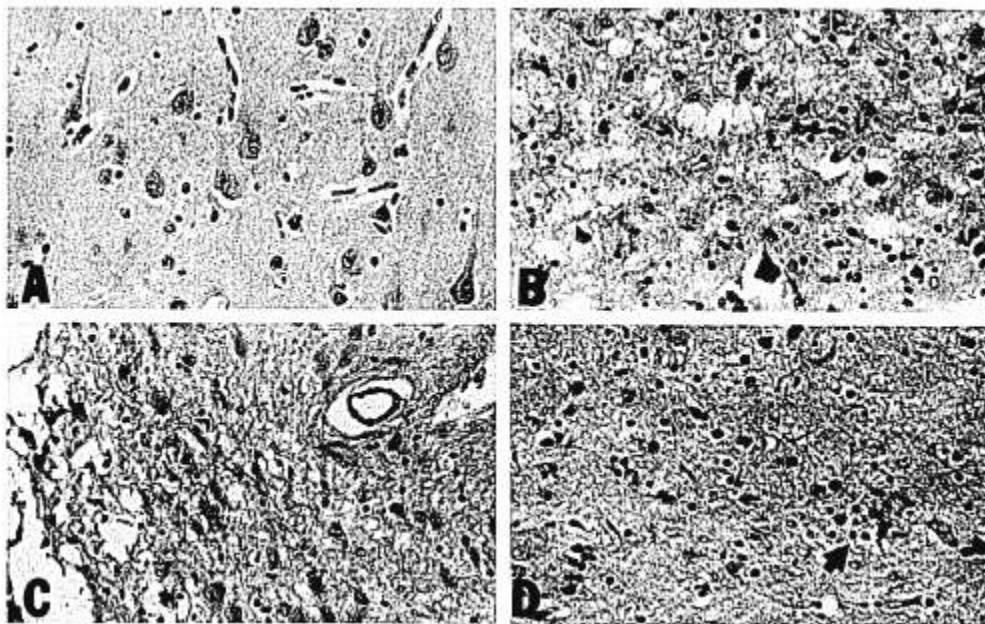
The other neuropathologic hallmark of AD is the NP. Plaques are composed of dilated neurites, presynaptic and postsynaptic, plus, in many instances, accumulations of extracellular amyloid (9). The dilated neurites may often contain paired helical filaments ultrastructurally identical to NFT. In the NP, however, the concentration of paired helical filaments is variable and always less than that comprising a perikaryal NFT. Linear regression analysis of NP counts and the degree of  $M_r$  60,000 protein phosphorylation does not yield a significant correlation ( $r = 0.54$ , Fig. 2).

#### **Increased $M_r$ 60,000 Protein Phosphorylation Is Not a Consequence of Reactive Gliosis**

The neocortical lesions of AD include, in addition to the NP and NFT, significant neuronal loss and accompanying reactive fibrous gliosis (Fig. 3D). This increased cortical gliosis could conceivably be responsible for the observed increase in  $M_r$  60,000 protein phosphorylation. To test this possibility, we measured  $M_r$  60,000 protein phosphorylation in brains which showed intense gliosis but did not possess the other neuropathologic stigmata of AD, i.e. they lacked NP and NFT. In PD (Fig. 3B), neuronal loss and gliosis are characteristically more severe than in AD but NP and NFT are not found. We also studied, as another instance in which gliosis was present without AD, a case of multi-infarct dementia (Fig. 3C) in which the neocortex contained multiple foci of minute infarction with associated fibrous gliosis.  $M_r$  60,000 protein phosphorylation in these cases did not significantly exceed that seen in the controls (Fig. 3A) and was about one-half that measured in brain tissues from Alzheimer patients with NFT (Table 1). We may conclude, therefore, that the increase in  $M_r$  60,000 phosphorylation was not the result of reactive gliosis.

### **DISCUSSION**

When these results are considered together, the increased level of  $M_r$  60,000 protein phosphorylation seems to be directly related to the neuronal perikaryal changes and not to NP formation or secondary gliosis. Because the correlation between NFT formation and  $M_r$  60,000 protein phosphorylation is highly significant, one may ask if the  $M_r$  60,000 protein is a constituent of NFT. Under our experimental conditions, the  $M_r$  60,000 protein as well as its kinase were found in the cytosol fraction only, suggesting the  $M_r$  60,000 protein is not a constituent of NFT per se. It is also possible, however, that a very weak association exists between the NFT and the  $M_r$  60,000



**Fig. 3.** A. Normal neocortical cytoarchitecture. H&E.  $\times 200$ . B. Pick's disease. The cortex shows extreme neuronal loss and gliosis with resulting microcystic degeneration of the neuropil. H&E.  $\times 200$ . C. Multi-infarct dementia. Numerous reactive astrocytes are seen in a rim of subpial tissue overlying a small cystic cortical infarct. H&E.  $\times 200$ . D. Alzheimer's disease. Extensive neuronal loss and gliosis can be seen in a severe example of AD. Note NP with amyloid core visible with H&E staining (arrow).  $\times 200$ .

protein, or that the cytoskeleton in AD is exceptionally vulnerable to postmortem changes (10) and the biochemical techniques employed are not adequate to detect the intact compartmentalization of the  $M_r$  60,000 protein.

Intact cytoskeletal structures, including loosely associated proteins, can be extracted using 2 M glycerol and a slightly acidic medium (11). Employing this condition where cytoskeletal structure is well preserved, we studied the association of  $M_r$  60,000 protein with cytoskeletal and NFT fractions from Alzheimer's brain homogenates. Again, the  $M_r$  60,000 protein and its kinase were found in the cytosol fraction.

In this context, it was important to test the possibility that the  $M_r$  60,000 protein was a tau protein, as the group of tau protein has been shown to be major antigenic components in NFT (12–15). We employed the technique of Lindwall and Cole (16) to purify tau protein. This resulted in a ladder of protein bands between  $M_r$  65,000 and 45,000 on SDS-polyacrylamide gels. However, the  $^{32}\text{P}$ -labeled  $M_r$  60,000 protein never copurified with tau proteins. Therefore, the  $M_r$  60,000 protein does not seem to be an authentic tau protein, although it is possible that it is a member of a tau superfamily having some unique character which does not allow its purification by this method.

The  $M_r$  60,000 protein phosphorylation is not increased in PD cases, despite the fact the Pick bodies share antigenic determinants with Alzheimer's NFT (17). It has recently been shown that, following dephosphorylation with alkaline phosphatase, Pick bodies are strongly labeled by an antibody to tau (18). Since tau is a component of Alzheimer NFT, and since Pick bodies apparently contain tau, the absence of

increased  $M_r$  60,000 protein phosphorylation in PD is further evidence that the  $M_r$  60,000 protein is unlikely to be a tau protein.

What then is the biological function of the  $M_r$  60,000 protein? The most attractive idea is that this protein is involved in the regulation of NFT formation. It is conceivable that abnormal tau protein phosphorylation is essential for NFT formation. It is not unreasonable to speculate that the  $M_r$  60,000 protein is a component of a kinase or a kinase regulatory protein, whose abnormal phosphorylation is an essential step in NFT pathogenesis. It has been reported that all kinases are also phosphoproteins (19). The study to test this hypothesis is under way in our laboratory.

One interesting finding of this study is the apparent dissociation between NFT and NP, the two lesions which typically characterize AD. The increased  $M_r$  60,000 protein phosphorylation was closely associated with NFT but not with NP. Masters and his colleagues (20) proposed, from their protein sequencing data, that in Alzheimer's disease the NP core protein derived from a component of the NFT. If so, it would be expected that biochemical correlates of the number of NFT would also correlate with counts of NP. Our present data demonstrate that one biochemical marker, increased  $M_r$  60,000 protein phosphorylation, correlates well with the number of NFT but not with the counts of NP, suggesting that NFT formation follows a distinct biochemical process which may not induce the pathogenesis of the NP. Support for this contention may be drawn from the observation that NFT can be seen in a wide variety of neuropathologic conditions other than AD, where they are not associated with NP. Furthermore, in a significant percentage of Alzheimer brains, neocortical NP may not be accompanied by NFT (6), a circumstance unlikely if the former are derived from the latter.

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