CHEMICAL PATHOLOGY OF NEUROFIBRILS

NEUROFIBRILLARY TANGLES OF ALZHEIMER'S PRESENILE-SENIILE DEMENTIA

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A subcellular fraction enriched in twisted tubules was obtained by differential centrifugation of a homogenate of neurons isolated from areas of the brain with many neurofibrillary tangles from patients with Alzheimer's presenile-senile dementia. A unique protein (molecular weight 50,000 daltons) which does not co-migrate with either of the two tubulin monomers or the major neurofilament protein, both purified from human brain, was found in this subcellular fraction on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Similarly processed tissue from areas of the brain poor in neurofibrillary tangles contained low levels of this new protein. The new protein band could not be seen in control patients.

The neurofilament is a linear 9-10-nm microfilament found in the neuronal cell body, the axon and the dendrites. It has a poorly defined lumen, short "side arms" protrude from it and it seems to be composed of globular subunits. Unlike neurotubules, the neurofilaments are stable and can be readily isolated by subcellular fractionation. Neurofilaments and their subunits can be disaggregated in detergents, guanidine hydrochloride or by succinylation (3, 11, 12). Davison and his colleagues (3, 6) have isolated a neurofilament subunit from the axoplasm of the Chilean giant squid. The isolation of a major neurofilament protein from calf brain has also been recently reported (4, 13, 14). This protein is acidic, and the mammalian subunit has a molecular weight of 50,000 as determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Although quite similar in amino acid composition to the microtubule protein, neither colchicine nor guanosine triphosphate binding activity could be found (2).

Several agents including aluminum, colchicine, vinblastine, podophyllotoxin, various nitriles and acrylamide have been demonstrated in animals to induce a proliferation of 10-nm filaments in neurons (16, 20, 21). In the case of aluminum these changes seem to be specific to the nervous system, while the mitotic spindle inhibitors cause similar changes in a wide range of cell types. In all of these intoxications, the filaments formed are morphologically identical to the normal neurofilaments. These induced filaments have not yet been isolated and characterized. It is not, therefore, known for certain if they are the same as the normal neurofilaments, although it seems likely.

In Alzheimer's presenile-senile dementia, one of the major lesions is the presence of many intraneuronal argentophilic fibrillary tangles, especially in the hippocampus. These tangles are composed of bundles of extraordinary fibrillar elements called twisted tubules (TT), found to date only in human brain (17, 18). The neurofibrillar tangles are also found in the brain of normal aged humans, but to a much lesser degree than in Alzheimer's presenile-senile dementia. Similar neurofibrillary changes are also found in Guam-Parkinsonism dementia complex, mongolism, postencephalitic Parkinsonism and tuberous sclerosis (5). The TT measure 22 nm at their widest and are constricted at about 80-nm intervals to a width.
of about 10 nm. Terry (17), on the basis of the ultrastructural studies of these structures in cross-section, which showed both arcuate and circular profiles, suggested that these are tubules with a “twist” every 80 nm. Kidd (9) to the contrary proposed that each twisted tubule is made of a pair of helically wound 10-nm neurofilaments. The protein subunits of neurotubules have been well studied. The major neurofilament subunit protein (molecular weight 50,000) has also been reported. It is not known whether these TT are composed of the normal neurofilament protein, one of the monomers of tubulin, neurotubule protein or some other protein or proteins. We therefore undertook the study to isolate and characterize these structures to resolve the above question.

MATERIALS AND METHODS

The tissue used for this study was autopsied human brain specimens obtained not later than 15 hr postmortem, and kept frozen at -70°C until use. Before being subjected to chemical analysis, one-half of each brain dissected along the midline was fixed in formalin and studied morphologically to assess the frequency of neurons showing neurofibrillary changes. Several cases of Alzheimer’s presenile-senile dementia, one of Guam-Parkinsonism dementia complex and several examples of normal (non-neurologic) young and age-matched adult patients were studied in this work.

The neurons were isolated from hippocampal cortex by the method of Iqbal and Tellez-Nagel (7). The tissue was chopped with a sharp blade to a fine mince, screened once through a 149-mesh nylon bolting cloth, and five times through a 74-mesh stainless steel mesh in a medium consisting of 1% Ficoll and 10% each of glucose and fructose in 10 mM phosphate buffer, pH 6.0. The filtrate was mixed with equal volume of 50% sucrose (1.461 M) sucrose in 0.03 M sodium potassium phosphate buffer containing 0.01 M KCl at pH 6.5. Homogenization was 15 strokes in a glass Dounce homogenizer with loose pestle, followed by another 4 strokes with a tight pestle. The homogenate was then centrifuged at 9000 × g for 35 min. The layer of myelinated axons which had floated to the top was carefully harvested. The floating layer was rehomogenized with 6 strokes in the Dounce homogenizer with a tight pestle in the original medium to a final volume of 520 ml, and the floatation procedure was repeated three times. The layer from the final floatation was homogenized in 0.32 M sucrose containing 1 mM ethylenediaminetetraacetate disodium salt (EDTA) to a final volume of 150 ml in a glass Dounce homogenizer by 6 strokes with a loose and 4 strokes with a tight pestle. The suspension was then stirred at 4°C for 60 min to shock the myelin of the axons, and then crystalline sucrose was added to bring its final concentration to 1.0 M. The suspension was centrifuged at 82,500 × g for 45 min. A filament enriched fraction was recovered as a pellet. The floating layer was rehomogenized in 1.0 M sucrose containing 1 mM EDTA in a blade-type homogenizer (Sorvall-Omni Mixer, setting 10) for 2 min, and filaments were obtained as a pellet above. The above step was repeated once more to get a third crop of filaments from the floating layer. The three batches of the filaments were then pooled.

Tubulin was purified from the freshly autopsied brain of a 5-year-old child without neurologic disease by the method of Shelanski et al. (15).

For electron microscopy, the fractions were fixed with 2.5% glutaraldehyde, postfixed in 2% buffered osmic acid and embedded in Epon.

Protein was determined in the fractions by the method of Lowry et al. (10). Protein patterns were studied by Tris-glycine discontinuous SDS-polyacrylamide slab gel electrophoresis using a 7.5-30% acrylamide gradient. Both buffer and gel contained 0.1% SDS. Samples for electrophoresis were prepared by heating the isolated fractions with SDS (1%), 2-mercaptoethanol (1%), glyceral (10%), with phenol red (0.0001%) as tracking dye. Gels were stained with 1% fast green FCF.

The molecular weights of the new protein and of the major filament protein bands were estimated by comparison of their mobility with that of proteins of known molecular weight on SDS-polyacrylamide gel using 15% acrylamide gel. The following proteins were used as references for molecular weight: bovine serum albumin (molecular weight (mol wt) 67,000), human brain tubulin (α-monomer, mol wt 56,000; β-mono-
The isolated neuronal perikarya were 95-98% pure by particle count (Fig. 1). A few cells had the axonal stumps still with the cell body. The plasma membrane was interrupted at several places, but most of the cytoplasmic organelles were still intact. The neurofibrillary tangles, when present in the affected cells, were well preserved. The contaminants in the TTF were rough endoplasmic reticulum, mitochondria and some lipofuscin (Fig. 2). Neurofilaments of the 10-nm type were very sparse in both the TTF and the isolated neurons.

The neurofilament fraction was highly purified and contained mostly loose bundles of filaments (Fig. 3). The contaminants in the fraction were small quantities of myelin, mitochondria and smooth membranes. Twisted tubules or neurontubules were not seen in this fraction. The neurofilament fraction on gel electrophoresis had one major band (mol wt 53,000), four minor bands and some trace bands. The major human neurofilament band co-migrated with human β-tubulin on SDS-polyacrylamide gels. The calf brain major neurofilament protein (mol wt 51,000) moves slightly ahead of the human major neurofilament protein.

The TTF and the neuronal fractions from brains with neurofibrillary pathology were found to contain a unique band (enriched fraction band or EFB) which moved slightly ahead of the fast moving monomer (mol wt 53,000) of tubulin and the major human neurofilament protein on both 15% and acrylamide gradient gels (Fig. 4). The molecular weight of the EFB protein was estimated to be 50,000 daltons. The EFB was derived from hippocampal neurons and TTF obtained from several cases of Alzheimer’s dementia and from one case of Guam-Parkinsonism dementia, another disorder in which similar neurofibrillary changes are found in great abundance.

**RESULTS**

The isolated neuronal perikarya were 95-98% pure by particle count (Fig. 1). A few cells had the axonal stumps still with the cell body. The plasma membrane was interrupted at several places, but most of the cytoplasmic organelles were still intact. The neurofibrillary tangles, when present in the affected cells, were well preserved.

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**DISCUSSION**

The EFB was observed in electrophoretic studies of the TTF and of the neurons isolated from areas of the brain rich in neurofibrillary tangles. Similarly processed tissue from minimally affected cortical areas of the same brains barely show the EFB.

![Image](https://example.com/image.png)

**Fig. 1.** A phase contrast micrograph of neuronal perikarya isolated from unfixed frozen normal human cortex. x400.
Fig. 2. A. an electron micrograph of a neuronal perikaryon with neurofibrillary tangle in its cytoplasm, isolated from hippocampus of a case of Alzheimer's dementia. x38,000. B. an electron micrograph of neurofibrillary tangle at higher magnification. x140,000.
Fig. 3. An electron micrograph of a neurofilament enriched fraction obtained from human white matter. A, ×7,000; B, ×63,000.
Fresh, unfrozen brain tissue taken at autopsy 4 hr postmortem from an aged normal (non-neurologic) human case was subjected to various treatments. Neurons were isolated from this tissue, kept at 4°C and room temperature for 4, 6, 22 and 30 hr, and from tissue frozen and thawed one, two and five times. The electrophoretic pattern of the total neuronal proteins on SDS-polyacrylamide gels did not show the EFB protein. We also did not observe any increase in the intensity of EFB derived from tissue taken 20 hr postmortem. It is unlikely, therefore, that the band is an artifact of freezing and thawing or postmortem changes. Neurons isolated from the hippocampus of control cases matched as to postmortem time do not show a similar band, and this also lessens the possibility of topographic or postmortem artifacts.

Cytologic localization of the EFB has not yet been determined, but is in process. Therefore, it is not certain that it is the TT subunit, although this seems likely. Another possibility is that the EFB may be another neuronal protein which appears with aging. If the EFB does represent TT, it remains to be determined whether this is modified neurofilament protein, neurotubular protein or a new protein. The electrophoretic mobility of EFB is very close to that of the major filament protein and the normal neurotubular protein, and they may be very similar, if not identical. Peptide maps of the tryptic digest of these proteins are still preliminary, but suggest that these proteins are similar. Thus, if the EFB corresponds to the neurofibrillary tangle, then the twisted tubules may be made of modified neurofilament (at least, the major subunit) or the neurotubular subunit proteins. TT, unlike neurotubules, resemble neurofilaments in being insoluble in buffer and in being structurally stable in autopsied tissue. Some ultrastructural studies have suggested that each TT is made up of a pair of helically wound neurofilaments (9). Tilt-stage electron microscopy is also in process.

The role of trace metals such as aluminum is important, since this metal is known to cause...
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neurofilamentous proliferation in experimental animals (20, 21). Furthermore, Crapper, Krishnan and Dalton (1) have recently reported a 3-4-fold increase over the normal level of aluminum in cerebral specimens of patients with Alzheimer's dementia.

The function of neurofilaments is not clearly understood. They may function as a part of the force-generating mechanism in axonal transport (19). Should the twisted tubules be really made up of a pair of helically wound neurofilaments, these pathologic structures may cause the malfunction of the axoplasmic transport and consequently the death of neurons.

We have recently observed that the human major neurofilament protein purified from fresh autopsy tissue, unlike that purified from frozen autopsy tissue, co-migrates with the corresponding protein purified from fresh calf brain on SDS-polyacrylamide gels. However, the peptide maps of the tryptic digest of the human major neurofilament protein purified from either fresh or frozen autopsy tissue are identical.

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LITERATURE CITED