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Phosphorylated tau immunoreactivity of granulovacuolar bodies (GVB) of Alzheimer's disease: localization of two amino terminal tau epitopes in GVB*

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Summary. An immunocytochemical study of Alzheimer's disease hippocampus with a panel of anti-tau antibodies revealed two antibodies that stained granulovacuolar bodies (GVB) in pyramidal neurons of Ammon's horn. These two affinity-purified anti-tau antibodies were raised in rabbits against synthetic peptides homologous to sequences (amino acids 44–55 and 75–87) in the 58 amino acid insert in the amino terminus of the longest form of human tau. This region is homologous to exons 2 and exon 3 of bovine tau. The exon 2 peptide contains a serine (amino acid residue 46), which has been shown to be a phosphorylated site in paired helical filaments. Antibodies to a nonphosphorylated exon 2 peptide failed to immunostain GVB, but those to the phosphopeptide consistently stained GVB. Staining, however, was most consistent with the antibody to the exon 3 sequence. As in previous studies, GVB were also stained by RT97, a neurofilament antibody whose epitope in tau appears to be a phosphorylated site in or near exon 2, perhaps at serine residue 46 (Brion et al. 1992). Antibodies to epitopes in the amino terminus, mid-region and carboxy terminus of tau failed to consistently stain GVB. More often they produced staining around the periphery of the GVB, giving the appearance of an "empty vacuole." Most GVB were also immunoreactive with an antibody to ubiquitin. The results are consistent with the hypothesis that GVB are derived from sequestered altered tau possibly mediated by ubiquitin. The failure to detect most regions of tau in GVB is consistent with the idea that tau is partially degraded or highly modified in GVB.

Key words: Alzheimer's disease – Granulovacuolar degeneration – Hippocampus – Paired helical filament – Tau protein

Granulovacuolar degeneration or granulovacuolar bodies (GVB) are neuronal alterations that are highly characteristic of Alzheimer's disease, but also detected in other degenerative diseases and to a lesser extent in human aging [1, 16, 24, 31]. Although GVB are most often detected in the hippocampus, they can also be found in neurons in other regions of the brain, particularly the limbic cortices and brain stem monoaminergic nuclei [31]. The molecular nature of GVB is not known with certainty, because GVB cannot be purified for biochemical evaluation. Enzyme histochemical studies suggested that they might be derived from lysosomes [30].

Ultrastructural studies suggested that they might arise from microautophagy [27]. More recently, several immunocytochemical studies of GVB have shed additional light on their biochemistry.

Immunocytochemical studies of GVB by Price et al. [28] suggested that GVB contained tubulin aggregates. Subsequent studies by Kahn et al. [18] and Dickson et al. [8] provided evidence that GVB contained epitopes shared with phosphorylated neurofilament proteins and tau. The report by Bondareff et al. [4] provided additional evidence for involvement of tau in GVB. On the other hand, several recent studies have failed to detect tau epitopes in GVB [23, 26]. The purpose of this report is to describe immunocytochemical studies of GVB that demonstrate additional tau immunoreactivities in GVB.

Materials and methods

Case material

Tissue was obtained from autopsies performed as part of aging and dementia studies at Albert Einstein College of Medicine. Alzheimer brains used in this study were from subjects who were clinically demented and met pathological criteria for Alzheimer's disease, including the presence of numerous senile plaques and neocortical neurofibrillary tangles (NFT).

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Tissue preparation

Tissue was fixed in formalin for 10 days to 2 weeks and embedded in paraffin. Sections (7 μm thick) were cut and mounted on poly-lysine-coated glass slides for immunostaining. In other studies hippocampal sections removed from the brain at the time of the autopsy were placed in OCT medium and frozen in liquid nitrogen. Sections (10 μm) were cut, picked up on glass slides, air-dried and then fixed in 100 % acetone for 10 minutes. In yet other studies, tissue obtained at the time of autopsy was fixed in Bouin's solution or periodate-lysine paraformaldehyde (PLP) for 1–5 h and kept in 30 % sucrose before sectioning on a vibratome at a thickness of 40 μm.

Immunoperoxidase

After removal of paraffin wax with xylene and alcohol, blocking of endogenous peroxidase with 0.3 % H_2O_2 and nonspecific antibody binding with 5 % normal serum, tissue was immunostained using monoclonal and/or polyclonal antibodies by incubating sections for 2 h at room temperature or overnight at 4 °C. Following incubation in the primary antibody, the sections were washed in phosphate-buffered saline (PBS) and then incubated with biotinylated secondary antibodies. The antibody binding was detected with avidin-biotin-peroxidase complex (ABC kit, Vector Labs, Burlingame, Calif.). The chromogen was 3,3-diaminobenzidine. In double-labeling experiments the second antibody was detected with β-galactosidase-conjugated secondary antibodies (Fisher Labs, N.J.). The chromogen was X-gal. Vibratome sections were immunostained with peroxidase-labeled secondary antibodies (Fisher Labs, N.J.), rather than the ABC method.

Phosphatase pre-treatment

To assess the phosphatase sensitivity of the antibody binding, a protocol similar to that originally described by Sternberger was used [29]. Sections were incubated in *E. coli* alkaline phosphatase (Sigma Chemicals, St. Louis, Mo.) (100 IU/ml) of 100 mM TRIS buffer, pH 8.0 with 500 mM phenylmethylsulfonyl fluoride prior to incubation with antibody. Sections incubated in PBS served as control.

Immunoelectron microscopy

Vibratome sections that had been stained were trimmed to areas of interest (usually the CA2/3 region) with a dissecting microscope. Sections were processed after post-fixing in osmium and also without osmication to differentiate immunostaining more clearly from other electron-dense, osmiophlic structures. The dehydrated tissue was subsequently embedded in plastic. Ultrathin sections were cut and mounted on copper grids and viewed with an electron microscope without additional staining.

Antibodies

Anti-tau monoclonal antibodies. A monoclonal antibody to an epitope in tau and neurofilament (RT97) [5] was used as a positive control for immunostaining of GVB, since it has been demonstrated by several groups to stain GVB [8, 18, 24]. RT97 recognizes a phosphorylated epitope in the high molecular weight neurofilament subunits and a phosphorylated epitope in the amino half of tau (serine 46; B. Anderton, personal communication). Other anti-tau monoclonal antibodies included Tau46 [21] and Tau-1 [3], which recognizes an epitope in tau that is blocked in paired helical filament (PHF)-tau by a phosphorylated site in or near the epitope. Tau-1 was used as a control for immunostaining after phosphatase pretreatment of sections, since previous studies have shown that it reacts with NFT in fixed tissue only after phosphatase treatment [15]. (Similar to previous studies, we found that Tau-1 consistently stained a significant number of intracellular NFT in cryostat sections [7] without any pretreatment.) Sections were also stained with a panel of anti-tau antibodies as described previously [9]. Unless noted otherwise, anti-tau antibodies failed to stain GVB. Alz-50, a monoclonal antibody to an epitope near the amino terminus of tau [14, 32] and Tau46, a monoclonal antibody recognizing an epitope near the carboxy terminus of tau [21], are representative anti-tau antibodies with epitopes at both extremes of the tau molecule. No immunostaining of GVB was noted with Tau46.

Polyclonal antibodies to synthetic peptides. Antibodies raised to amino acid sequences according to the largest form of human tau [13] were also generated. These included antibodies to the sequences encoded by exons 1 (E1 [6], exon 2 (E2), tau amino acid residues 44–55 (Lys-Glu-Ser-Pro-Leu-Gln-Thr-Pro-Thr-Glu-Asp-Gly) and exon 3 (E3), tau amino acid residues 75–87 (Val-Thr-Ala-Pro-Leu-Val-Asp-Glu-Gly-Ala-Pro-Gly-Lys) [22]. An antibody was also raised to a serine-phosphopeptide of exon 2 (E2p) (Lys-Glu-Ser[P]-Pro-Leu-Gln-Thr-Pro-Thr-Glu-Asp-Gly). All tau antibodies used in these studies recognize normal tau and PHF-tau in Western blots (Fig. 1a). As shown previously [22], E2 recognized the two higher molecular mass isoforms (64 and 68 kDa) of PHF-tau and E3 recognized only the highest molecular mass (68 kDa) PHF-tau isoform (Fig. 1a).

Polyclonal antibodies were affinity purified using activated CH-sepharose 4B conjugated with synthetic peptide to which they were raised. E2p was purified by passing the antiserum generated by immunizing rabbits with phosphopeptide through an affinity column containing the non-phosphorylated peptide. The flow

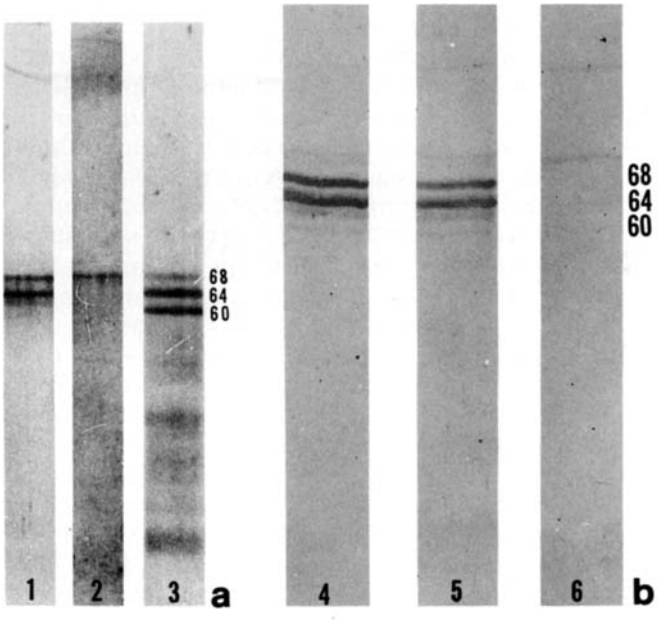


Fig. 1. a Western blots of paired helical filament (PHF) preparations stained with exon 2 (E) (*lane 1*), E3 (*lane 2*) and Tau46 (*lanes 3*). Both E2 and E3 recognize polypeptides in the 60–68 kDa range that co-migrate with proteins stained by a well-characterized anti-tau monoclonal antibody. As previously shown [22], E2 epitopes are detected in two, E3 in one and Tau46 in all three PHF-tau polypeptides. **b** Western blots of PHF preparations from a different brain (*lanes 4–6*) stained with E2 (*lane 4*) and E2p (*lane 5*). Specificity of phosphorylated E2 (E2p) immunoreactivity is demonstrated by lack of staining in *lane 6* after absorption with E2p synthetic peptide

through contained antibody to phosphopeptide, while the bound antibody recognized the non-phosphorylated epitope. After elution from the column, the E2, similar to E2 generated by immunization of rabbits with the non-phosphorylated peptide, could be specifically immunoabsorbed with synthetic peptide to the E2 sequence. Similarly, immunostaining of E2p could be immunoabssorbed by pre-incubating the antibody in E2p synthetic peptide (Fig. 1b) Although it might be expected that RT97 would recognize the E2p synthetic peptide, no binding was detected in immunoblots with varying amounts (5 μg, 0.5 μg and 50 ng) of E2p synthetic peptide spotted on nitrocellulose paper. Both E2 and E2p recognized the peptide at all these concentrations, with more intense reactivity with E2p than E2 at the lowest concentration (not shown).

Further tests of specificity of staining were immunostaining in the absence of primary antibody or with non-immune rabbit serum. Immunostaining was also performed with a polyclonal antibody to ubiquitin, which has been previously characterized [10].

Results

GVB were immunostained with antibodies to phosphorylated neurofilament epitopes (RT97) and ubiquitin as previously described (Fig. 2a, 2b). Both antibodies stained the dense center of the GVB and often a thin rim of cytoplasm or membrane around the GVB. Phosphatase treatment diminished, but did not completely eliminate, RT97 staining of GVB as previously noted [8], whereas axonal staining was absent under these conditions. Sections processed concurrently with Tau-1 demonstrated the efficiency of the phosphatase treatment, since Tau-1 immunostaining of NFT was only seen after phosphatase treatment. Alz-50 did not consistently stain GVB, but frequently stained the cytoplasm of neurons with GVB in either a fibrillar or granular pattern [17]. This produced the appearance of empty vacuoles (Fig. 2c). In a few cases, Alz-50 gave weak and inconsistent immunoreactivity of some GVB.

The antibody to exon 3 of tau (E3) gave the most consistent and intense immunoreactivity of GVB. The staining was detected in tissue processed by all methods, including cryostat sections, paraffin sections and vibratome sections. GVB were more intensely stained than any other reactive structure, including NFT and neurites in senile plaques and the neuropil (Fig. 3a) when the antibody was increasingly diluted. The staining was shown to be specific, since it was not detected when antibody was pre-incubated with exon 3 synthetic peptide (Fig. 3b) or with preimmune serum. Phosphatase pretreatment had no appreciable effect on E3 immunoreactivity.

Staining of GVB was particularly intense in Bouins or PLP-fixed vibratome sections (Fig. 4a(inset)]. As is characteristic of other anti-tau antibodies, E3 stained

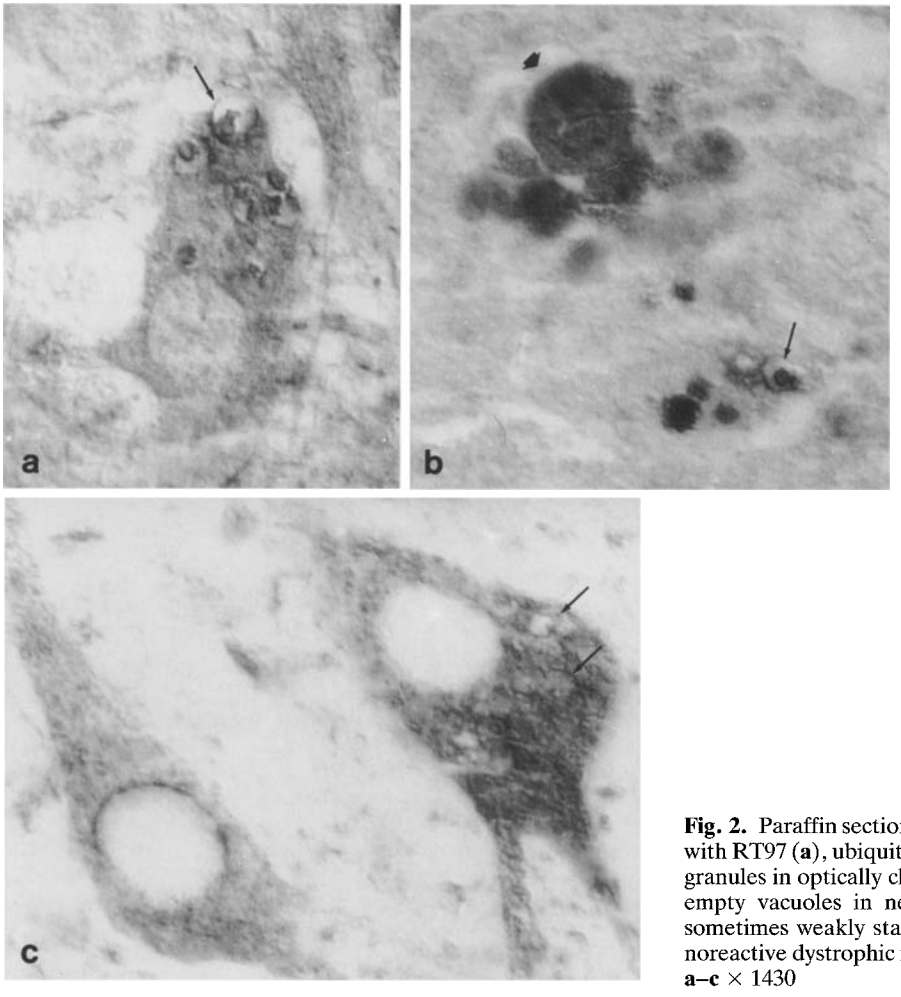


Fig. 2. Paraffin sections of Alzheimer hippocampus immunostained with RT97 (**a**), ubiquitin (**b**) and Alz-50 (**c**). Note immunostaining of granules in optically clear vacuoles by RT97 and anti-ubiquitin, but empty vacuoles in neurons stained with Alz-50. Granules were sometimes weakly stained with Alz-50. Note also ubiquitin-immunoreactive dystrophic neurites in a senile plaque (*arrowhead*) in (**b**). **a–c** × 1430

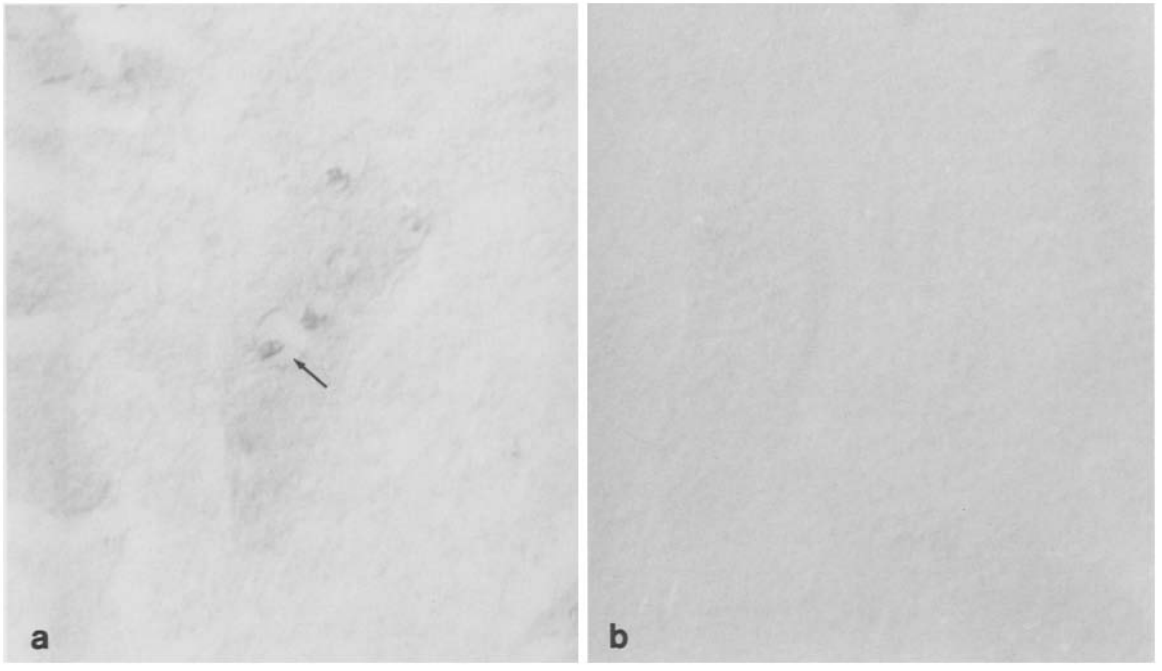
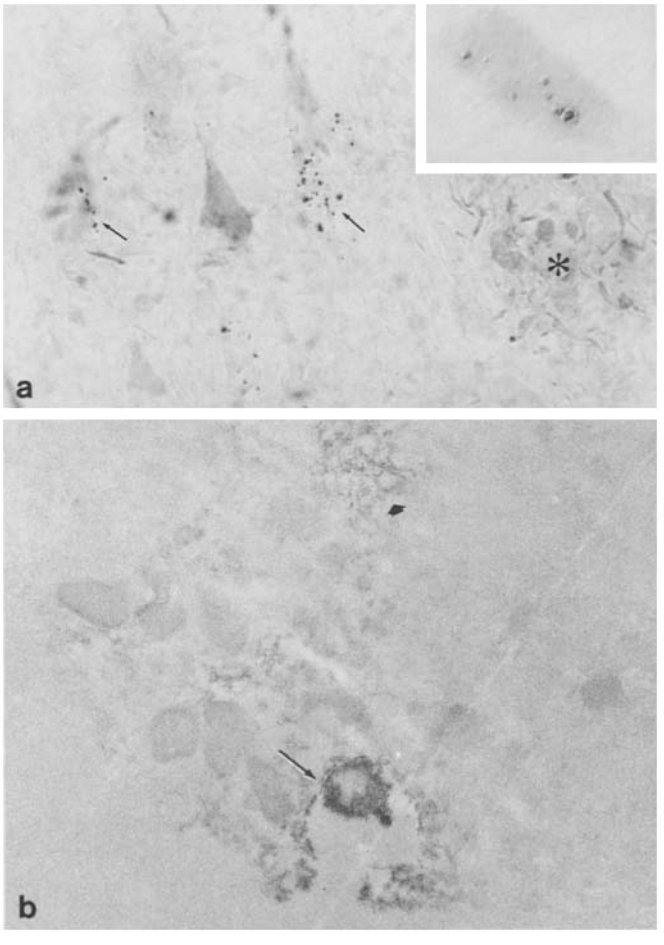


Fig. 3. Paraffin sections stained with E3 demonstrates immunoreactivity of granulovacuolar bodies (GVB) (*arrow*) (**a**), but not after absorption with synthetic peptide to E3 (**b**). **a,b** × 1430



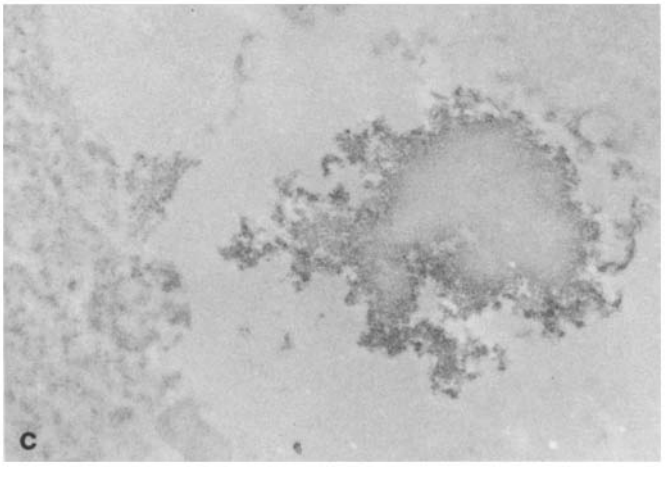


Fig. 4a–c. Vibratome sections of hippocampus stained with E3. At the light microscopic level, GVB in neurons (*arrows*), neurofibrillary tangles (NFT) and neurites in senile plaque (*asterisk*) are stained with E3 (**a**). *Inset* shows higher magnification of GVB. At the electron microscopic level (**b,c**), E3 immunoreactivity is localized to foci in the neuronal cytoplasm (*arrowhead*) and in GVB. The GVB staining includes a thin rim around the vacuole in addition to the irregular dense granule in the vacuole (**b**). At highest magnification the E3 immunoreactive granule shows sparse staining in its center and irregular granular processes (**c**). **a** × 285; *inset* × 570; **b** × 12 000; **c** × 30 000

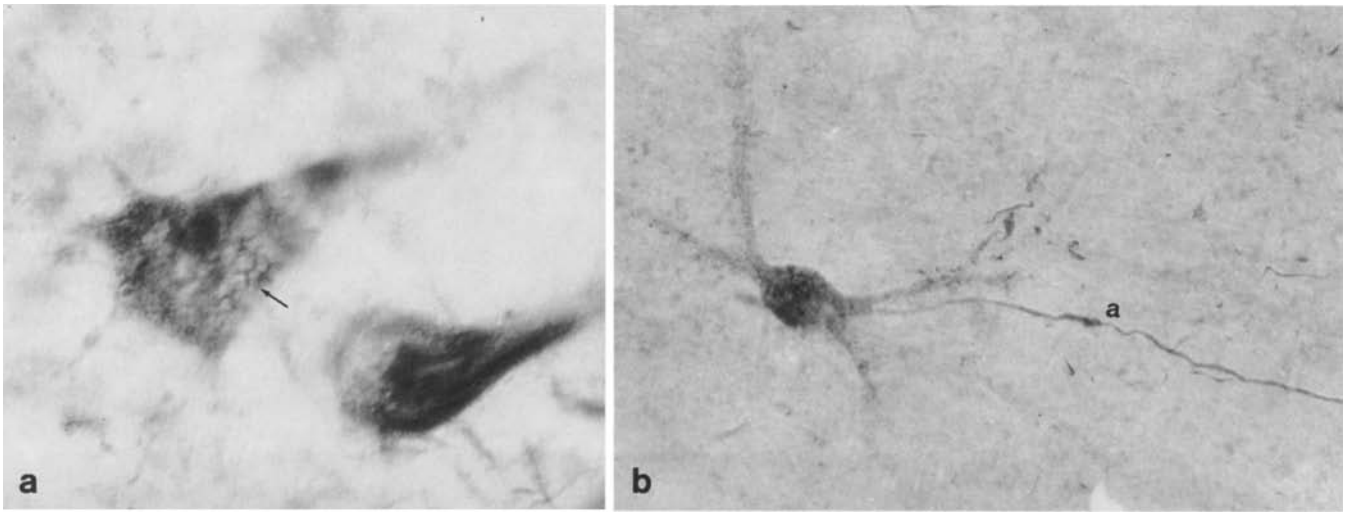


Fig. 5. Immunocytochemistry with E2 shows somatodendritic neuronal staining (**a,b**) as well as staining of neuronal processes that are consistent with both axons (labeled *a*) and dendrites (**b**). The neuron on the left has cytoplasmic staining and (**a**) empty vacuoles, while the neuron on the right has an intracellular NFT. *a* × 1430; **b** × 570

NFT and neurites in senile plaques in addition to GVB (Fig. 4a). Sections stained with E3 only, without additional contrasting and without osmium post-fixing were processed for electron microscopy. The cytoplasm of neurons with E3 reactivity often had foci of staining that in most, but not all cases, corresponded to PHF (Fig. 4b). The immunostained granules were dense irregularly shaped particles in optically clear vacuoles. There was often increased staining of a rim of cytoplasm around the vacuole (Fig. 4b). The granules were not spherical, but rather irregular and fuzzy with densely immunostained granular protrusions (Fig. 4c). The center of the granule usually had only weak immunoreactivity (Fig. 4b,c). Other round cytoplasmic organelles were not stained, including lysosomes, lipofuscin and mitochondria.

The antibody to E2 gave a staining pattern somewhat similar to Alz-50. Neurons with GVB often had cytoplasmic immunoreactivity, but the GVB were empty vacuoles (Fig. 5a). E2 also stained a subpopulation of hippocampal neurons in the endplate and CA3 region in a somatodendritic and axonal pattern (Fig. 5b). There was also frequently increased staining along the nuclear membrane as described with other tau antibodies [2]. E2 was also a sensitive antibody for detecting neuritic profiles in the neuropil, as previously noted [22]. Only occasionally were granules in GVB immunostained with E2. The immunostaining of all structures, including the subpopulation of neurons with somatodendritic staining, was eliminated by pre-incubating antibody with synthetic peptide to E2.

The antibody to the serine-phosphopeptide of E2 (E2p) showed consistent immunostaining of GVB (Fig. 6a); staining was abolished by pre-incubating the antibody in E2p synthetic peptide (Fig. 6b). In contrast to E3, which preferentially stained GVB over NFT and neurites, E2p staining of GVB was comparable in intensity to staining of NFT and neurites. E2p staining of GVB, NFT and neurites was usually decreased after phosphatase pretreatment of the sections; however, the pathological structures showed less-marked phosphatase effect than normal structures. This phenomenon has been previously noted for other antibodies (such as antibodies to neurofilaments) that detect phosphatase sensitive epitopes; staining of normal structures (e.g., axons) is diminished, but pathological structures (e.g., NFT) are resistant to phosphatase treatment [20]. Double staining with RT97 and E3 or E2p showed co-localization of immunoreactive granules. (not shown)

Discussion

The nature of GVB has been a mystery since they were first described in the hippocampus of Alzheimer's disease and other neurodegenerative diseases. The presence of tubulin immunoreactivity suggested that they might be derived from sequestered tubulin [28], but

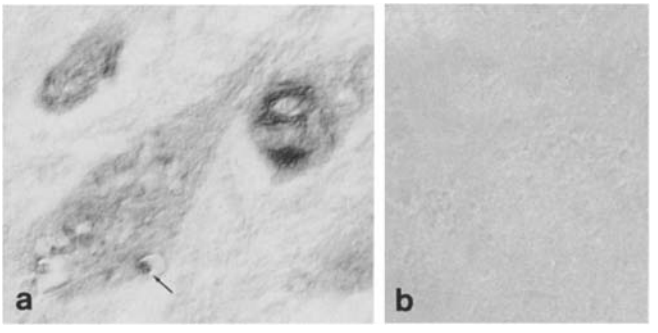


Fig. 6. a GVB (*arrow*) are reactive with antibodies to the phosphopeptide of exon 2 (E2p). No staining is detected with pre-immune serum (**b**) or with E2p after immunoabsorption. **a,b** × 1430

immunoreactivity with antibodies that recognized neurofilament epitopes suggested that GVB might be composed of other proteins [18]. Still other work based upon electron microscopic observations of well-preserved autopsy tissue suggested that GVB might be derived from autophagy [25, 27]. Depending upon the local constituents of the cytoplasm, autophagic vacuoles might be predicted to be heterogeneous.

Studies in this laboratory demonstrated that GVB also had epitopes shared with neurofilament, tau and NFT [8]. Moreover, the epitope was phosphorylated. This suggested that GVB contained phosphoproteins and that the specific nature of the protein(s) in GVB would require further studies using additional immunochemical probes. Several studies have now shown that GVB are also immunoreactive with antibodies to ubiquitin [23, 25]. Whether this represents a specific process of ubiquitin-mediated targeting of protein for lysosomal degradation or merely autophagy of ubiquitinated proteins [12] remains undetermined, but increasing evidence suggests that ubiquitinated structures in human brains are frequently membrane bound and consistent with lysosomal residual bodies [10].

The results of the present study show two additional epitopes shared between tau and GVB. At least one of these epitopes (E2p) is phosphorylated, since an antibody to the nonphosphorylated peptide (E2) failed to stain GVB, while the antibody to the phosphopeptide stained GVB. Whether E2p represents a different immunoreactivity than that detected by RT97 is not certain. The tau epitope recognized by RT97 has not been determined, but recent studies suggest that it may also recognize a phosphorylated site in the second exon [5]. The fact that RT97 did not recognize the E2p synthetic peptide on dot blots raises doubts as to the localization of the RT97 epitope; however, technical considerations may be responsible, since the epitope in a short synthetic peptide may not have the same configuration as the epitope in the intact tau molecule.

The reactivity of two independent anti-tau antibodies with GVB argues that tau protein, or a fragment of tau protein is present in GVB. Bondareff et al. [4] also described tau epitopes in GVB. Their results have been difficult to repeat in other laboratories [15, 23]. In particular Lubke et al. [23] examined a large panel of tau antibodies and could not detect immunoreactivity of

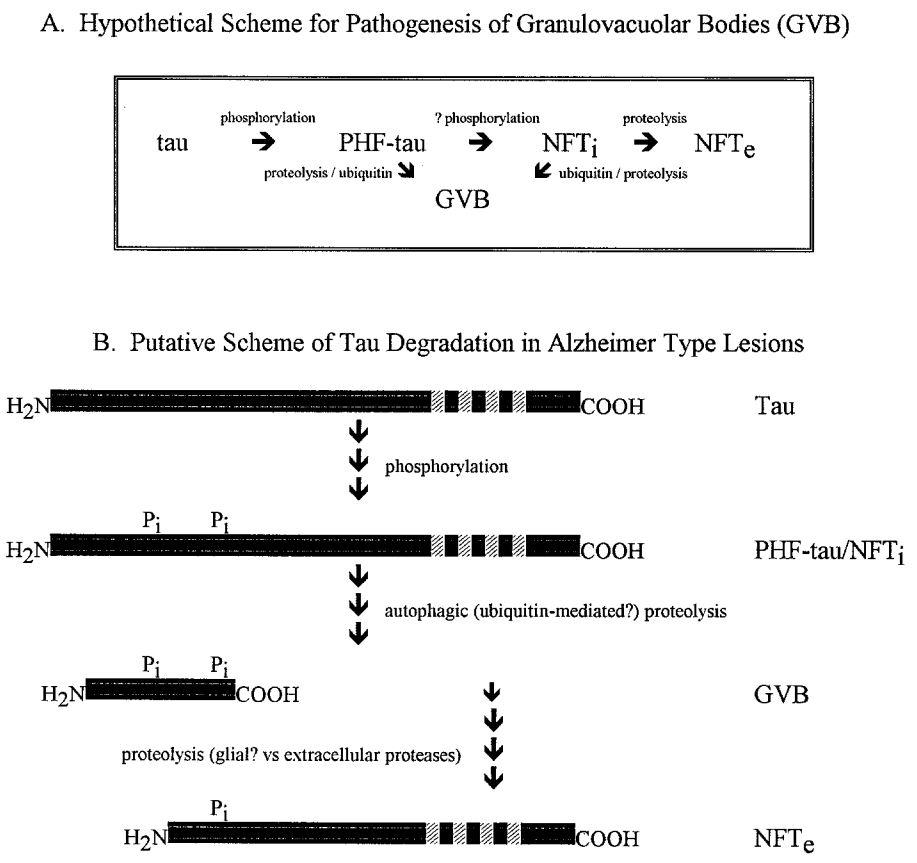


Fig. 7. A schematic representation of a hypothetical pathogenesis of GVB (**A**) and the degradation of tau protein in pathological lesions (**B**). Due to factors that are currently unknown tau proteins undergo excessive phosphorylation creating PHF-tau. Other post-translational or physicochemical processes may also be involved in the formation of intracellular NFT from PHF-tau. Since GVB are detected in neurons with and without NFT, they may be derived from autophagic sequestration of "pre-tangle" PHF-tau or NFT components. It is hypothesized that this may be mediated by ubiquitin and that proteolysis of tau leaves only a small fragment of tau (containing at least E2 and E3) in GVB. Separate proteolytic events, possibly mediated by glial or extracellular enzymes are involved in the generation of extracellular NFT (NFT_e) from intracellular NFT (NFT_i). The microtubule-binding domains [white boxes in (**B**) and the carboxy terminus of tau appear to be preserved in NFT_e

GVB with any anti-tau antibody. Immunoelectron microscopic localization was obtained with antibodies to ubiquitin and with RT97, however.

Results of our study suggest a possible explanation for some of the discrepancy in the literature. We also failed to detect immunoreactivity of GVB with a panel of antibodies to tau that spanned the length of the molecule [10], but we did detect immunoreactivity with anti-tau antibodies to the amino-terminal half of tau (E2p and E3). These results suggest that at least a fragment, if not all, of tau is present in GVB.

If tau protein or an altered form of tau protein is present in GVB, it remains to be determined why most anti-tau antibodies fail to stain GVB. This lack of immunoreactivity may be due to proteolysis or extensive modifications of tau. The preservation of this region of tau is paradoxical, since the amino half of tau is more sensitive to proteolysis than the carboxy half. For example, immunoreactivity with antibodies such as Alz-50 and E1 that recognize epitopes near the amino terminus is readily lost from tau and PHF proteins in vitro and in situ [19]. Also, extracellular NFT rarely display immunoreactivity with antibodies to this region of tau [10, 17], which suggests that this portion of tau has been lost in the cellular degradation accompanying neuronal death and the evolution of an extracellular NFT.

The failure to detect GVB by other anti-tau antibodies may also be related to masking of tau epitopes in GVB. We have been unable to unmask these epitopes with trypsin, phosphatase or hydrated autoclaving treatments [10].

Electron microscopic studies of GVB have provided evidence that GVB may be derived from autophagocytosis or sequestration of foci of neuronal cytoplasm [25, 27]. It seems reasonable that since phosphorylated forms of tau are present in the somatodendritic domain of neurons in Alzheimer's disease that such elements may be incorporated into GVB (Fig. 7). Antibodies to the amino half of tau seem to be particularly capable of detecting "pre-tangle" cytoplasmic alterations [2]. Alz-50 is a notable antibody for staining neurons that may be vulnerable to NFT formation in the hippocampus [17]. The present results demonstrate that antibodies to E2 produce similar immunoreactivity.

The stimulus for autophagy in hippocampal pyramidal neurons is unknown. In Alzheimer's disease the cause of this injury is open to speculation. The fact that GVB are largely limited to pyramidal neurons in Ammon's horn suggests that features common to this neuronal population be considered, among which is sensitivity to excitotoxic amino acids. The role of aberrant or excessive phosphorylation of tau proteins also needs to be considered as a factor in formation of GVB. Recent evidence suggests that mitogen-activated protein kinases (MAP kinases) may play a role in aberrant phosphorylation of tau protein in NFT [11]. A number of different agents have been reported to stimulate MAP kinase in vitro [11]. Whether one or more of these agents may also promote GVB formation remains to be determined.

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