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# Markers of axonal injury in post mortem human brain

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**Abstract** β-Amyloid precursor protein (βAPP) can be detected immunocytochemically at sites of axonal injury in the brain, and has recently been found to be a useful marker for injured axons in patients who survived for only 3 h after head trauma. It is transported by fast axonal transport and is thought to accumulate in detectable levels where the cytoskeleton breaks down. If this theory is correct, other substances should accumulate here in the same way, so we have used antibodies to other neuronal proteins to compare their efficacy as markers of axonal injury. SNAP-25, chromogranin A and cathepsin D also marked injured axons at all survival times studied (2.5 h–2 weeks), although they were not as sensitive or specific as βAPP. Immunolabelling for the 68-kDa neurofilament subunit (NF68) was present in most uninjured axons, and allowed axonal swellings to be seen in some cases. Synaptophysin, GAP-43, ubiquitin or tau did not label any normal or injured axons in this study. We, therefore, suggest that βAPP should be the immunocytochemical marker of choice for the detection of injured axons. This study also showed that microwave antigen retrieval significantly enhances the immunoreactivity of SNAP-25, chromogranin A, synaptophysin, GAP-43, ubiquitin and tau, in addition to that of βAPP, in formalin-fixed, paraffin-embedded tissue, and reveals NF68 antigenicity where it was not previously detectable.

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## Introduction

In formalin-fixed, paraffin-embedded sections of human brain, it has recently been shown that β-amyloid precursor protein (βAPP) immunocytochemistry can be used to detect axonal injury in the white matter after head trauma [16] after only 3 h survival [40]. It is also a marker of axonal damage in other types of brain lesion [10, 31, 40]. The fact that βAPP only marks injured and not normal axons gives this method considerable advantages over silver staining [40]. βAPP is normally present in neurons and is thought to have diverse roles (see [39]), as well as giving rise to β-amyloid deposited in the brain in Alzheimer's disease. There is evidence that it is associated with the endosomal/lysosomal system [5, 13, 17, 20,] and it is known to be carried by fast anterograde axonal transport [26]. In injured axons, the cytoskeleton breaks down, possibly due to calcium influx [2], causing interruption of axoplasmic flow and the subsequent accumulation of organelles [34]. Normal levels of axonal βAPP are not detectable by standard immunocytochemistry in formalin-fixed tissue, and its demonstration at sites of axonal injury is probably due to this accumulation mechanism. It is likely that other proteins accumulate at the site of axonal injury in the same way, so the following study was undertaken to compare the efficacy of antibodies to other neuronal proteins to that of βAPP as a marker of axonal injury.

Antibodies to chromogranin A and synaptophysin were used to label large, dense-core synaptic vesicles [3, 14, 45] and small synaptic vesicles [49, 50], respectively, and anti-cathepsin D was used to label lysosomes [4]. Antibodies were also employed to: SNAP-25, which plays a role in synaptic exocytosis [32, 44]; the growth protein GAP-43, involved in synaptic plasticity in the mature CNS [43]; and tau, the major component of

paired helical filaments in Alzheimer's disease [27]. These were compared with antibodies to ubiquitin and the 68-kDa neurofilament subunit (NF68), which have previously been found to be markers of axonal injury [18, 38].

Microwave antigen retrieval is a novel technique which has been used to enhance or unmask the antigenicity of many proteins in neural and non-neural tissue that has been routinely processed by formalin fixation and paraffin embedding [9]. We have recently shown that it significantly enhances βAPP immunoreactivity without making tissue sections friable [41]. In this study, the effectiveness of this method in enhancing or retrieving the immunoreactivity of the other neuronal antigens was assessed.

## Materials and methods

Six of the cases for study were derived from the collection of post-mortem brain tissue from the Neuropathology Laboratory at the University of Leeds. To compare the effectiveness of labelling in tissue processed elsewhere, additional cases were obtained from the Medico-Legal Centre at Sheffield (two pediatric cases) and the Department of Forensic Medicine, Leeds (three adult cases). Eight cases had known axonal injury (diffuse axonal injury or infarcts), one had died more or less instantly from multiple injuries so any axonal injury that was present would not be detectable, and two were cases of sudden death with no known axonal injury (Table 1). In all cases, the brain had been fixed in 10% formalin for 3–4 weeks and blocks embedded in paraffin wax.

Immunocytochemistry was carried out on 6-mm serial sections of corpus callosum with attached cingulate cortex. Endogenous peroxidase activity was blocked by incubation in 1% $H_2O_2$ in methanol for 30 min. Some sections then underwent antigen retrieval by microwave pretreatment [41] or trypsinisation. For the microwave treatment, they were heated in citrate buffer (pH 6) in a standard 650 W microwave oven (Deltawave, Toshiba), which was turned off as soon as the solution boiled. The sections were allowed to cool for 5 min, then reboiled in the same way and allowed to cool to room temperature for about 20 min. Additional sections were placed in 0.1% trypsin in 0.1% $CaCl_2$ chloride (pH 7.8) at 37 °C for 15 min.

**Table 1** Details of cases studied (*PM* post mortem delay, *LGI* Leeds General Infirmary, *NAI* non-accidental injury, *MLC* Medico-Legal Centre, Sheffield, *FML* Department of Forensic Medicine, University of Leeds)

| Number | Cause of death | Age | PM (days) | Survival | Source |
|---|---|---|---|---|---|
| 1 | Head injury | 81 years | 2 | 2½ h | LGI |
| 2 | Head injury | 17 years | 4 | 4 h | LGI |
| 3 | Head injury | 21 years | 2 | 30 h | LGI |
| 4 | Head injury | 11 months | 3 | 2 weeks | LGI |
| 5 | Reye's syndrome[a] | 22 months | 1 | < 12 h | MLC |
| 6 | Head injury (NAI) | 9 weeks | 1 | few h | MLC |
| 7 | Head injury | 44 years | 3 | 7 days | FML |
| 8 | Head injury | 78 years | 1 | 2½ days | FML |
| 9 | Multiple injuries | 38 years | 2 | 0 h | LGI |
| 10 | Cardiac failure | 62 years | 1 | 0 h | FML |
| 11 | Cardiac failure | 37 years | 2 | ½ h | LGI |

[a]This child had also suffered a mild trauma the previous day

All sections were then rinsed in TRIS buffer (pH 7.6) incubated for 30 min in 20% normal rabbit serum (where monoclonal primary antibodies were used) or 20% normal swine serum (where polyclonal primary antibodies were used). They were then incubated for 3 h in primary antibody in TRIS buffer containing 0.2% 'tween 20' (TRIS/tween). The following mouse monoclonals were used: βAPP (Boehringer, clone 22C11) 1/50; bAPP (Zymed, clone LN27) 1/200; synaptic protein, thought to be SNAP-25 (Sternberger, clone SMI 81) 1/5000; synaptophysin (DAKO, clone SY 38) 1/50; GAP-43 (Sigma, clone GAP-7B10) 1/2000; and NF68 (Sigma, clone NR4) 1/400. The following rabbit polyclonals were used: ubiquitin (DAKO) 1/300; chromogranin A (DAKO) 1/500; tau (Sigma) 1/200; and cathepsin D (gift from Dr. W. A. Reid; [35]) 1/500. The sections were then rinsed three times for 5 min in TRIS/tween and incubated for 0.5 h in biotinylated 1/200 rabbit anti-mouse IgG (DAKO) or swine anti-rabbit IgG (DAKO), as appropriate. After rinsing in TRIS/tween, an ABC detection system was used (Vectastain) for 0.5 h. Peroxidase activity was revealed with 0.025% diaminobenzidine, 300 mg Imidazole and 0.25% $H_2O_2$ for 10 min, then the reaction product was intensified with 0.5% $CuSO_4$ for 5 min. The sections were dehydrated and coverslipped with Eukitt (Kindler).

## Results

The variation in staining intensity between cases was very slight, and the results for all antibodies were comparable in tissue from all sources, of all ages. For all antibodies except cathepsin D, microwave antigen retrieval improved specific staining intensity without increasing non-specific background levels. Negative controls in which the primary antibody had been omitted showed no staining.

In agreement with our previous findings [40], immunostaining for βAPP labelled only injured axons while all others were unstained. In this study, injured axons were labelled in a case with only 2.5 h survival after head injury. No axonal labelling was found in any of the three control cases (cases 9–11). Comparable numbers of βAPP-immunoreactive axons were seen with antibodies 22C11 and LN27 (Fig. 1A, B). With both antibodies, microwave treatment increased the staining intensity of labelled axons as described in Sherriff et al. [41]. Granular neuronal staining could be seen in some cases of head injury which had survived for several hours, in accordance with previous findings [16, 36], particularly with antibody LN27. In the older patients (cases 1 and 8), βAPP-positive neurites were occasionally seen around plaques in the cortex, in accordance with Joachim et al. [22].

Anti-SNAP-25 strongly labelled injured axons in all eight cases, even without microwave treatment (Fig. 1C). In adjacent sections, comparable numbers of labelled axons could be seen to when anti-ßAPP was used, although the rest of the white matter also showed granular axonal staining, even in cases where no known axonal injury was present. In the grey matter, the neuropil was positively stained but neuronal cell bodies were not. Diluting this antibody to 1/80,000 slightly reduced the staining intensity of both normal white matter and injured axons.

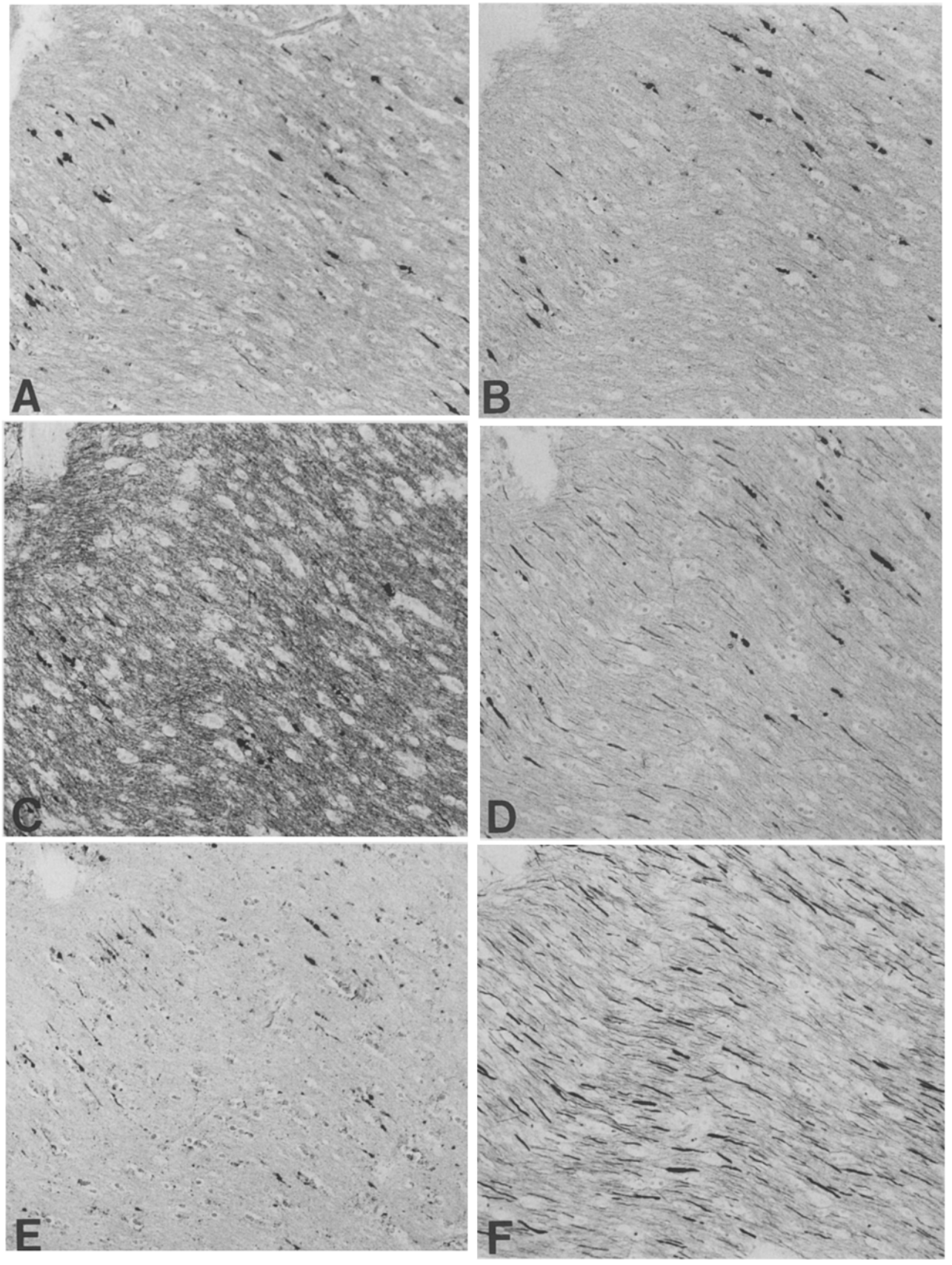


**Fig. 1A–F** Comparison of axonal injury labelling using different antibodies in adjacent sections of corpus callosum from case 2 (4-h post-traumatic survival). **A** ßAPP (22C11), only injured axons labelled, staining can be seen for some distance along axons; **B** ßAPP (LN27), as for 22C11; **C** SNAP-25, marks injured axons but normal white matter also stained; **D** chromogranin A, also stains some uninjured axons; **E** cathepsin D, pale labelling of injured axons and granular staining in white matter; **F** neurofilament subunit (68 kDa), labels many axons, reduced staining at site of injury in this case. (ß*APP* ß-amyloid precursor protein) **A**–**F** × 140

Chromogranin A labelling was present in injured axons in all eight cases, but without antigen retrieval, the staining was often paler and more granular than that seen with βAPP. Trypsinisation slightly increased the number and intensity of labelled axons, although microwave treatment provided the most intense staining and allowed more axons to be labelled. In many cases, fewer immunoreactive axons were seen than with antibodies to βAPP or SNAP-25. In some injured and uninjured brains, the anti-chromogranin A antibody faintly labelled normal axons after microwave pretreatment (Fig. 1D); in these cases where injured axons were also present, their number and staining intensity was very similar to that of βAPP. Many neuronal cell bodies were positively stained, as were plaque neurites in cases 1 and 8, in accordance with Munoz [30].

The cathepsin D antibody labelled injured axons, although the staining was sometimes pale (Fig. 1E). Strong granular cathepsin D immunoreactivity was seen in neuronal and glial cell bodies in both injured and control brains, and dot-like staining was seen in the white matter, presumably representing lysosomes within axons or glial cells. In some cases these positively stained structures in the white matter made injured axons difficult to see clearly. Microwave treatment reduced the strength of all cathepsin D immunoreactivity.

Without antigen retrieval or when trypsin was used, staining for NF68 was absent. However, after microwave treatment, the immunoreactivity was seen in almost all axons in both injured and control brains. Swollen axons were sometimes labelled, but in many cases NF68 immunoreactivity was actually reduced at sites of axonal injury (Fig. 1F). Labelling for both synaptophysin and GAP-43 was confined to the grey matter neuropil in all cases, but axons were never labelled with either antibody. Ubiquitin also did not label swollen axons in any case, even when trypsin or microwave pretreated, although immunoreactive dot-like structures were seen in older cases as previously described [33]. Tau positivity was not found in injured axons, although in case 1, a few dystrophic neurites in the cortex were stained. Synaptophysin, GAP-43, tau and ubiquitin staining were all enhanced by microwave treatment.

## Discussion

We have shown that the most effective marker for axonal injury in formalin-fixed, paraffin-embedded human brain is βAPP, which labels injured axons specifically, being undetectable in normal white matter. A model of the accumulation of βAPP and other substances is shown in Fig. 2. βAPP immunocytochemistry does not rely upon morphological changes, i.e. the appearance of axonal swellings or 'retraction balls' [1] (Fig. 2D), to identify injured axons, as is the case with other methods, such as silver staining or neurofilament immunocytochemistry. Indeed, at short survival times, many βAPP-positive axons do not appear obviously swollen (Fig. 2B). The concentration of the protein may, therefore, reach a detectable level before obvious signs of the axoplasmic swelling, due to its delivery by fast axonal transport (Fig. 2B). βAPP immunocytochemistry also enables axonal injury to be detected where the site of damage is not necessarily in the plane of section, as immunoreactivity generally extends for some way back along the axon, where swelling may not have occurred (Fig. 2C). Post-mortem delay is known to cause artefactual increases in axonal diameter which can lead to misdiagnosis of axonal injury at short survival times when methods which stain all axons such as silver stains are used (see [11]). However, the fact that βAPP accumulation is an energy-requiring process means that its presence indicates damage to axons during life.

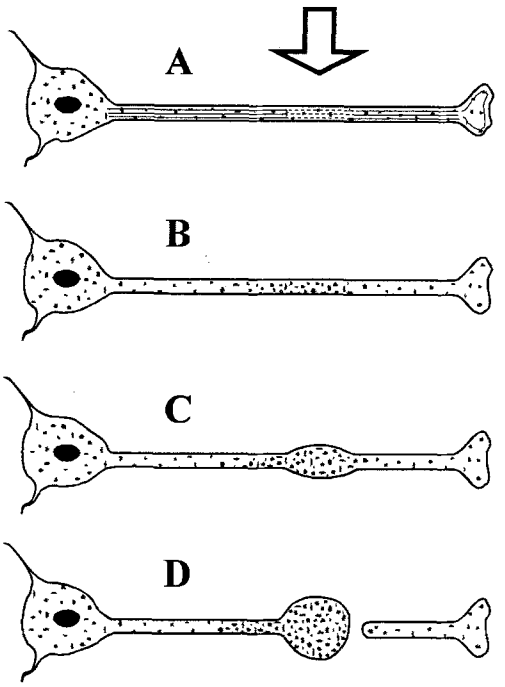


**Fig 2A–D** Accumulation of βAPP and other substances carried by fast anterograde axonal transport, in injured axons. **A** Cytoskeletal breakdown, possibly due to calcium influx [2] (*parallel lines* represent cytoskeleton, *arrow* indicates site of damage). **B** Hold-up of axonal flow and concentration of organelles due to delivery by fast axonal transport (cytoskeleton not shown for clarity). **C** Further accumulation and axonal swelling. **D** Disconnection of the distal part of the axon. For simplicity, any accumulation of proteins carried by retrograde transport has been omitted from this diagram

This study has shown that βAPP immunocytochemistry detects axonal injury in cases of all ages, from different laboratories, independent of post-mortem delay. In our experience (unpublished observations), the levels of staining in adjacent blocks of tissue which have been fixed in formalin for 1 week to 6 months are almost identical, and any discrepency can be removed by the use of microwave antigen retrieval. Slight variations in tissue fixation do not, therefore, seem to seriously affect results, making βAPP immunocytochem-

istry ideal as a routine laboratory technique. Microwave antigen retrieval is not a necessary part of the immunocytochemical procedure, but it enhances staining and may be most useful in detecting βAPP immunoreactivity where the concentration of βAPP is low or formalin fixation has severely masked the immunoreactivity [41].

The minimum survival time for the detection of axonal injury has been reduced to 2.5 h in this study. Despite the fact that animal studies have shown that βAPP accumulates in axonal swellings in the aged brain [24], this has not been found in the human brain studied so far [40], thus, despite the advanced age of the patient with 2.5 h survival, the extensive axonal injury seen here is probably the result of trauma. Animal studies have shown that the rate of fast anterograde axonal transport decreases considerably in old age [15, 48], so if this phenomenon occurs in human brain, βAPP may, therefore, concentrate even more quickly in younger cases. As the accumulation of βAPP is an active process, relying on an energy-requiring, kinesin-based motor [13], it is conceivable that, despite axonal injury being present, βAPP may not accumulate in certain circumstances, e.g. in patients who had suffered hypoxia or brain death. For this reason, it may be possible to underestimate the extent of axonal injury in these cases.

It must be noted that βAPP marks axonal injury due to factors other than trauma [10, 31, 40]. In some cases, βAPP immunoreactivity marked damage of the diffuse axonal injury type, while in some of the cases with several days' survival, βAPP-positive axons were associated with infarcts. In case 5, it is unclear whether the brain lesion was a consequence of a minor fall sustained the previous day or the oedema associated with Reye's syndrome. The possible roles of factors such as oedema and hypoxia in the development of white matter damage and, hence, βAPP immunoreactivity are unclear, and are the subject of further study. βAPP immunocytochemistry has the potential to become a diagnostically useful technique for the early visualisation of axonal injury after head trauma and it may be indicative of white matter damage in other settings. However, until the technique has been validated on larger numbers of cases, in a wide variety of circumstances, caution in the interpretation of the absence or quantity of staining should be used, with the above points in mind.

This study has also shown that antibodies to SNAP-25, chromogranin A and cathepsin D label injured axons, although they were less useful as markers of axonal injury than βAPP for various reasons. SNAP-25 strongly labelled swollen axons, but many apparently normal axons were also stained, which probably represented the protein undergoing axonal transport. As stained axons could not be identified unequivocally, this precluded the categoric detection of injury at short survival times where gross swelling had not occurred. For the same reason, the quantity of axonal injury could be underestimated at longer survival times. Extreme dilution of the antibody only slightly reduced the intensity of background white matter staining.

The pattern of axonal labelling with chromogranin A and cathepsin D was very similar to that of βAPP, but even with microwave enhancement, fewer axons were labelled and the staining intensity was usually weaker. This weak axonal staining by these two antibodies may have been because there are relatively small numbers of large dense-core vesicles and lysosomes in neurons, compared to some other organelles, so only a low concentration is detected at the sites of axonal injury. In view of the fact that part of the βAPP molecule is secreted, it has been suggested that it may be associated with secretory vesicles. However, it is known that synaptophysin-containing vesicles show no βAPP immunoreactivity and it was suggested that βAPP-positive vesicular structures may correspond to dense-core vesicles [37]. Electron microscopical studies have shown that in salivary glands it is associated with both large and small dense-core vesicles but in the adenohypophysis it only localises with the small type [8]. The present study supports the idea that, in brain, βAPP is associated with a different organelle than the large, dense-core vesicle, as the characteristics of βAPP labelling in this study are slightly different to that of chromogranin A. βAPP is thought to be processed via the endosomal/lysosomal system [6, 13, 17, 20]. However, it does not appear to be associated with lysosomes undergoing anterograde transport because, as with chromogranin A, the staining characteristics with antibodies to cathepsin D and βAPP are different.

Previous reports [18, 52] have suggested that antibodies to NF68 can be used in the detection of axonal injury because the labeling was uniquely axonal, and immunoreactivity is increased at the site of axonal injury, enabling swellings to be readily seen. We did not find any increase in NF68 immunoreactivity at sites of axonal injury or that NF68 was a specific marker for injured axons, but found that it labelled most axons, and staining was often absent at sites of axonal injury. Anti-NF68 showed up inconsistencies in axonal diameter even in uninjured brains, and injured axons could only be identified with certainty if they were obviously swollen. The extent of axonal injury was severely underestimated using this antibody.

Synaptophysin, GAP-43, tau and ubiquitin did not label any normal or injured axons in this study. The presence or absence of neuronal proteins at sites of axonal injury in the brain may tell us something about their cellular processing here, in the same way as nerve crush can be used to explore the peripheral nervous system. As synaptophysin labels small synaptic vesicles [49, 50] which are carried by fast axonal transport to the terminal [12], we were initially surprised that injured axons were not labelled with anti-synaptophysin, considering a recent report that synaptophysin accumulates at the site of peripheral nerve crush in the rat [28]. The fact that synaptophysin immunoreactivity is found only in terminal regions and not in the neuronal cell body or axon may mean that, in the brain, synaptophysin: (1) only associates with small synaptic vesicles after they

have undergone axonal transport; (2) is post-translationally modified at the synapse, and the antibody only recognises this form; or (3) is present in such small quantities in the axon that the amount which accumulates at sites of axonal injury may be undetectable. The precise cellular site at which synaptophysin associates with small synaptic vesicles is unclear [12] and the results of this study support findings that no small synaptic vesicles are found proximal to nerve block, although large, dense-core vesicles are [46, 47], and that small synaptic vesicles may be generated in nerve endings from large membraneous intermediates [12].

High levels of GAP-43 immunoreactivity are found in developing neurons [42], although in the mature brain, it is present in much smaller amounts as a component of the presynaptic membrane [5]. GAP-43 is known to be present in the human brain [7], and the distribution is similar to that of synaptophysin. GAP-43 accumulates on both sides of a sciatic nerve crush in the rat [28]. The absence of the protein at sites of axonal injury could be a result of GAP-43 being post-translationally modified at the synapse or not being present in the pyramidal cells which contribute most of the axons to the white matter of the corpus callosum. The rate of anterograde translocation of GAP-43 is known to be increased during regeneration [5], although the lack of significant regeneration in the brain may mean that this increase does not occur here. However, GAP-43 is probably carried by fast axonal transport in the brain due to its proposed synaptic function.

The absence of tau immunoreactivity in injured axons, even in older patients, shows that paired helical filaments did not form at the survival times studied. Their presence in tangles and dystrophic neurites in Alzheimer's disease [25] and dementia pugilistica [51] is, therefore, not an immediate consequence of axonal injury.

To our knowledge, ubiquitin is not known to be transported by fast axonal transport, so is unlikely to accumulate by the same mechanism as that proposed for βAPP and other proteins (Fig. 2). However, it has previously been found in axonal swellings after trauma in man [19, 29] and animals [23, 38]. It was, therefore, surprising that ubiquitin was not found in any of our cases, especially those with long survival times, as it is associated with targeting proteins for degradation [21]. We have carried out ubiquitin immunostaining on many cases of axonal injury and consistently find that axonal swellings are not labelled, although we do find dot-like ubiquitin positivity in the aging brain.

This study also demonstrated the effectiveness of using microwave antigen retrieval in routinely processed neuropathological tissue. In addition to enhancing the staining intensity of βAPP [41], it was found to be more effective than trypsin digestion for SNAP-25, chromogranin A, synaptophysin, GAP-43, tau and ubiquitin. In agreement with previous findings [9], NF68, which is normally undetectable in routinely processed tissue, was unmasked and became strongly labelled using this method. The fact that this technique enhances the immunoreactivity of these neuronal antigens means that it is a useful research tool and has a potentially important role in routine diagnostics.

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