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GUSTAV FISCHER

American Foundation, the British Council and the Lady Tata Foundation. Thanks to this support, he was able to study successively in Paris (Collège de France), Zurich (Canton Hospital), Boston (Massachusetts General Hospital) and after the war in London (Chester Beatty Institute). Retrospectively, it is clear that such a diverse international training was certainly central for broadening his scientific education, enlarging his general culture and developing his capacity to communicate.

It was early in the course of his medical studies that, in the Laboratory of Prof. Pol. Gerard, Pierre Dustin began his first morphological studies. They were focused on the complex periarterial structures of the spleen. These first steps were already a sign of his lifelong interest in blood diseases, an interest which never failed and subsequently led to work on human red blood cells and virus induced leukemias. However, one of the most remarkable orientations of his scientific career was his fascination for the ever-increasing capacities of the electron-microscope and morphological analysis of this new ultrastructural world. This was indeed a very exciting time when he monitored with enthusiasm and curiosity the work of his pupils and studied with them a number of virus-related diseases such as progressive multifocal leuco-encephalitis, necrotizing encephalitis, murine friend leukemias and others.

It was also at this time that his Belgian colleague Christian de Duve, threw light on the concept of lysosomes and initiated the ultrastructural analysis of the storage and metabolic diseases, a vast domain in which Pierre Dustin and his school made invaluable contributions.

Likewise, the changing patterns of the cytoskeleton and particularly the microtubule system have always absorbed him. In 1978, he wrote a 452 page monograph on this subject in which he analysed the ultrastructural modifications which occur during normal and abnormal mitoses and the effects of certain cell poisons. It is meaningful that his last scientific article, as he wrote me, was devoted to the significance of the neurofibrillary tangles observed in Alzheimer's disease. This article was written with the collaboration of his closest pupils: J. Flament-Durand and J. P. Brion and was published in PRP (1992; 188; 248–253). In acknowledgement of their faithful friendship, the present Festschrift opens with a contribution written by these authors and devoted to his favorite subject.

The teaching skills of Pierre Dustin were soon recognized as he became a 'Professeur agregé' at the early age of 31, and was appointed a full Professor at 42. His teaching was clear and convincing. These qualities can still be appreciated in his remarkable book entitled "*Leçons d'Anatomie Pathologique Générale*" published in 1966. This book was met with great success and was re-edited in 1981 with the collaboration of his pupil N. Dourov. It is in my opinion, with the introduction to pathology by Payling Wright, one of the best manuals of General Pathology.

Pierre Dustin was a tireless worker and an active member of several scientific societies and editorial boards. For many years, he was the Chief-Editor of the "Revue Médicale de Bruxelles." Prior to etablishing the European Society of Pathology, he founded the "Société Belge d'Anatomie Pathologique" with his colleagues from Liege and Louvain, Professors Betz and Maisin. Since 1982, he had been a permanent member of the *Académie Royale de Médecine de Belgique* and an affiliated member of the French *Académie de Médecine (Paris)*.

Behind his strict and somewhat severe side, Pierre Dustin was in fact a friendly and welcoming person, searching for personnal contacts and intellectual exchanges. He was particularly keen on art and an expert on modern Belgian painters. He was always delighted to present his own remarkable collection to the friends that he and his wife Marie-Jeanne warmly welcomed in their home in Brussels. On occasion, he would unhesitantly undertake a long plane flight so as not to miss an exceptional collection of paintings, as the recent retrospective exhibition of Matisse's work in Washington. He also liked listening to music with an evident preference for the classical composers. He sometimes used to sit down at his piano and play for his own pleasure. Above all, he was always an inveterate reader not only of scientific papers, but also of English, as well as French literature. To a large degree, this passion accounted for his great erudition. By the extent of his culture and his tastes, he was a man in the tradition of the Renaissance, a man whose opinions and advice were carefully heeded.

Pierre Dustin passed away in Brussels in September 1993 – he was 79 years old. His family, his wife and children, all his friends and students miss him sorely. His memory will certainly remain alive for a long time. This Festschrift, I hope, will help to keep him from oblivion as well as acknowledge his crucial role in the foundation of the European Society of Pathology.

I'm indebted to Mrs. Marie Jeanne Dustin and Professors Nicolas Dourov, Jacqueline Flament-Durand and Jean-Pierre Brion for providing information and documents.

I also express my thanks to Miss Emmanuelle Le Roy and Mrs. Mary Hegarty for their help in collecting and preparing the manuscripts.

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Original Papers

Distribution and Expression of the α-Tubulin mRNA in the Hippocampus and the Temporal Cortex in Alzheimer's Disease[1]

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SUMMARY

The distribution of the messenger RNA for α-tubulin has been investigated by in situ hybridization in the human hippocampus and temporal cortex in normal subjects and in Alzheimer's disease. The α-tubulin mRNA was strongly expressed in neurons in the gyrus dentatus, in the Ammon's horn and in cortical layers of the temporal cortex. The same distribution was observed in Alzheimer's disease. An important reduction of the hybridization signal was apparent, however, in areas rich in neurofibrillary lesions, e.g. as in layer II of entorhinal cortex. Neurons containing neurofibrillary tangles exhibited a weaker hybridization signal than adjacent neurons devoid of neurofibrillary tangles. The immunoreactivity for α-tubulin was drastically reduced in tangles-bearing neurons. These results indicate that tubulin transcription is reduced in tangles-bearing neurons, a reduction which might play a role in the reported decrease in the number of microtubules in neurons containing neurofibrillary tangles.

Introduction

Alzheimer's disease, the most common cause of dementia in aged people, is characterized by the presence of typical neuropathological lesions, i.e. neurofibrillary tangles and senile plaques. Neurofibrillary tangles (NFT) are composed of bundles of abnormal filaments which are found in neuronal pericarya, dendrites and axons. Ultrastructural studies have demonstrated that these abnormal filaments have unique morphological features which differenciate them from microtubules, intermediate filaments or microfilaments; they show regular constrictions and a twisted appearance and are generally designed by the term "paired helical filaments" (PHF). Both immunocytochemical and biochemical studies have demonstrated that PHF are composed of the microtubule-associated protein tau[4, 7, 10, 18, 21, 25, 26, 33, 39], a protein which plays a role in the stabilisation of axonal microtubules.

Microtubules are essential components of neuronal and non-neuronal cells and are known to have multiple roles[9]. In neurons, microtubules play an important role in the formation and maintenance of cell shape, and in the phenomenon of intracellular traffic known as axoplasmic transport. For instance, microtubules are vital to ensure the fast axoplasmic transport of membranous organelles along dendrites and axons.

In Alzheimer's disease, a decrease in the number of neuronal microtubules has been observed, especially in neurons containing PHF[11, 13, 14, 19, 34]. This loss of microtubules is associated with accumulations of membranous organelles: degraded mitochondria, multivesi-

[1] This paper is dedicated to the memory of Professor P. Dustin (1914–1993), with whom we had the privilege to work for many years and to benefit from his enthusiasm for fundamental research in Pathology.

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cular bodies, lysosomal bodies and endoplasmic reticulum[11, 35]. Accumulation of these organelles might be a consequence of the disturbance of axoplasmic flows due to the disappearance of microtubules. A similar accumulation of membranous organelles in neurons can be experimentally produced by treatment with colchicine or vinblastine, two microtubule poisons which disrupt the microtubule network[15, 16]. Such a disturbance of axoplasmic flows in Alzheimer's disease would compromise the maintenance of distal parts of neuronal extensions and play an important role in the degeneration and death of neurons. These observations and others, point to the potential impairment of the microtubule system in Alzheimer's disease[31].

The disappearance of microtubules in affected cells might be a consequence of a reduction in their stability or of the ability of tubulin to polymerise in microtubules. Alternatively, a reduction of tubulin synthesis might also impair the formation and turn-over of microtubules in these neurons. In this study, we have investigated by *in situ* hybridization the distribution and level of expression of α-tubulin messenger RNA in the hippocampus and the temporal cortex of control subjects and in patients with Alzheimer's disease.

Results

Loco-regional Distribution of Tubulin mRNA

In both normal subjects and Alzheimer's disease, tissue sections hybridized with the radioactive anti-sense tubulin riboprobe (i.e. nucleotide sequence complementary to the sequence of the tubulin mRNA) exhib-

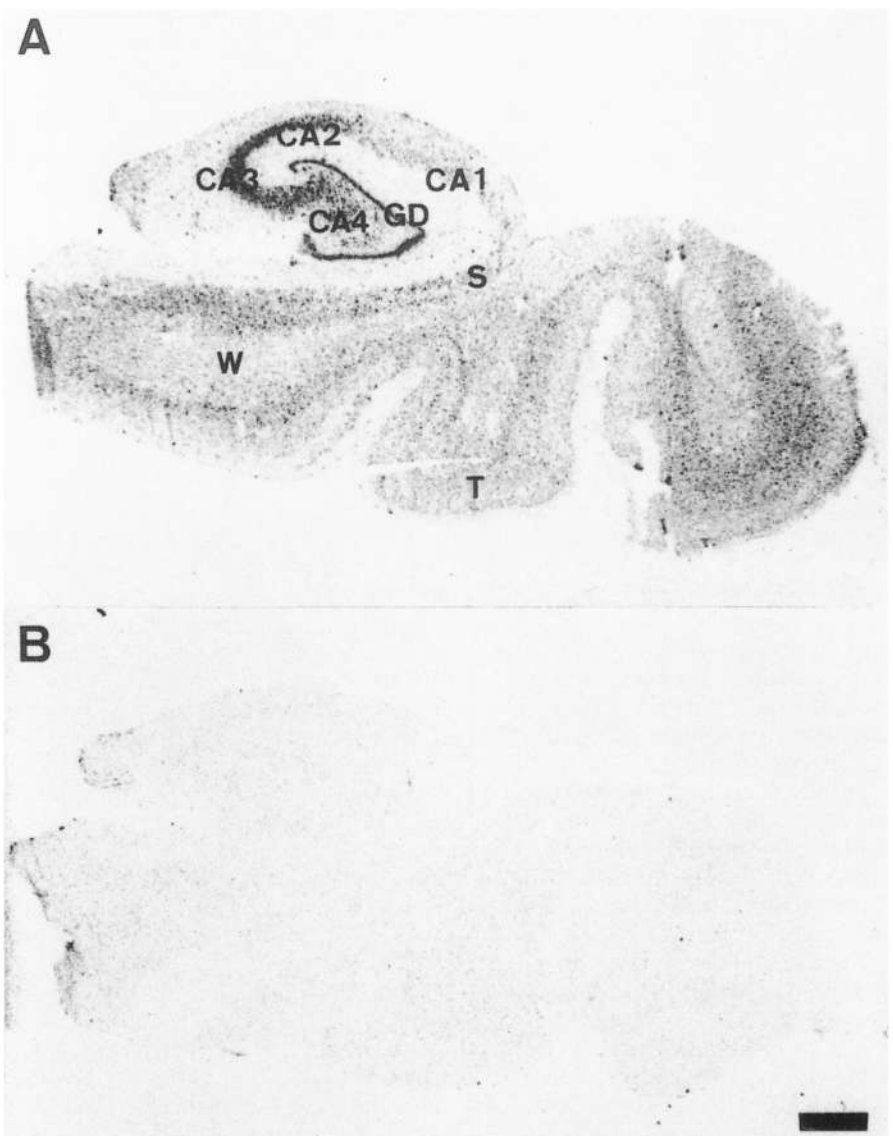

Fig. 1. *In situ* hybridization (autoradiography on X-ray film) showing the distribution of tubulin mRNAs on tissue sections of the hippocampus and the temporal cortex in a patient with Alzheimer's disease. The sections were hybridized with an anti-sense RNA probe (A) or a sense RNA probe (B). CA1, CA2, CA3, CA4: sectors 1 to 4 of Ammon's horn; GD: gyrus dentatus; S: subiculum; T: temporal cortex; W: white matter. Scale bar: 2 mm.

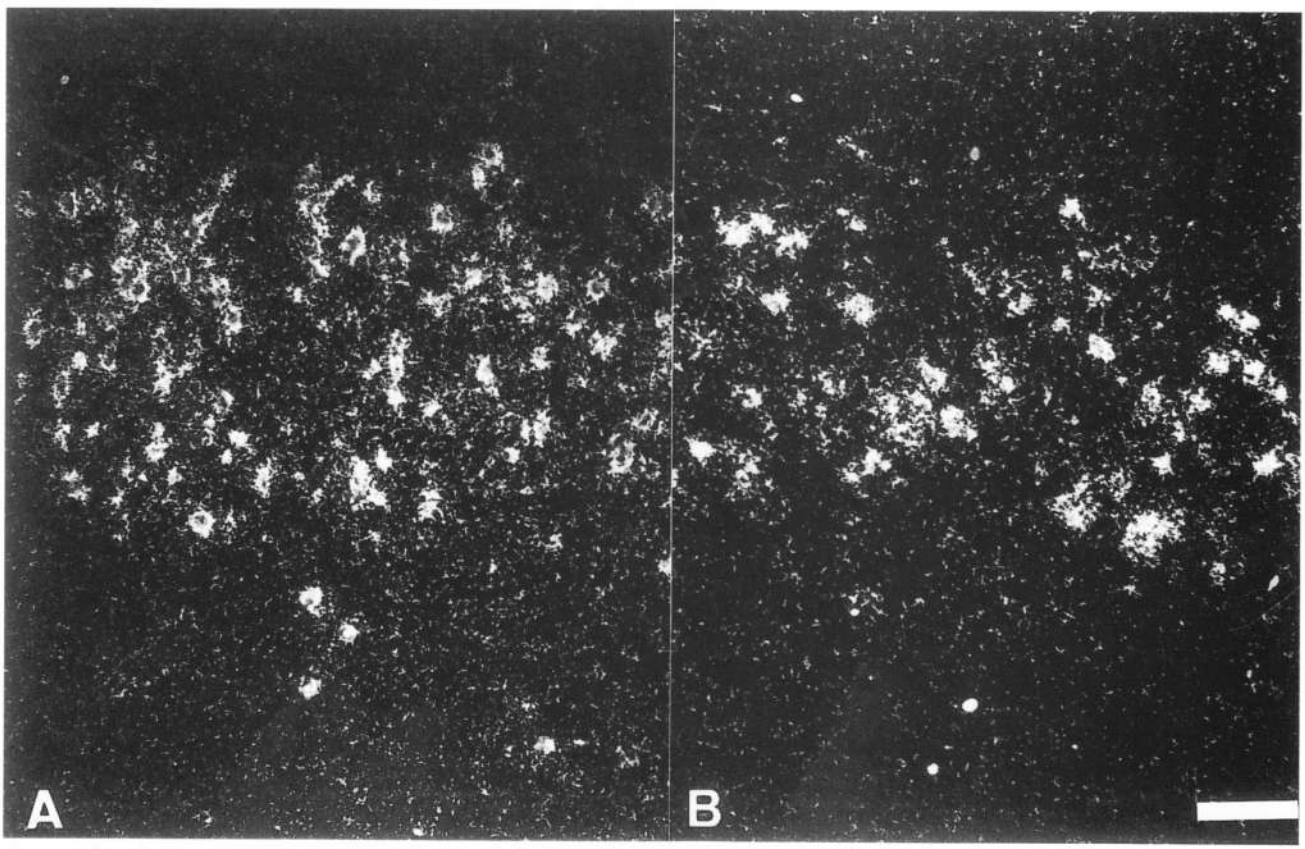

Fig. 2. *In situ* hybridization (emulsion autoradiography, dark field illumination) showing the distribution of tubulin mRNAs in the CA3 sector in the hippocampus, in a control subject (A) and in a patient with Alzheimer's disease (B). The tissue sections were hybridized with antisense RNA probes. There is a strong hybridization signal in pericarya of pyramidal neurons, both in Alzheimer's disease and in the control subject. Scale bar: 100 μm.

ited a strong autoradiographic signal on X-ray film. A methodological control of the hybridization technique was performed by hybridization of tissue sections with a radioactive sense tubulin riboprobe (i.e. a sequence similar to the nucleotide sequence of the tubulin mRNA); these tissue sections did not show any significant autoradiographic signal (compare Figs. 1A and 1B). In control subjects, a strong hybridization signal was present in the granule cell layer and the pyramidal cell layer of the Ammon's horn. The signal was particularly strong in the CA4, CA3 and CA2 sectors of the Ammon's horn. An intense hybridization was also observed in the subiculum and the presubiculum (where the islands of cells in layer II are well demonstrated). All cortical layers in the entorhinal and the temporal cortex showed a hybridization signal. The signal was much weaker in white matter.

The general distribution of tubulin mRNA was preserved in Alzheimer's disease. A low magnification view (X-ray film) of the distribution of tubulin mRNA in the posterior hippocampus and adjacent temporal cortex of a patient with Alzheimer's disease is shown in Fig. 1A. As in controls, a strong hybridization signal was present in the granule cell layer of gyrus dentatus and in CA4, CA3 and CA2 sectors of pyramidal cell layer. A decrease of the hybridization signal was however observed in the CA1 sector, in the subiculum and in the adjacent entorhinal and temporal cortex. In the entorhinal cortex, there was an almost complete disappearance of the hybridization signal in the superficial cortical layers.

Cellular Distribution of Tubulin mRNA

The examination at higher magnification of tissue sections processed for emulsion autoradiography confirmed that a strong hybridization signal was present only in neuronal layers. The expression of tubulin mRNA was especially important in pyramidal neurons in the Ammon's horn. In all neurons, tubulin mRNAs were mainly localized in pericarya.

A relative preservation of tubulin mRNA expression was observed in several sectors of the hippocampus and the temporal cortex in patients with Alzheimer's disease; e.g. pyramidal neurons in CA3 sector displayed a hybridization signal similar to the one observed in the same area in control subjects (compare Figs. 2A and 2B).

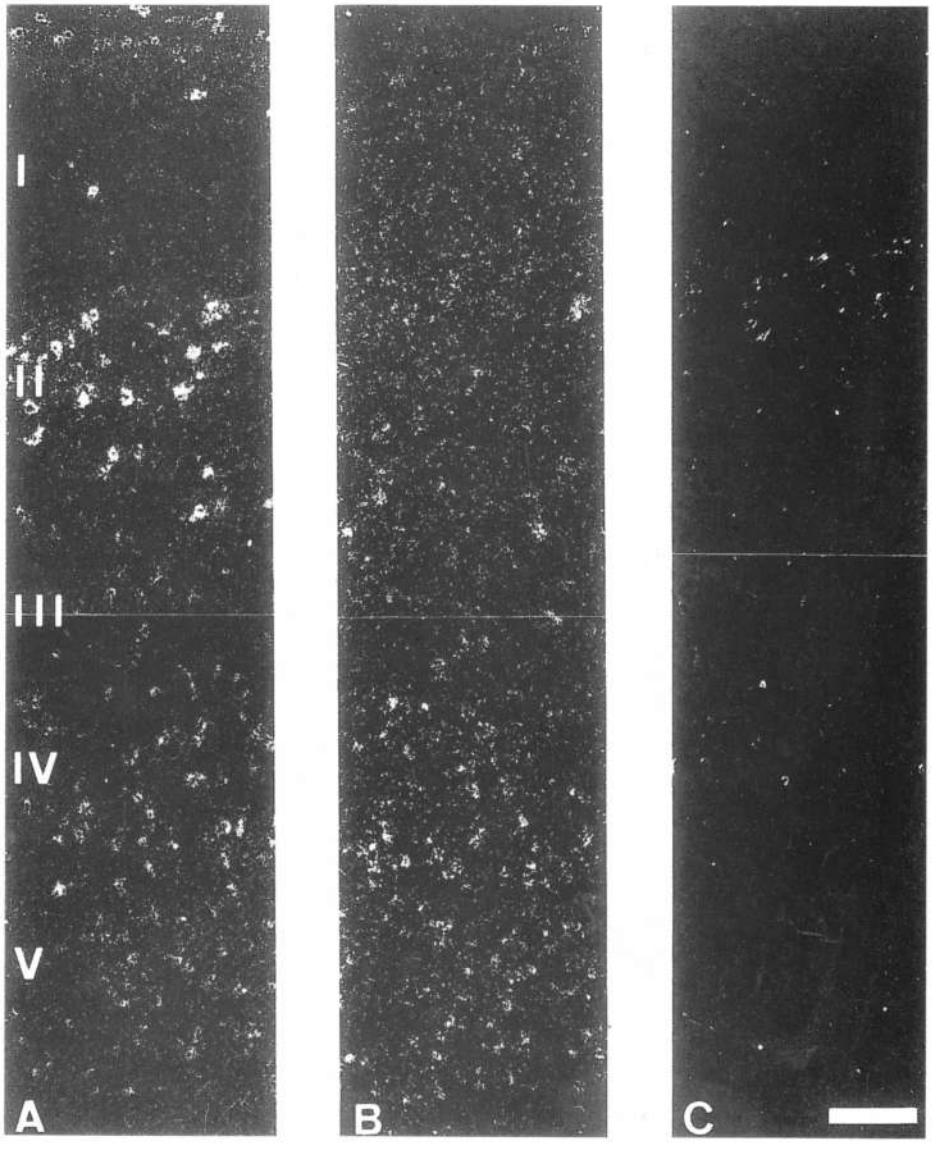

Fig. 3. *In situ* hybridization (emulsion autoradiography, dark field illumination) showing the distribution of tubulin mRNAs in the entorhinal cortex, in a control subject (A) and in a patient with Alzheimer's disease (B). The hybridization signal seen on cell bodies in deep cortical layers is relatively similar in the control and in Alzheimer's disease. In the control, a strong hybridization signal is present in neurons in layer II. In Alzheimer's disease, the hybridization signal in layer II is drastically reduced. The tissue section in (B) was counterstained with Congo red and viewed under polarization filters (C) to demonstrate neurofibrillary tangles, abundant in layer II. I–V: cortical layers. Scale bar: 200 μm.

On the other hand, areas rich in neurofibrillary lesions (the latter being demonstrated by Congo red staining) showed a general reduction of the hybridization signal. This was most clearly seen in regions such as the CA1 sector, the subiculum and in layer II of the entorhinal cortex. In the latter cortical layer, a strong hybridization signal was observed in control subjects (Fig. 3A) but not in patients with Alzheimer's disease (Fig. 3B). This layer contains numerous NFT in Alzheimer's disease (Fig. 3C).

Most neurons containing NFT (arrows in Figs. 4A and 4B) were observed to exhibit a reduced hybridization signal, as compared to adjacent normal neurons not containing NFT (arrowheads in Figs. 4A and 4B). Extracellular NFT did not display any hybridization signal.

α-Tubulin Immunoreactivity in Neurons containing NFT

In the hippocampus and the temporal cortex, normal neurons showed a strong pericaryal and dendritic labelling with the α-tubulin antibody (Fig. 5A). On the contrary, most neurons containing NFT (arrows in Figs. 5A and 5B) showed a drastic reduction in tubulin immunoreactivity. This reduction in tubulin immunoreactivity was contrasting in areas where the NFT-bearing neurons were adjacent to neurons devoid of NFT. In some NFT-bearing neurons, a weak perikaryal tubulin immunoreactivity was still noticed; these neurons often did not show a strong tubulin immunoreactivity in dendrites, as seen in adjacent normal cells.

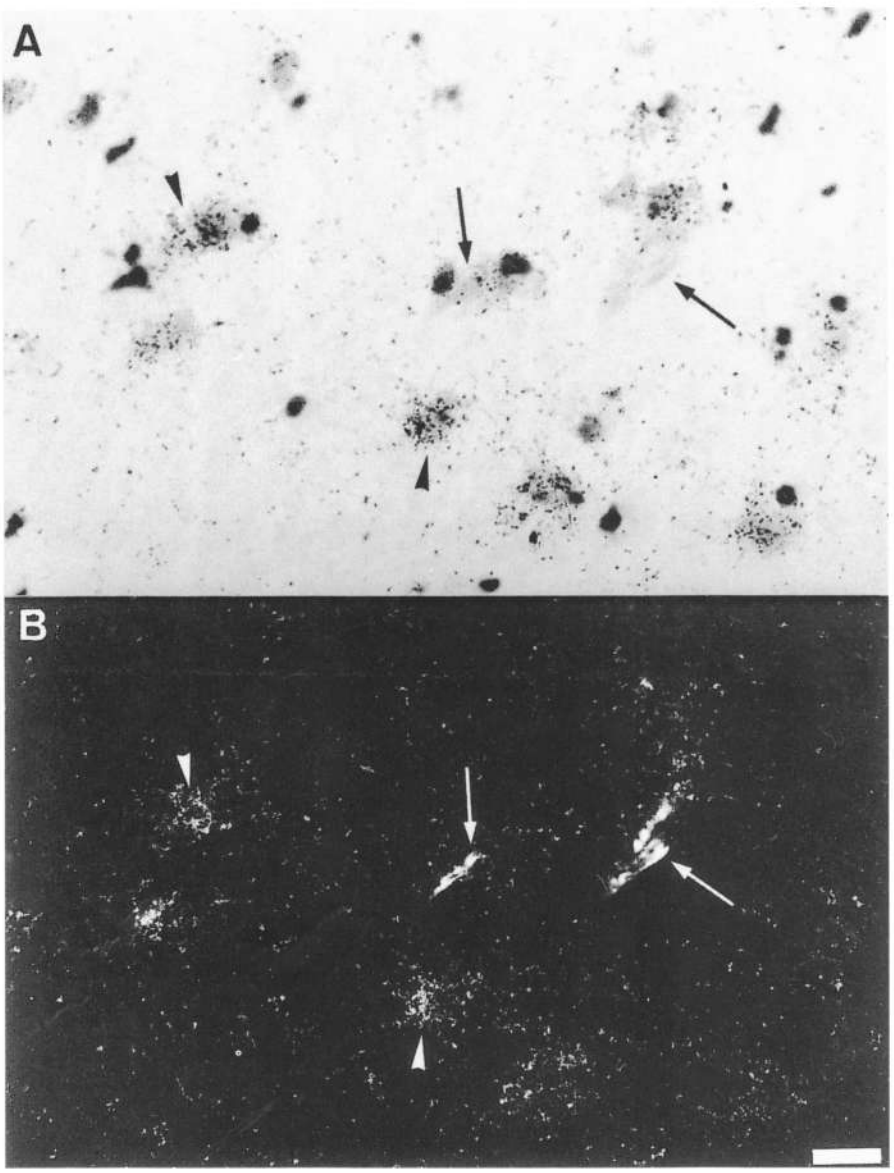

Fig. 4. *In situ* hybridization (emulsion autoradiography) showing the distribution of tubulin mRNAs in the CA1 sector of the Ammon's horn in a case of Alzheimer's disease. After hybridization, the tissue section was counterstained with Congo red to demonstrate neurofibrillary tangles. The same area was examined under bright-field illumination (A) or between polarization filters (B). Neurons containing neurofibrillary tangles (arrows) show a less intense hybridization signal than adjacent neurons which do not contain neurofibrillary tangles (arrow heads). Scale bar: 25 μm.

Discussion

The level of expression of α-tubulin transcripts in the human brain has been investigated in this study using *in situ* hybridization. In normal subjects, a high expression of α-tubulin mRNA was found in all neurons in the hippocampus and in the temporal cortex. This high expression of tubulin mRNA in neuronal cells has been previously described in various species[9].

A similarly high neuronal expression of tubulin transcripts in neurons was observed in Alzheimer's disease, although significant loco-regional differences were present. Some areas clearly showed a reduction in the hybridization signal; these regions, such as the CA1 sector and layer II of the entorhinal cortex, correspond to areas which contain abundant neurofibrillary lesions[2]. Most probably, this reduction in tubulin expression also reflects the loss of neurons affecting these areas. A reduction of tubulin mRNA in the cortex in Alzheimer's disease has also been observed by Northern blot analysis[5].

The observation at the cellular level confirmed that neurons containing neurofibrillary tangles exhibit an important reduction in the hybridization signal for α-tubulin mRNA. Extracellular NFT were not associated to any hybridization signal; this is not unexpected since extracellular NFT are considered as cell remnants, persisting in the neuropil after neuronal death. The persistance of a weak hybridization signal in a few neurons containing NFT suggests that the process of NFT formation is not preceded by a complete defect in tubulin transcription and that the decrease of tubulin mRNA expression might rather accompany the formation of NFT.

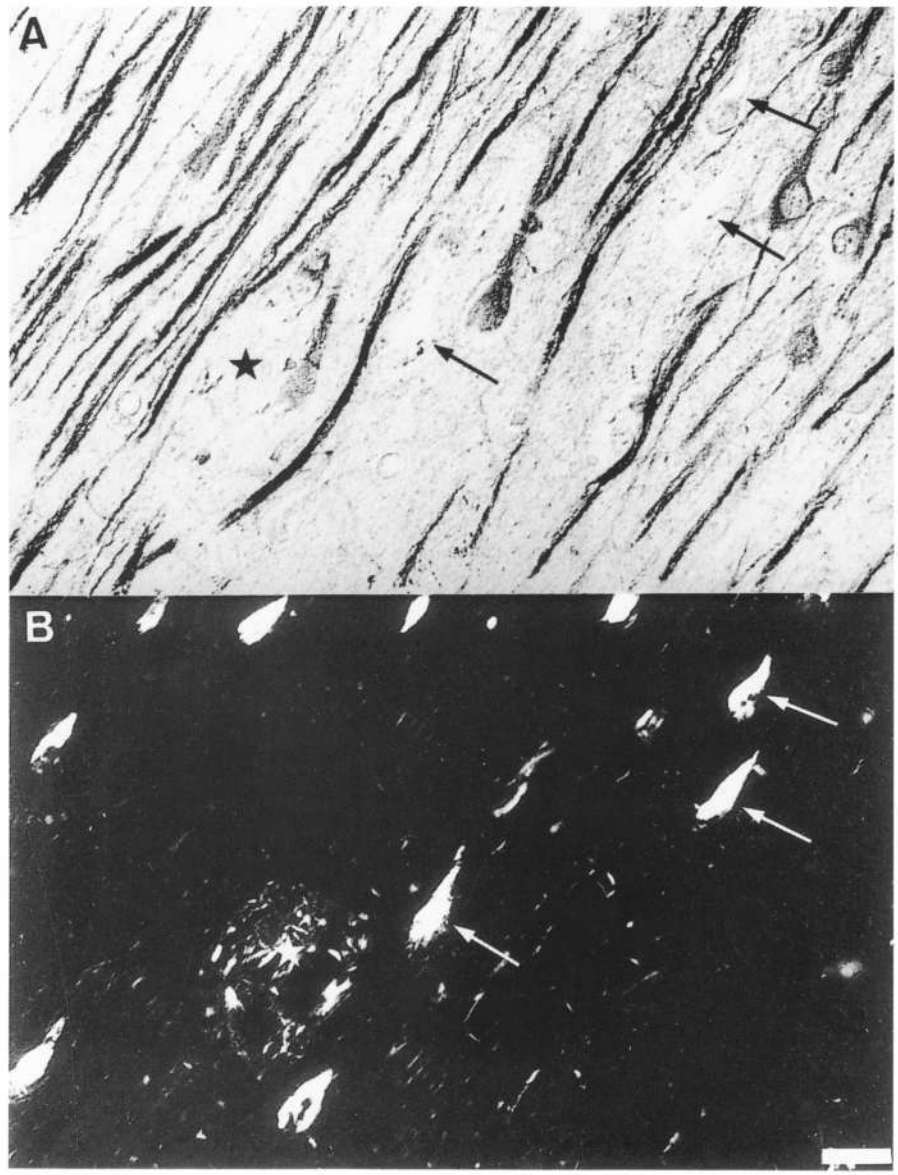

Fig. 5. Immunolabeling (PAP method) with the anti-α tubulin antibody on tissue section of the hippocampus (CA1 sector) in a case of Alzheimer's disease. After immunolabeling, the tissue section was counterstained with Congo red to demonstrate neurofibrillary tangles. The same area was examined under Nomarski optic (A) or between polarization filters (B). Most neurons containing neurofibrillary tangles (arrows) show an important decrease in tubulin immunoreactivity. The amyloid deposits in a senile plaque (asterisk) are also birefringent after Congo red staining. Scale bar: 25 μm.

On the other hand, a drastic reduction in tubulin immunoreactivity was observed in most neurons containing NFT, contrasting with the weak persistence of tubulin transcripts in some of the NFT-containing neurons. This might also suggest that tubulin mRNAs are not efficiently translated in NFT-containing neurons. Accordingly, a reduction in the translational activity of mRNA in Alzheimer's disease has been reported in some studies[36]. A reduction in tubulin immunoreactivity in NFT-containing neurons has also been observed previously[32] and is in agreement with previous ultrastructural studies indicating an inverse relationship between the presence of PHF and microtubules in dendrites[13, 14, 20]. Previous biochemical analyses have demonstrated a reduction in the level of tubulin proteins in the brain in Alzheimer's disease and in Down's syndrome[1]. Patients with Down's syndrome develop neuropathological lesions similar to those observed in Alzheimer's disease.

The mechanism responsible for the observed decrease of tubulin mRNA expression remains hypothetical. This decrease might result from a general decrease of DNA transcription in neurons in Alzheimer's disease. This hypothesis is supported by the observations that chromatin is abnormally condensed in the cortex in Alzheimer's disease[28] and that levels of total[30] and/or messenger RNA are reduced in the disease[23, 37]. The loss of cellular RNA seems especially important in NFT-containing neurons, e.g. as judged from RNA staining with the dye azure B[8].

Alternatively, a selective decrease of tubulin mRNA expression might occur in NFT-containing neurons. By Northern blot analysis[5], it was reported that the deficit in tubulin mRNA in Alzheimer's disease cortex was

more pronounced than the deficit in the message for the amyloid peptide precursor. In normal cells, there is an autoregulation of tubulin synthesis through the intracellular concentration of free tubulin, which modulates the stability of tubulin mRNA[6, 40]. An increase in the pool of free tubulin subunits in affected neurons would thus be expected to decrease tubulin mRNA stability and to lead to a decrease in tubulin synthesis. A transient increase in tubulin subunits might be the consequence of microtubule instability, resulting in microtubule depolymerisation.

Several factors might induce a shift of the equilibrium between polymerised and unpolymerised tubulin. Among these factors, the microtubule-associated protein tau is thought to play a major role. In addition of being the major building block of NFT, soluble tau proteins are highly phosphorylated in Alzheimer's disease[3, 12, 22, 27]. The phosphorylation of tau proteins is known to affect its ability to interact with microtubules; i.e. phosphorylated tau is less able to induce tubulin polymerisation and microtubule stability. A defect in brain microtubule assembly in Alzheimer's disease has been reported[24] and phosphorylated tau species isolated from Alzheimer's brain have been found to be inefficient in polymerizing microtubules[29]. These phosphorylated tau proteins most probably induce or contribute to microtubule instability. Microtubule stability could also be modulated by other factors such as the apolipoprotein E genotype[38].

In conclusion, this study demonstrates a reduction of tubulin mRNA expression in neurons containing neurofibrillary tangles. We suggest that this reduction contributes to the loss of microtubules in these cells.

Materials and Methods

Tissue Samples

Blocks of human hippocampi were taken from two cases of Alzheimer's disease (65 and 81 years, post-mortem delays of 4.5 and 8 hours, respectively) confirmed by the presence of numerous neurofibrillary tangles and senile plaques in the hippocampus and the neocortex and from two normal subjects (61 and 82 years, post-mortem delays of 8 and 4.5 hours, respectively). For *in situ* hybridization, the tissue samples were snap-frozen in isopentane cooled to − 80 °C. Tissue sections (10 µm thick) were cut on a Leitz cryostat and immediately fixed for 5 min in 4% (w/v) paraformaldehyde in 0.1 M PBS, dehydrated in graded ethanol solutions containing 300 mM ammonium acetate and kept frozen at − 80 °C. For the immunocytochemistry, tissue samples were fixed in methacarn (methanol: chloroform:acetic acid) for 48 hours, dehydrated and embedded in paraffin.

Preparation of Nucleic Acid Probes

A rat α-tubulin cDNA[17], kindly provided by Dr. Ginzburg (Israel), was used to generate RNA probes for *in situ* hybridization. This cDNA has been subcloned in Bluescript plasmid. This plasmid was linearized by digestion with BamH1 or EcoR1, run on a 0.8% agarose gel and the DNA fragments isolated from pieces of agarose using a silica matrix (Bio101). The linearized plasmids were used to generate sense or antisense RNA transcripts with T3 or T7 RNA polymerases (Boehringer). RNA probes were radioactively labelled by inclusion of 50 µCi^{35} S-UTP (new England Nuclear) in the transcription solution. The DNA template was removed by digestion with DNase1 (Pharmacia) and the mixture extracted twice with phenol/chloroform and precipitated with sodium acetate and cold ethanol. The probes were resuspended (10^6 cpm/µl) in 10 mM Tris (pH 7.6), 1mM EDTA and 30 mM dithiothreitol.

In situ hybridisation

The radioactive RNA probes (10^6 cpm/section) were added to the following hybridization solution: 50% (v/v) formamide, salmon sperm DNA (1 mg/ml), tRNA (0.6 mg/ml), dithiothreitol (10mM), dextran sulfate (10%, w/v), 2XSSC. The hybridization was carried out for 6 h at 50 °C. Sections were washed in 50% (v/v) formamide, 2XSSC at 52 °C, followed by a wash in 2XSSC at 52 °C. Sections were finally treated with ribonuclease (100 µg/ml) for 30 min at 37 °C and dehydrated in graded ethanol solutions. Slides were dipped in K5 autoradiographic emulsion (Ilford, diluted 1/2) and exposed for 1 to 2 weeks. They were developed in D19 (Kodak) and fixed in sodium thiosulfate (30%, w/v). Some slides were counterstained with Congo red and examined under crossed polarization filters. Others sections were exposed in contact with a X-ray film (Hyperfilm-βmax, Amersham).

Immunocytochemistry

Tissue sections were immunolabelled with a mouse monoclonal antibody to α-tubulin (Sigma, clone DM1A) using the peroxidase-antiperoxidase method. After dewaxing and rehydratation, tissue sections were treated with H_2O_2 to inhibit endogenous peroxidase and incubated with the blocking solution (20% normal goat serum in TBS: 0.01 M Tris, 0.15 M NaCl, pH 7.4). They were then incubated overnight with the α-tubulin antibody diluted 1/1000. The following day, the sections were incubated with a goat anti-mouse antibody (Nordic) followed by a mouse PAP complex (Nordic). The peroxidase activity was revealed using diaminobenzidine as chromogen. After labelling, tissue sections were counterstained with Congo red and examined under crossed polarization filters to demonstrate neurofibrillary tangles.

Acknowledgements

We thank Drs. Ginzburg and Littauer (Weizmann Institute, Israel) for providing us with the tubulin cDNA and Mrs. A. M. Couck and Mr. J. L. Conreur for valuable technical assistance. This study was supported by grants from the Belgian FRSM (3.4504.91), the "Fonds de Recherche Divry", the "Foundation M. T. de Lava" and the European Neuroscience Program.

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