In vivo metabolism of mammalian neurofilament polypeptides in developing and adult rat brain

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Neurofilaments *Monoclonal antibodies* *Immunoblotting* *Development* *Proteolytic degradation*

1. INTRODUCTION

A calcium protease which degrades neurofilaments was first observed in the fan worm *Myxicola infundibulum* and in the squid *Loligo forbesi* [1] and subsequently in a mammalian system [2,3]. Many of the polypeptides in *Myxicola* axoplasm are degradation products of the 160 000 and 150 000 M_r polypeptides as has been shown by careful peptide mapping. Moreover, there is a high probability that they result from in vivo processes since axoplasm can be extruded and solubilized in SDS in 10 s. A two hour incubation of axoplasm in the absence of calcium chelators does not alter the gel pattern [4].

In this paper we have used a monoclonal antibody which binds to the 200 000 M_r neurofilament polypeptide to investigate possible in vivo processing of mammalian neurofilaments.

2. MATERIALS AND METHODS

2.1. *Preparation of rat nervous system samples*

Rats were killed by stunning and/or decapitation and samples of cerebral cortex, cerebellum, brain stem and spinal cord were made into 10% w/v homogenates in 5% w/v SDS, 0.125 M Tris (pH 6.8), 5 mM EGTA, 5 mM EDTA, 1 mM PMSF, 1 mM PCMB within 5 min, to minimise post mortem autolysis. With sciatic nerve, desheathing followed by a 40 s sonication at 150 watts in a Braunsonic 1510 sonicator was needed to solubilize the preparation.

2.2. *Electrophoresis*

Samples were run on 10% w/v polyacrylamide gels according to the method of Laemmli [5,6]. Alternatively, neurofilaments were irreversibly reduced with NEM before electrophoresis. 1 mM DTT was added to neurofilaments in 5% w/v SDS and 0.185 M Tris (pH 8.1). Nitrogen was bubbled through the solution before the addition of 10 mM NEM. After 1 h at room temperature, 2-mercaptoethanol was added to a final concentration of 50 mM to block the excess NEM and samples were reboiled before application to gels. The proteins were blotted on nitrocellulose using the method of Towbin et al. [7] with modifications. Nitrocellulose paper was obtained from Schleicher and Schuell. Protein was transferred over-night using a voltage gradient of 5 V/cm. 3% w/v haemoglobin was used in place of bovine serum albumin to saturate protein binding sites. ^{125}I-labelled rabbit anti-mouse immunoglobulin was used as a second antibody.

2.3. *Monoclonal antibody RT97*

This was prepared according to Wood and Anderton [8] and labels only neuronal cells on frozen sections of rat cerebellum.

3. RESULTS

The evidence that RT97 is a neurofilament-specific antibody is 3-fold. Firstly, only fibrillar stain-

Abbreviations: SDS, sodium dodecyl sulphate; PMSF, phenylmethyl sulphonyl fluoride; PCMB, *p*-chloromercuribenzoic acid; NEM, *N*-ethylmaleimide; DTT, dithiothreitol.

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ing of neuronal cells is observed on frozen cerebellar sections as reported previously [8]. Secondly, on the immunoblot of Triton-insoluble neurofilament-enriched material from rat brain, RT97 labels predominantly the 200 K and 145 K neurofilament polypeptides, although other minor bands are visible (figs.1 and 2). Because RT97 is a monoclonal antibody, the assumption has been made that these other bands and the additional bands seen in whole rat brain proteins are neurofilament related polypeptides. Thirdly, on immunoblots of 10% w/v homogenates of whole rat liver, kidney and muscle, prepared using the same conditions as those shown in figs.1 and 2, a band at 200 K is clearly visible although much fainter than in brain homogenates when the autoradiograph is exposed for the same time; the area of diffuse staining containing the other bands is absent. This is the labelling pattern to be expected due to the innervation of these tissues and here there is no evidence that RT97 binds to any polypeptides unrelated to neurofilaments in these tissues (data not shown). The minor bands assumed to be degradation products of neurofilaments and seen in the immunoblots of neural tissues are not seen in liver, kidney, etc., because the level of neurofilaments due to innervation is low. If these additional bands in brain preparations were not related to neurofilaments, then they might be expected to be also present in non-neural tissues in easily observable amounts.

3.1. *Neurofilament polypeptides during development*

Figure 1a shows a Coomassie blue stained gel of

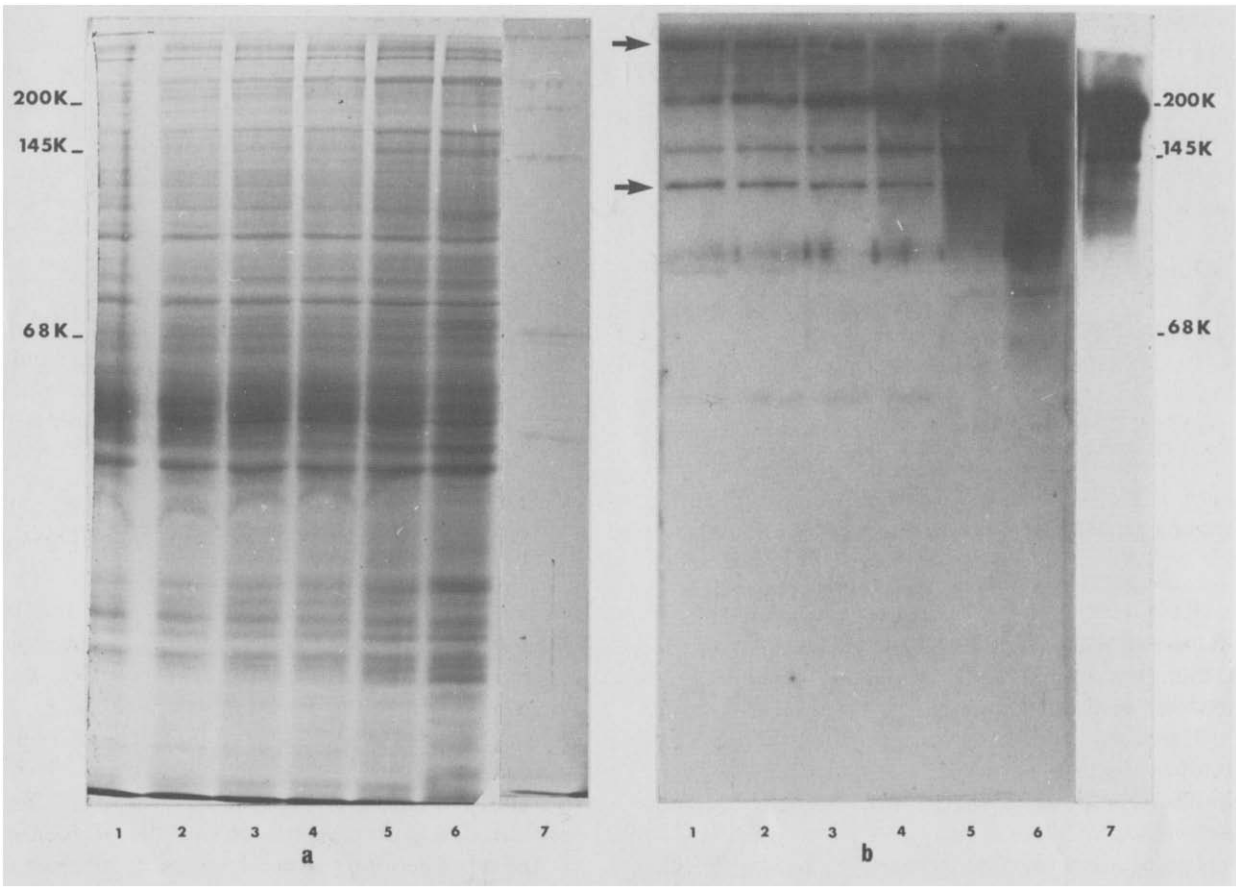

Fig.1. A Coomassie blue stained gel (a) and an immunoblot with RT97 antibody (b) of an homogenate of whole rat brain 0,2,4,8,16 days after birth and then from an adult animal (tracks 1–6) followed by Triton X-100-insoluble material from adult rat brain (track 7).

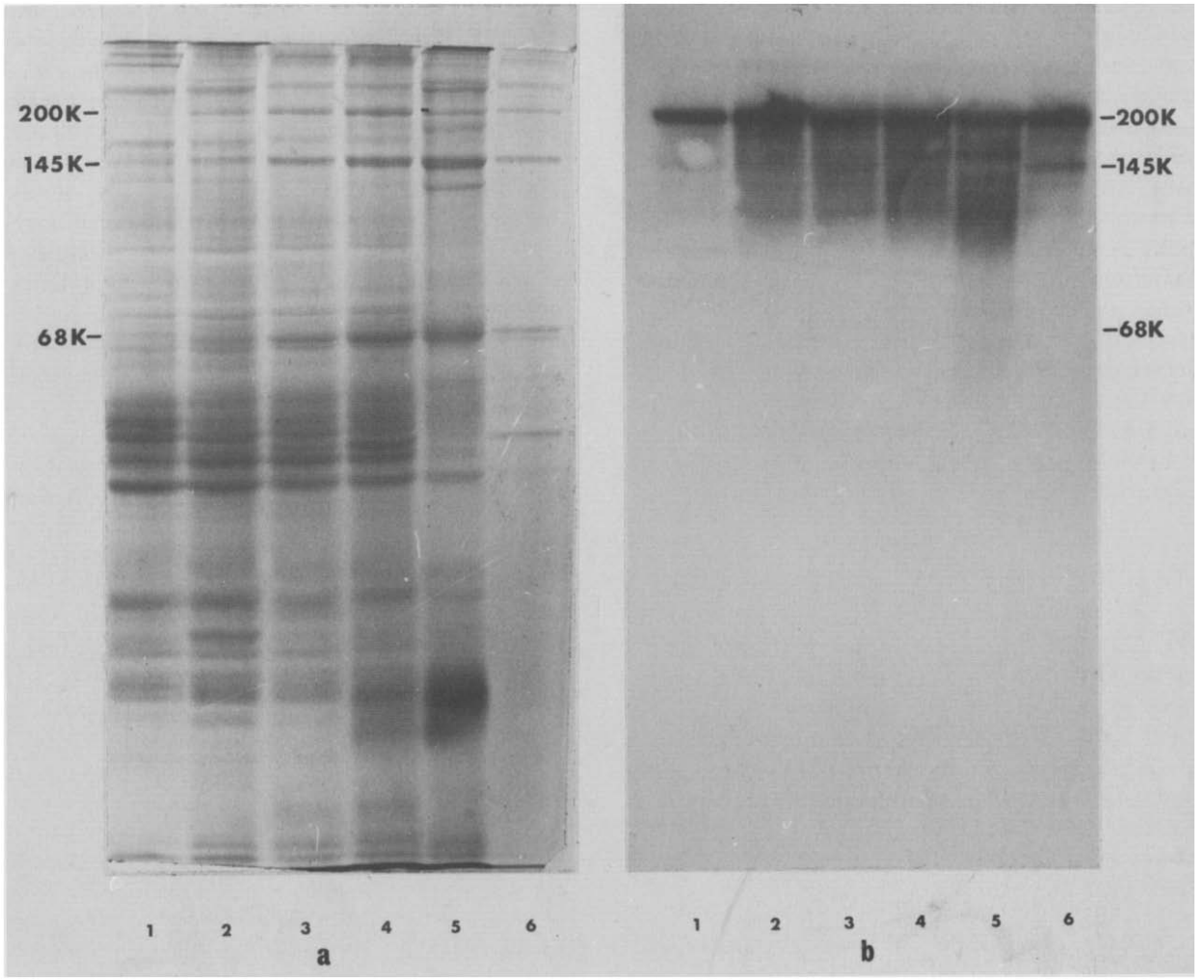

Fig.2. A Coomassie blue stained gel (a) and an immunoblot with RT97 antibody (b) of an homogenate of adult rat cerebral cortex, cerebellum, brain stem, spinal cord an sciatic nerve respectively (tracks 1–5) followed by Triton X-100-insoluble material from adult rat brain (track 6).

whole rat brain at different stages after birth, followed by a sample of Triton X-100 insoluble matter from adult rat brain (track 7).

Figure 1b shows an immunoblot of the same samples, from a gel run under identical conditions, incubated with RT97 and then radiolabelled rabbit anti-mouse IgG.

The binding of RT97 to the 200 K and 145 K polypeptides increases steadily during development unlike the binding to the two prominent polypeptides of M_r greater than 200 K and less than 145 K respectively (see arrows on fig.1), which decrease relative to the 200 K during development. The absolute amount of the high M_r polypeptide also appears to decrease with age. A large number of neurofilament derived polypeptides, mostly with M_r-values spanning from 200 K to about 80 K become apparent in adult brain. The same binding pattern occurs in samples of neonatal and adult rat brain which have been pretreated with NEM.

3.2. *Different areas of the adult rat nervous system*

Figure 2a shows a Coomassie blue stained gel of

homogenates from different parts of the rat nervous system. Figure 2b shows an immunoblot of the same samples from a gel run under identical conditions incubated with RT97 and then radiolabelled rabbit anti-mouse IgG. A rat brain homogenate made from an animal which had been left after sacrifice at room temperature for 2 h before removal of the brain did not show further degradation (data not shown), suggesting that the observed staining pattern is due to in vivo processing of the polypeptides rather than a postmortem artefact. However, the possibility of a biphasic proteolytic action cannot be excluded. The similar pattern of degradation observed in all the adult samples suggests that the mechanism of breakdown is similar in different parts of the nervous system.

4. DISCUSSION

A monoclonal antibody against a neurofilament polypeptide shows similar binding patterns to Western blots of polypeptides from different parts of the adult rat nervous system and different patterns to rat brain at different stages of development. Parts of the nervous system used to make gel samples were homogenized and boiled in SDS quickly enough for in vitro degradation to be an unlikely source of the binding patterns observed. Moreover the clear band of higher M_r than the 200 000 M_r polypeptide observed during development could not have been produced in this way.

For the moment, it cannot be excluded that binding to bands other than the 200 000 and 145 000 M_r polypeptides arise from capricious cross-reactions of the monoclonal antibody with other polypeptides, otherwise unrelated to neurofilaments. However, the absence of any labelling apart from a very faint band of M_r 200 000 in immunoblots of homogenates of muscle, liver and kidney make this an unlikely explanation of the results.

We suggest therefore that the multiple additional bands below the 200 K protein which appear during development are the result of physiological processing of neurofilament polypeptides. This might occur by the known neurofilament calcium-activated protease [9] and represent the normal pattern of neurofilament turnover when these apparently insoluble fibrous proteins reach the nerve terminals, as has been suggested previously [10]. As maturation of the brain proceeds and more neuronal connections are established, neurofilament catabolism might therefore progressively increase. In individual processes, neurofilament breakdown might be absent or minimal during neurite extension but necessary once elongation has ceased.

The pattern of polypeptides binding to RT97 in neonatal and adult rat brain is retained in samples which have been pretreated with NEM. Alkylation of all protein thiols by NEM has not been proven here although standard conditions were employed which are usually assumed to give complete blocking, thus sulphydryl group oxidation remains a possible but unlikely explanation for the presence of, for instance, the high-M_r protein in neonatal brain. Assuming that this protein is not an artefact, its presence suggests that neurofilaments are metabolised in a manner not previously detected. Two possible explanations for the observations are considered here. Firstly, this may be an example of expression of an alternative foetal neurofilament gene which is later switched off as the 200 K and 145 K genes are turned on. Secondly, it is possible that the 200 K or 145 K neurofilament proteins are produced by posttranslational processing from a larger precursor protein. Synthesis in a cell free system indicates that this is not the case in adult neural tissue [11] although purified messenger RNA was not available for these experiments. Furthermore in fig.1 there is a decrease in this high-M_r species relative to the two neurofilament polypeptides so that the mechanism of neurofilament biosynthesis could change during development.

We are now examining more extensively the relationship between the established neurofilament proteins and the additional cross-reactive polypeptides described here.

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