# 14-3-2 PROTEIN IN RAT BRAIN SYNAPTOSOMES: AN IMMUNOCHEMICAL STUDY

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## 1. Introduction

14-3-2 Protein is an acidic protein of monomeric molecular weight about 46 000 ± 2000 dalton, purified from beef brain [1]. Studies have been carried out on its specificity to the nervous tissue and have demonstrated that this protein is brain specific and present in all mammalian brains studied [2]. Further studies have been carried out on the localization within the nervous system, of 14-3-2 protein. It was concluded that it is primarily neuronal [3], as confirmed later by studies on the expression of this protein in neuroblastoma cells grown in culture [4].

A recent study [5] on the proteins pattern obtained from five subcellular fractions of the mouse brain substantiate a previous hypothesis of Packman et al. [6], on rabbit brain subfractions, suggesting 14-3-2 protein as a major protein component among soluble proteins of synaptosomes.

The present report intends to answer with quantitative data this hypothesis indicating that 14-3-2 protein is highly concentrated among buffer soluble proteins of synaptosomes prepared from rat brains.

## 2. Experimental

Bovine 14-3-2 protein was prepared according to Grasso et al. [1], by an adaptation for this protein of the general purification scheme of acidic brain-specific proteins described by Moore and McGregor [7].

Antisera (Ra 35 B6) to beef brain 14-3-2 protein were obtained from Dr. B.W. Moore [3].

For complement fixation assay we used the modification of Moore and Perez [8] of the method described by Wasserman and Levine [9]. Microcomplement fixation reactions were performed in a final volume of 0.61 ml, using a 1:500 dilution of antisera to beef brain 14-3-2 protein.

For the preparation of synaptosomes from brains of young male Wistar rats we followed essentially the procedure of Cotman and Matthews [10], using a sucrose–Ficoll (Pharmacia, Uppsala Sweden) gradient. The synaptosomal layer was freed from Ficoll by resuspending it several times in 0.32 M sucrose followed by centrifugation. After the third wash the synaptosomal pellet was resuspended in 5 mM Tris buffer (pH 8.1) and the soluble proteins extracted by overnight dialysis at 0–4°C against the same buffer. The insoluble material was pelleted by centrifugation and re-extracted with the same buffer containing 5 mM EDTA [11].

Synaptosomal fractions were prepared for electron microscopy by fixation with 3% glutaraldeyde in phosphate buffer pH 7.2 containing 1.75% sucrose. Samples were post-fixed in 2% osmic acid, dehydrated and embedded in Epon resin. Sections were stained, after being cut on a Sorvall MT2, with uranyl acetate and lead citrate and examined in a Philips 300 electron microscope.

Proteins were measured by the method of Lowry [12], after precipitation with 5% TCA in the cold (0.4°C).

## 3. Results

As experienced by Cotman and Matthews [11], there are some variations in different Ficoll lots, therefore we checked the results of our preparations



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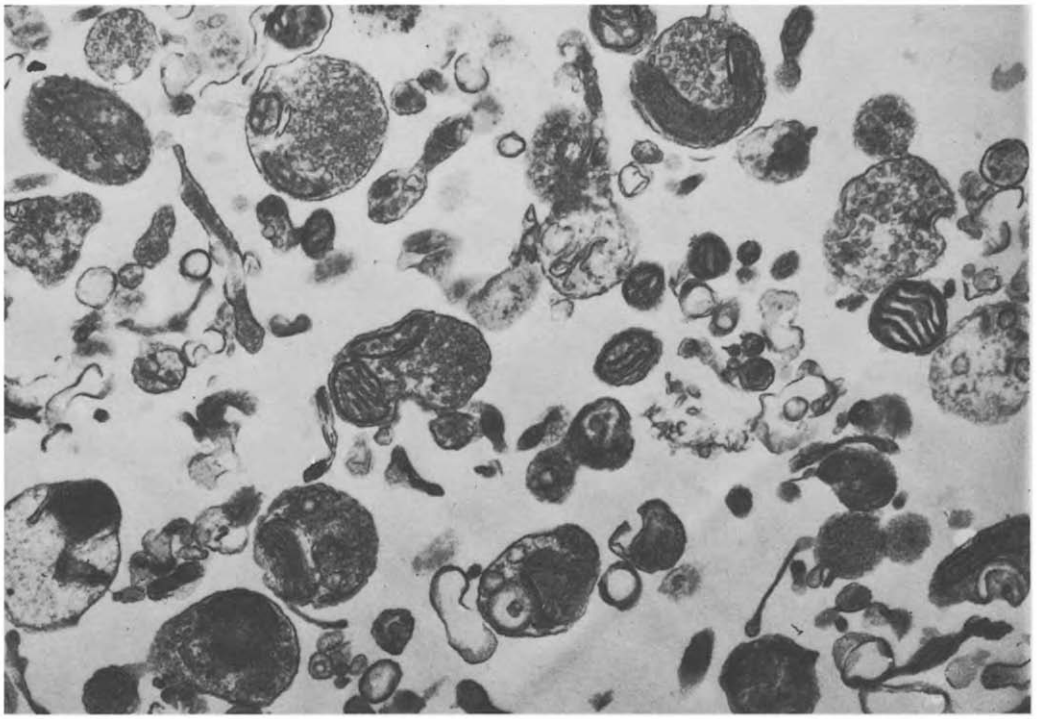

Fig. 1. Electron micrograph (70 000 ×) of synaptosome fraction from 7.5–13% band of Ficoll–sucrose gradient. Several synaptosomes are seen.

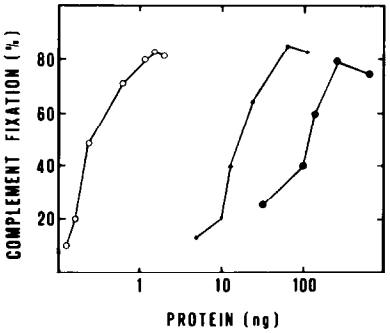


Fig. 2. Microcomplement fixation reaction between antiserum to beef-brain 14-3-2 protein and buffer extracts of rat synaptosomes (*), total brain (•) and purified beef-brain 14-3-2 protein (○). A dilution of antiserum 1:500 was used. In the experiment reported here the amount (0.4 ng) of reacting beef-brain 14-3-2 protein required to give 50% fixation is assumed to be present as beef-brain equivalents, in the amount of cross-reacting rat-brain antigen giving 50% fixation. (30 ng and 150 ng).

at the electron microscope. As shown in fig. 1 the population of synaptosomes we obtained is relatively pure and the estimated purity falls within the limits set by these authors for the method, that is more than 70% of the subcellular structure are synaptosomes.

A complement fixation reaction between extracts of rat brain synaptosomes and antisera to beef-brain 14-3-2 protein is demonstrated (fig. 2). The values given for proteins extracted from synaptosomes have to be intended not in absolute, but relative terms, because of the serological cross-reaction between antiserum to beef brain 14-3-2 protein and the heterologous antigen present in the extract from rat brain.

With this consideration in mind quantitative data on 14-3-2 protein distribution in different subcellular fractions is given in table 1. We considered it significant to our working hypothesis to measure 14-3-2 protein in the following fractions: a) the supernatant fraction from which the mitochoncrial fraction, con-

Table 1
Content of 14-3-2 protein relative to the content of soluble protein in various subfractions of rat brain.

| Fraction | μg of 14-3-2 Protein*/ mg of soluble proteins |
|---|---|
| a) Rat brain cytoplasm | 4.09 ± 2.23 |
| b) Synaptosomes cytoplasm | 17.46 ± 2.32 |
| c) EDTA extractable proteins from synaptic plasma membranes | 2.05** |

The values for 14-3-2 protein represent means (± S.E.M.) for 5 assays.
* In terms of beef-brain equivalents.
** This value is based on 3 assays.

taining synaptosomes, has been removed; b) the supernatant resulting from Tris buffer extraction of synaptosomes; c) the supernatant resulting from a second extraction of the synaptosomal plasma membranes in presence of EDTA. The results reported in table 1 clearly indicate that 14-3-2 protein is present, in synaptosomes, in higher concentrations than in any of the fractions considered. In fact the values (4 μg/mg protein) obtained for soluble proteins are similar to those established for 14-3-2 protein in extracts of brain cytoplasm (4–6 μg/mg) [3]. On the contrary 14-3-2 protein measurable in extracts of synaptosomal cytoplasm (17 μg/mg protein) is more than four times higher than that of total brain extract. We think that to this value should be added the data for EDTA extractable proteins (2 μg/mg protein) since we think it may reflect trapped 14-3-2 protein more than membrane bound one.

## 4. Discussion

Among others, two groups have reported electrophoretic studies on the soluble proteins composition of subcellular fractions of the brain [5, 6]. Both Packman et al. [6]; describing the electrophoretic pattern of proteins extracted from subcellular fractions of rabbit brain and Waehneldt et al. [5], studying those of the mouse, suggested the presence in synaptosomal extract of 14-3-2 protein in relatively high concentrations. Furthermore, it may well also be that one of the bands (probably band no. 2) present in quantities higher in the nerve endings than in the cell bodies of rat brain, as described by McBride and VanTassel [13], is 14-3-2 protein. However bands with identical mobility in polyacrylamide gel do not necessarily belong to identical proteins. Therefore the analyses, by microcomplement fixation assay of the specific neuronal marker, 14-3-2 protein, confirm and also give quantitative answers to the above mentioned hypothesis.

Whether the difference in 14-3-2 protein concentration between synaptosomal buffer extracts and total brain extract is real, one must be in doubt. 14-3-2 Protein is a neurinal component, therefore its concentration is expected to be higher in subcellular structure which should come from neurons. However by comparison of our data and data obtained measuring 14-3-2 protein in discrete areas of human brain [2], or in neurons dissected from freeze-dried sections using Lowry's methods [2], the hypothesis of 14-3-2 protein preferentially localized in neurons, still stands. The protein is synthesized in the perykarion [14], transported to the nerve endings and there eventually accumulated.

## References

[1] Grasso, A., Cicero, T. and Moore, B.W. (1969) Abstr. 2nd Int. Meeting Inter. Soc. Neurochem., pp. 201–202, Milan.
[2] Moore, B.W. (1972) Intern. Rev. Neurobiol. 15, 215–225.
[3] Cicero, T.J., Cowan, W.M., Moore, B.W. and Suntzeff, V. (1970) Brain Res. 18, 25–34.
[4] Augusti-Tocco, G., Casola, L. and Grasso, A. (1973) Cell Differentiation 2, 157–161.
[5] Waehneldt, T.V., Ralston, H.J. and Shooter, E.M. (1973) Neurobiology 3, 215–224.
[6] Packman, P.M., Blomstrand, C. and Hamberger, A. (1971) J. Neurochem. 18, 1–9.
[7] Moore, B.W. and McGregor, D. (1965) J. Biol. Chem. 240, 1647–1653.
[8] Moore, B.W. and Perez, V.J. (1966) J. Immunol. 96, 1000–1005.
[9] Wasserman, E. and Levine, L. (1960) J. Immunol. 87, 290–295.
[10] Cotman, C.W. and Matthews, D.A. (1971) Biochim. Biophys. Acta 249, 380–394.
[11] Reynolds, J.A., and Trayer, H. (1971) J. Biol. Chem. 246, 7337–7342.
[12] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 266–275.
[13] McBride, M.J. and VanTassel, J. (1972) Brain Res. 44, 177–187.
[14] Zomzely-Neurath, C., York, C. and Moore, B.W. (1973) Arch. Biochem. Biophys. 155, 58–69.