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Pelizaeus-Merzbacher Disease: Biochemical Analysis of Isolated Myelin (Electron-Microscopy; Protein, Lipid and Unsubstituted Fatty Acids Analysis)

J.M. Bourre, C. Jacque, J. Nguyen-Legros, J.H. Bornhofen, C.A. Araoz, O. Daudu and N.A. Baumann

Laboratoire de Neurochimie, Inserm U 134, Hôpital de la Salpétrière, Paris and Departments of Pediatrics and Neurology, University of Arkansas Medical Science Campus, Little Rock, Ark.

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Abstract. Analysis of myelin from a leukodystrophic brain was performed (Pelizaeus-Merzbacher disease, classical type). Myelin recovery was 7% of normal, when isolated by ultracentrifugation. Electron microscopy showed a great amount of loose lamellae, with less thick sheaths and periodicity close to normal. This myelin contains fewer lipids than normal, sphingolipids and plasmalogens being reduced. Fatty acids from phospholipids are essentially normal, however enols from plasmalogens are largely reduced. Purified sphingolipids (cerebrosides, sulfatides and sphingomyelin) present a considerable diminution in very long chain fatty acids; the ratio of very long chain fatty acids (over C18) on shorter chains is 1% of the normal value for saturated fatty acids and 2% for the monounsaturated homologues. Protein analysis showed that basic protein and proteolipids were reduced, Wolfgram proteins being relatively increased.

Pelizaeus (1885) and *Merzbacher* (1910) described a dysmyelinating disease of 1 family. A number of identical or similar cases have been reported all over the world and classified under the same heading as the original case because of their clinical and neuropathological resemblance to this disease. However a distinction was proposed (*Seitelberger*, 1970) between 'congenital Seitelberger type' (*Seitelberger*, 1954), 'classical Pelizaeus-Merzbacher type' and 'adult Lowenberg-Hill type' (*Lowenberg and Hill*, 1933). 15 clinical reports (61 diseased) have been performed on the classical type (*Bourre et al.*, 1977b). Chemical investigations have been performed on 2 cases, but the brains were formalin fixed. *Norman and Tingey* (1963) and *Allegranza et al.* (1968) showed a reduction of total lipids including cholesterol, cerebrosides and sulfatides. *Watanabe et al.* (1973) reported two cases related to Pelizaeus-Merzbacher disease showing normal content in sphingolipids or only a 50% decrease (*Watanabe et al.*, 1969). We have performed a neurochemical analysis in whole brain on a case of the classical type (*Bourre et al.*, 1978) showing that a lesser amount of solid material was

found, lyophilized weight being 76% of the normal value; the amount of lipid was even more decreased. Among lipids, plasmalogens were slightly diminished; cerebrosides and sulfatides were drastically reduced (8% of the normal value in whole brain), sphingomyelin being less affected. As very long chain fatty acids (in total brain) are largely diminished, one aspect of this disease seems to be a defect in the synthesis of myelin very long chain fatty acids as these acids are reduced by 99.2%.

The purpose of this work was to analyze myelin in this case of leukodystrophy.

Materials and Methods

Case Report

This case, Angela Sp., had a brother and both children were affected in the same way. Clinical and anatomopathological investigations have been reported elsewhere (*Bornhoffen et al.*, 1978).

Materials and Myelin Preparation

The biochemical studies were carried out on fresh autopsy material that was kept frozen at – 60 °C for 18 months. The frontal lobe was analyzed. The control brain was a normal 11-year-old girl; her brain was kept frozen at – 60 °C for 10 months. The specimens were kept at – 30 °C for 24 h and 6 h at 4 °C. Myelin was isolated according to *Norton and Poduslo* (1973) with slight modification for the diseased brain: three layers from the first gradient were pooled to increase the yield of the preparation. The myelin was eventually lyophilized after suspension in water and weighed.

Electron Microscopy

For electron microscopical observations, the pellets of both normal and Pelizaeus-Merzbacher myelin were fixed for 1 h in 3.5% glutaraldehyde in 0.1 *M* Millonig buffer containing glucose (pH = 7.4). The pellets were then cut into small blocks and rinsed for the night in Millonig buffer. The next day, the blocks were post-fixed for 1 h in 2% buffered osmic acid, dehydrated in ethyl-alcohol and propylene oxide and embedded in epon.

The ultra-thin sections were stained with uranyl acetate and lead citrate and observed through an Hitachi HU-11 A electron microscope.

Lipid Analysis

The technique used for lipid extraction as well as the methods used for quantitative lipid analysis have been described previously (*Baumann et al.*, 1968; *Jacque et al.*, 1969). Briefly, myelin was homogenized in chloroform-methanol (2:1 v/v); total lipid extract was chromatographed using chloroform-methanol-water (70:30:4, by vol). The chromatograms were sprayed with bichromate. They were also viewed under ultraviolet light (245 nm) or stained with molybdic reagent for phospholipids, α-naphthol for glycolipids, 2–4 dinitrophenyl hydrazine for plasmalogens, antimony trichloride for sterols. The proportion of the galacto and glyco moiety in cerebrosides was determined by the method of gas liquid chromatography after estimation on thin-layer chromatogram impregnated by borate.

For quantification, separation of lipids was performed on TLC glass plates (10 × 10 cm) coated with silica gel 60 F 254 Merck. About 500 μg of lipid extract were spotted at 2 cm from the bottom and the left of the plate (*Pollet et al.*, 1978; *Bourre et al.* 1977b). The solvents used in succession were the following: (1) chloroform-methanol-water 70:30:4 (v/v/v) migrating from the bottom to the top of the plate; (2) at right angle to the first, chloroform-methanol 2:8 (v/v) on 2/3 of the plate; (3) in the same direction, chloroform-methanol 2:1 (V/V); (4) finally, chloroform-methanol 2:1 (v/v) in the same direction as that from the bottom to the top of the plate.

Lipids were detected by iodine vapors. After complete sublimation of the iodine, the areas of individual lipids were scraped off and submitted to quantification. So as to eliminate interferences, each assay has been performed with a constant amount of lipid to which was added increasing amounts of the standard substance to be determined. The slope of the curve obtained is compared to one consisting of the standard scale only. This method is called 'standard additions method'. Most of the methods used for cholesterol, galactolipid, phosphorus, NANA determination are classical (*Pollet et al.*, 1978).

Isolation of Lipids and Fatty Acids Analysis

A large part of the lipid extract was used to isolate cerebrosides, sulfatides and sphingomyelin. Phos-

pholipids were eliminated by selective methanolysis (*Hajra and Radin*, 1963) slightly modified: lipids dissolved in chloroform-methanol (2:1) (200 mg) were evaporated to dryness and dissolved in 2.5 ml chloroform and 1 ml of methanolic sodium hydroxide 0.5 *N*. Methanolysis was allowed to proceed for 30 min at 37 °C with mechanical stirring; the mixture was acidified to pH 1 with concentrated HCl and left for 2 h at room temperature to obtain complete methanolysis of the plasmalogen fraction of phosphatides. The methanolysate was washed three times by 0.2 vol of Folch upper phase. The lower phase was checked for the absence of phospholipids by thin-layer chromatography and viewing by molybdic reagent. Each individual sphingolipid was then purified by a column chromatography. This procedure consisted of 4 g of unisil (100–200 mesh) in chloroform. The column was washed 2 times by chloroform before applying the lipids dissolved in this solvent. 75 ml chloroform-eluted cholesterol and fatty acids, 125 ml chloroform-methanol 95-5 (v/v) eluted cerebrosides, 138 ml of chloroform-methanol 80/20 eluted sulfatides, 75 ml of methanol-eluted sphingomyelin. The purity of each fraction was checked by thin-layer chromatography sprayed by α-naphthol and molybdic reagent. No cross-contamination was detected. Each lipid fraction was methylated with 14% boron trifluoride in methanol. A preparative thin-layer chromatography separated unsubstituted fatty acids methyl esters from the other fatty acids (solvent: 20% ether in petroleum ether b.p. 45–60 °C).

Fatty acid analysis was performed by gas liquid chromatography on 3% SE 30 column (on chromosorb WAW 100/120) with temperature programming at 2 °C/min between 155–288 °C. Fatty acids were identified by retention time and equivalent chain lengths and cochromatography with commercial standards.

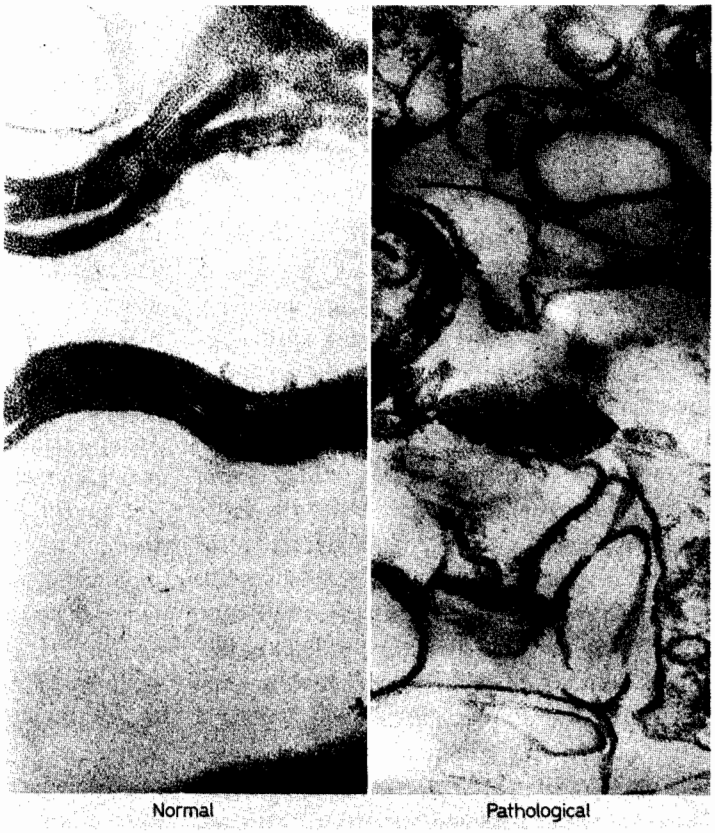

Fig. 1. Aspect of isolated myelin under electron microscopy.

Protein Analysis

Polyacrylamide gel electrophoresis was run for qualitative analysis of proteins. The freeze-dried pellets of myelin were solubilized in 1 *M* acetic acid (pH 2.4) gels containing 8 *M* urea. Electrophoresis in 7.5% acrylamide (W/V) was carried out for 2 h at 2.5 mA per gel. The electrolyte was 1 *M* acetic acid in both tanks. After staining with amido black 0.1% (W/V) in 7% acetic acid, the gels were destained in 10% acetic acid.

Results

Electron Microscopy of Isolated Normal and Leukodystrophic Myelin (fig. 1)

Myelin from this case presents a dissociated aspect when comparing with normal myelin prepared under the same conditions. Myelin sheaths are less thick, and leaflets frequently separated by some apparently wide volumes. When measurable the periodicity is close to normal.

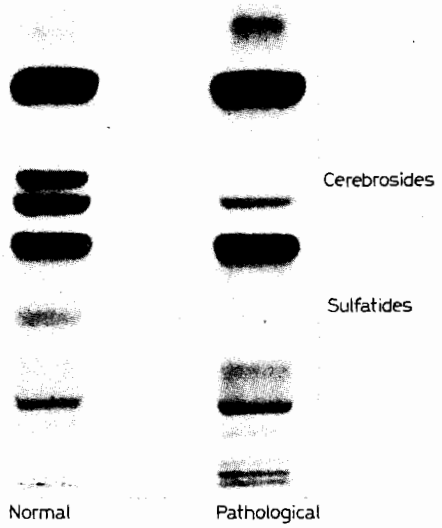

Fig. 2. Thin-layer chromatography of myelin lipids. Precoated TLC plates silica gel 60 F 254 from Merck. Solvent: chloroform-methanol-water (70:30:4, v/v/v), 0.600 mg lipid extract was spotted. Sprayed with bichromate reagent.

Thin-Layer Chromatography of Myelin Lipids and Protein Profile

Qualitative abnormalities of cerebrosides, sulfatides and sphingomyelin are detected in leukodystrophic material (fig. 2). Normally cerebrosides give two spots, the upper corresponds to the kerasin type (with non-hydroxylated fatty acids) the lower one to the phrenosin type (with hydroxylated fatty acids). In the leukodystrophic material, the slower moving spot is largely reduced and the faster spot has almost completely disappeared. Sulfatides are nearly undetectable. These results are confirmed when spraying with α-naphthol; when using molybdic reagent or antimony chloride no abnormality is detected. However when using 2-4-dinitro-phenyl-hydrazine, ethanol amine plasmalogen is reduced.

The normal pattern of myelin proteins shows 2 main bands and numerous faint ones. The most cathodic band is colored in blue green whereas the others are blue; it comigrates with a sample of purified myelin basic protein. The other main component is the Folch proteolipid. The high molecular weight Wolfgram proteolipid appears as a faint band in this system. The protein pattern of the leukodystrophy is strongly modified when compared to normal. The myelin basic protein and the Folch proteolipids are reduced whereas a band in the high molecular weight area (the Wolfgram proteolipid?) is strongly increased.

Quantitative Analysis

The results of quantitative analysis are presented in tables I and II. Myelin recovery is largely reduced in the leukodystrophic brain. Myelin quantity is 7% of the normal value.

Table I. Myelin recovery in normal and leukodystrophic brain

	Normal	Pathological
Lyophilized brain		
Wet brain, mg/g	218	165
Myelin recovery		
Wet brain, mg/g	33	2.5
Lyophilized brain, mg/g	151	15.1
Myelin total lipid extract		
Lyophilized myelin, mg/g	0.75	0.62
Wet brain, mg/g	24.7	1.4
Lyophilized brain, mg/g	113.2	8.4

Values are mean values from 4 experiments.

Table II. Lipid analysis in normal and leukodystrophic myelin

Lipids, mg/100 mg lyophilized myelin	Normal	Pathological
Cholesterol	15.2	12.4
Diacylphosphatides	29.3	36.1
Sph	5.3	9.1
PE	11.6	4.0
PC	7.6	18.9
PS	4.2	1.8
PI	0.6	1.8
PA	0.1	1.9
Plasmalogens	8.6	4.2
Glycolipids	21.6	8.5
Cerebrosides	14.2	3.7
Sulfatides	2.2	0.4
Gangliosides	5.4	4.5
NANA	0.33	0.41
Galactose in total glycolipids, %	97.2	78.5
Glucose in total glycolipids, %	2.4	21.5

Values are mean from at least 3 experiments.
Sph = Sphingomyelin; PE = phosphatidyl ethanolamine; PC = phosphatidyl choline; PS = phosphatidyl serine; PI = phosphatidyl inositol; PA = phosphatidic acid; NANA = N-acetyl-neuraminic acid.

Pathological myelin contains fewer lipids than normal material, and, conversely, more proteins. Thus one of the characteristics of myelin (to contain much more lipids than any other membrane) is lost. If based upon the brain wet weight, myelin lipids are 6% of the normal value. The glycolipid content is reduced approximately 2.5 times in the leukodystrophic myelin; more over this membrane contains abnormally large amounts of glycolipids (20% instead of 3%, galactolipids being conversely 80 and 99%). Lipid analysis (table II) shows that gangliosides are slightly increased (as determined by NANA measurement); cholesterol is close to normal. Phospholipids are slightly increased (mainly at the level of sphingomyelin and inositol phosphatides), but ethanolamine phosphatides and plasmalogens are largely reduced.

Fatty Acid Analysis of Myelin Lipids

Fatty acid profile from phospholipid is essentially normal in the leukodystrophy (including polyunsaturated chains). However dimethylacetals with 16 and 18 carbon atoms are largely reduced (this result corresponds to the reduction of plasmalogens).

In cerebrosides (table III) the fatty acid profile is completely disturbed in the leukodystrophy, with considerable reduction in fatty acids with very long chains: the C_{24} fatty acids are reduced (20 and 17% of the normal value respectively for saturated and monounsaturated chains) when considering the ratio R: fatty acids with chain lengths over 18 carbon atoms to fatty acids with chain lengths below 19, this

Table III. Cerebrosides unsubstituted fatty acids in normal and leukodystrophic myelin

		Chain length								Total %	$R = \frac{\geq C_{19}}{< C_{19}}$
		16	18	20	22	23	24	25	26		
Saturated fatty acids	N%	1.7	4.4	0.9	3.6	3.5	17.5	5.0	3.3	39.9	25.5
	P%	15.5	23.6	2.0	0.7	0.8	3.5	0.8	0.9	48.8	0.24
Monounsaturated fatty acids	N%	–	3.2	1.3	1.2	0.9	38.0	7.8	7.7	60.1	18.6
	P%	–	35.4	6.1	0.5	0.2	6.4	1.5	2.4	52.5	0.32

Mean values from 3 experiments and 15 chromatograms. C_{19} = Fatty acid with 19 carbon atoms.

Table IV. Sulfatides unsubstituted fatty acids in normal and leukodystrophy myelin

		Chain length								Total %	$R = \frac{\geq C_{19}}{< C_{19}}$
		16	18	20	22	23	24	25	26		
Saturated fatty acids	N%	2.3	4.5	0.8	2.4	4.6	15.5	5.2	4.3	39.4	4.8
	P%	13.5	25.1	2.1	0.6	0.7	3.4	0.8	0.8	47.0	0.18
Monounsaturated fatty acids	N%	–	3.6	1.3	1.0	0.8	38.5	7.5	6.9	59.6	15.5
	P%	–	38.1	5.2	0.3	0.2	5.8	1.3	1.6	52.5	0.27

Values are mean from at least 3 experiments. C_{19} = Fatty acid with 19 carbon atoms.

Table V. Sphingomyelin unsubstituted fatty acids in normal and leukodystrophic myelin

		Chain length								Total %	$R = \frac{\geq C_{19}}{< C_{19}}$
		16	18	20	22	23	24	25	26		
Saturated fatty acids	N%	5.6	28.8	1.9	1.4	2.3	8.7	3.1	1.4	53.2	1.4
	P%	13.0	51.0	2.2	–	t	1.0	t	t	67.2	0.05
Monounsaturated fatty acids	N%	–	13.7	1.6	0.5	t	19.8	5.3	4.8	45.5	2.3
	P%	–	26.1	3.7	–	t	2.7	t	t	32.5	0.24

Mean values from 3 experiments and 15 chromatograms. C_{19} = Fatty acid with 19 carbon atoms.

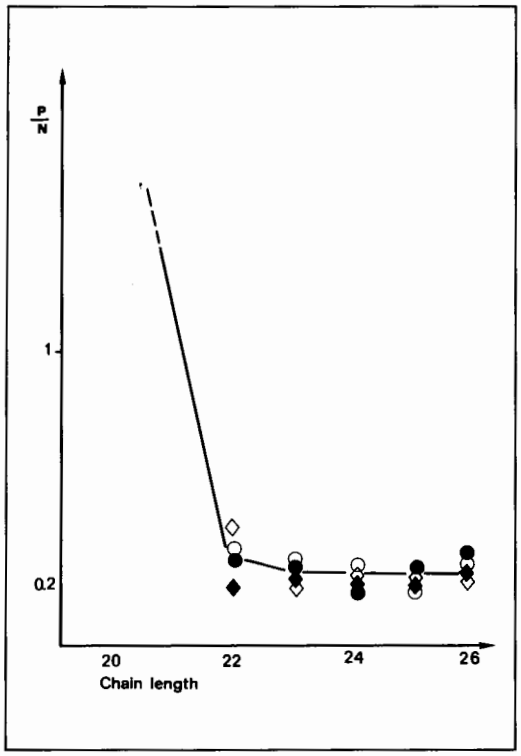

Fig. 3. Ratio R = pathological/normal for each chain length of fatty acids in cerebrosides and sulfatides. • = Saturated cerebrosides; ○ = monounsaturated cerebrosides; ◆ = saturated sulfatides; ◇ = monounsaturated sulfatides.

ratio is 1% of the normal value for saturated fatty acids and about 2% for monounsaturated. In sulfatides (table IV) essentially the same defects are seen, very long chain fatty acids being reduced. For instance 24 carbon atom fatty acids are 20 and 16% of the normal value when respectively saturated and monounsaturated. As for cerebrosides, the ratio R is also drastically reduced. Figure 3 shows the value of the ratio pathological/normal for each chain length, for saturated and monounsaturated fatty acids in cerebrosides and sulfatides. It must be pointed out that this ratio is constant for all chain lengths over 22 carbon atoms in both lipids.

In sphingomyelin (table V) very long chain fatty acids are reduced in the same order of magnitude as in the two other sphingolipids. 24 carbon atoms fatty acids are both approximately 16% of the normal value.

Discussion

We have previously shown that in the total leukodystrophic brain there is a reduction of galactolipids, paralleling the diminution of myelin, with an unexpected drastic reduction in the very long chain fatty acids (*Bourre et al.*, 1978). The present study shows that myelin contains a smaller amount of glycolipids, formed from lower quantities of very long chain fatty acids: even in 'purified' leukodystrophic myelin, the lack of very long chain fatty acids must be pointed out. It is logical that both saturated and monounsaturated fatty acids are reduced, as these fatty acids share a common synthetic pathway (*Bourre et al.*, 1976). Moreover myelin fatty acids are synthesized in endoplasmic reticulum and not in mitochondria (*Bourre et al.*, 1973b, 1977a; *Paturneau-Jouas et al.*, 1976). Among the three microsomal systems, the elongating system synthesizing very long chain fatty acids is affected in a murine leukodystrophy, the Quaking mutant (*Bourre et al.*, 1973a, 1977a) with defective myelination. This system is also probably abnormal in this leukodystrophy which is a Pelizaeus-Merzbacher disease as shown by clinical and anatomopathological investigations. But is it genetically distinct, as this case is under recessive autosomal heredity (this girl had an affected brother), and not sex-linked? It is known that myelination is managed by at least two chromosomes in mice, as

two mutants have been described, Quaking and Jimpy, with hereditary defective myelination due to mutation on autosome and gonosome respectively (*Sidman et al.*, 1964). The main biochemical findings in man and murine mutant are collected in table VI. There is a large analogy between these two dystrophies, when considering different parameters. However, the very long chain fatty acid defect is much more important in man than in mice. In the latter the defect is in the same order of magnitude for fatty acids and specific myelin components: the mutation seems to be a defect in the cell differentiation expressed by a stop in the my-

Table VI. Data on this patient and murine (Quaking) leukodystrophy

	Patient		Mice	
	ratio = $\frac{\text{leukodystrophy}}{\text{normal}}$	reference	$\frac{\text{Quaking}}{\text{normal}}$	reference
Lyophilized brain weight	0.75	*Bourre et al.* (1977b)	0.85	*Baumann et al.* (1968)
Brain total lipid extract	0.65	*Bourre et al.* (1977b)	0.70	*Baumann et al.* (1968)
Galactolipids	0.28	*Bourre et al.* (1977b)	0.54	*Baumann et al.* (1968)
Cholesterol	1.0	*Bourre et al.* (1977b)	0.91	*Baumann et al.* (1968)
Phospholipids	1.3	*Bourre et al.* (1977b)	1.2	*Baumann et al.* (1968)
$C_{24} + C_{24:1}$ in sphingolipids				
cerebrosides	0.1	*Bourre et al.* (1977b)	0.22	*Jacque et al.* (1969)
Sulfatides	0.1	*Bourre et al.* (1977b)	0.24	*Jacque et al.* (1969)
Sphingomyelin	0.15	*Bourre et al.* (1977b)	0.21	*Jacque et al.* (1969)
Myelin yield	0.08		0.1	*Baumann et al.* (1973)
Myelin lipids				
mg/mg myelin	0.82		0.83	*Baumann et al.* (1973)
mg/brain	0.07		0.08	*Baumann et al.* (1973)
Galactolipids	0.34		0.46	*Baumann et al.* (1973)
$C_{24:0} + C_{24:1}$ in cerebrosides	0.2		0.55–0.46	*Baumann et al.* (1973) *Singh et al.* (1971)
$C_{24:0} + C_{24:1}$ in myelin	*0.07*		0.23	*Baumann et al.* (1973)

elinogenesis. In man the drastic reduction in the very long chain fatty acids is much more important than the reduction of any other component (and the decrease in sphingolipids is possibly due to the lack of substrate, i.e. fatty acids). One aspect of this case of leukodystrophy is possibly a defect in the biosynthesis of very long chain fatty acids. Moreover, isolated myelin in man is more abnormal than in the Quaking: this is possibly due to the more important lack in very long chain fatty acids, as it is now well known that these acids are fundamental in myelin structure and stability.

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Dr. Jean-Marie Bourre, Laboratoire de Neurochimie, Inserm U 134, Hôpital de la Salpétrière, F–75634 Paris Cédex 13 (France)