Effects of vigabatrin and of GABA on myelinated rat cerebellar cultures: preliminary data

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1 The aim of this study was to evaluate the effect of high concentrations of vigabatrin (γ -vinyl GABA) and of GABA on myelin of the central nervous system cultures.

2 Explants of rat cerebellum were cultured for 14–19 days *in vitro* on collagen-coated coverslips in Leighton tubes. They were exposed for up to 14 days to 500 nmol ml⁻¹ vigabatrin or to 1000 nmol ml⁻¹ GABA.

3 Qualitative and quantitative blind examination of living cultures and of Sudan black B-stained slides showed mild toxicity of both drugs for myelinated fibres. No clear-cut differences could be demonstrated between the two compounds, although vigabatrin seemed slightly more toxic than GABA at these doses.

4 In electron microscopy, no patent intramyelinic oedema nor primary demyelination were seen. On the contrary, some degenerating myelinated fibres and astrocytic gliosis were seen in both experimental conditions. The changes involved axons as well as myelin sheaths.

5 The toxicity of GABA and vigabatrin was surprisingly mild in this very sensitive model.

Keywords vigabatrin γ -vinyl GABA toxicology GABA intramyelinic oedema tissue culture myelin

Introduction

The discovery of vacuoles in the white matter of some animals under chronic intoxication with high doses of vigabatrin (γ -vinyl GABA) raised many debates about the mechanism of these changes. They predominate in some species (rats, dogs and monkeys in decreasing order of severity as far as the best studied species are concerned) and in some specific areas of the central nervous system, depending on the species, for example the cerebellum in rats. They have never been seen in human specimens (Pedersen *et al.*, 1987; Trotter *et al.*, 1977; Hauw *et al.*, 1988). They appear mainly related to intra-myelinic oedema (John *et al.*, 1987; Hauw *et al.*, 1988). They could be due to the rise of GABA concentration in the central nervous system induced by the structural analogue of

GABA, which acts as an irreversible inhibitor of GABA transaminase. They would thus be linked to the pharmacological effect of the drug which is related, in all likelihood, to elevation of the levels of GABA and GABA metabolites in the central nervous system: free GABA, conjugated GABA, or homocarnosine, a dipeptide of GABA histidine (Palfreyman *et al.*, 1983). Another hypothesis would be that intra-myelinic oedema might be secondary to a side effect of the inhibition of GABA transaminase. For example, since the omega-amino acids β -alanine and β -aminoisobutyric are also substrates for GABA transaminase, inhibition of the enzyme should increase their concentration—and has indeed been reported to do so (Grove *et al.*, 1981). Similarities between the brain histo-

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pathology of congenital hyper- β -alaninemia (which includes spongiform leukodystrophy) and intramyelinic oedema induced by vigabatrin have been emphasized (Brandt & Christensen, 1984). It could also be related to the transamination of enzymes other than GABA transaminase, such as ornithine aminotransferase (John *et al.*, 1987) or to a direct toxic effect of vigabatrin. This is less likely, since microvacuolation is induced by two different inhibitors of GABA transaminase of very different structure, namely vigabatrin and ethanolamine-*O*-sulphate (John *et al.*, 1987). We tried to answer some of these questions by assessing the effects of vigabatrin and of GABA on myelinated central nervous system cultures.

Methods

Tissue cultures

Rat cerebellar cultures were performed on collagen-coated coverslips in Leighton tubes as described for cerebellum of guinea-pig (Hauw *et al.*, 1974), and mice (Hauw *et al.*, 1980) and for rat spinal ganglia (Hauw *et al.*, 1972), with slight modifications. Briefly, rat collagen-coated coverslips were prepared according to Bornstein (1958). Cerebella from newborn Wistar rats, provided by C.E.R.J., were dissected out. Two explants (2×2 mm each) from cerebellar cortex and deep nuclei were set on each coverslip. Nutrient medium (0.25 ml in each tube) consisted of Earle's MEM: 28.06%, Hank's BSS: 36, 70%, glutamine 200 mM: 58% glucose 200 mg ml⁻¹: 3.5%, Hepes: 1.16%, fetal calf serum: 30%. It was changed every 2 or 3 days. Living cultures were examined daily and checked for myelin from the 14th day on in bright field light microscopy, using a $\times 40$ long-working distance objective and an inverted microscope.

Highly myelinated cultures were selected and used for experiments from the 14th to the 19th day *in vitro*.

Solutions of vigabatrin and GABA

Vigabatrin and GABA were generously donated by Merrell International Research Centre, Strasbourg, France. Vigabatrin and GABA were used at concentrations of 500 nmol ml⁻¹ and of 1000 nmol ml⁻¹ respectively. The solutions were added to the nutrient medium at every renewal. Sterility was obtained by filtration on Millipore filters (0.22 μ m). In one experiment, the levels of vigabatrin and of GABA in the supernatant of cultures were measured (Table 1).

Assessment of the effect of vigabatrin and GABA on myelinated cultures

Living cultures were examined daily and checked for myelin in bright field light microscopy. Scores for the density and the quality of myelinated fibres were established. At the end of the experiments, cultures were fixed in buffered 4% formalin and stained with Sudan Black B for the study of myelin in light microscopy, as described (Hauw *et al.*, 1974). Scores of the density and of the quality of myelinated fibres (presence of irregularities of the diameter of the fibres, and/or of ovoids indicating degenerative fibres) were assessed blind by two different examiners. In addition, some cultures were processed for electron microscopy by fixation with Sorensen's buffered 2.5% glutaraldehyde and 1% OsO₄.

Experiments

Four experiments have been performed. In each, three groups of cultures (comprising three to seven tubes each) were compared: vigabatrin-

Table 1 Assay of total GABA and vigabatrin (GVG) in the supernatants of rat cerebellar cultures

Day	Controls	GABA-treated (1000 nmol ml ⁻¹)	Vigabatrin-treated (500 nmol ml ⁻¹)	
	GABA	GABA	GABA	GVG
0	6.64	6.47	7.71	0
2	5.56	387	9.05	177
4	5.51	—	10.22	333
7	5.56	—	9.86	322
9	6.46	—	9.32	389
11	6.50	—	9.14	328
14	6.32	—	10.54	305

Concentrations are given in nmol ml⁻¹;—not measured.

treated, GABA-treated and control untreated cultures.

Short-term studies included two experiments (5 and 6 days long). Two long-term experiments were performed for 14 days.

Results

Short-term studies

No significant differences between the experimental and the control cultures could be seen by light microscopic examination.

Long-term studies

Slight differences between the experimental and the control cultures could be found with light microscopy. This was not obvious on daily assessment of living cultures. However, it was seen by qualitative and quantitative blind examination of Sudan black B-stained slides. Mild toxicity of vigabatrin and of GABA was indicated by the presence of more numerous irregularities in the diameters of the myelinated fibres, of some ovoids and the increasing density of macrophages. No clear-cut differences could be demonstrated between the effects of the two compounds: although vigabatrin seemed a little more toxic than GABA at these doses, the differences were too slight to be significant in these experimental conditions (Figure 1 and Table 2). In electron microscopy, some degenerating myelinated fibres and astrocytic gliosis were seen in the controls as well as in the experimental cultures. However, they seemed milder in the control cultures. The changes involved axons as well as myelin sheaths. No marked primary demyelination, and no obvious intramyelinic oedema were seen. Neuronal cell bodies did not show any patent degeneration (Figure 2).

Discussion

The low toxicity of both vigabatrin and GABA in this very sensitive model (Bornstein, 1973; Raine, 1973; Hauw *et al.*, 1983) is surprising. The concentrations of the drugs added to the

nutrient medium (and measured after the experiment in the supernatant) were markedly higher than those found in the central nervous system of experimental animals (for example, 2.4–7.2 nmol g⁻¹ cerebral cortex in monkeys 6 h after a 16 month 300 mg kg⁻¹ day⁻¹ oral dosing schedule: (Heagele & Schwartz, personal communication)). These data can also be compared with the levels found in the CSF of patients treated with vigabatrin (for example, total GABA: from 8 to 20 nmol ml⁻¹ and vigabatrin: from 1.8–5 nmol ml⁻¹ after vigabatrin 2 g day⁻¹ for 14 days in the study of Schechter *et al.* (1984)).

Two main reasons might explain these negative findings: 1) the inadequacy of the model: cultures of myelinated central nervous tissue are usually susceptible to the same agents as those that induce demyelination *in vivo* (Bornstein, 1973; Raine, 1973; Hauw *et al.*, 1983). We lack some positive controls demonstrating that they can be affected by intra-myelinic oedema induced by vigabatrin. Experiments with higher doses of GABA and vigabatrin are in progress. However, the use of triethyl-tin, another drug known to induce intra-myelinic oedema (Stam, 1985; Aldridge *et al.*, 1987) have shown that this lesion could indeed be seen in tissue culture conditions (Graham *et al.*, 1975); 2) the short duration of the experiments in culture, as compared with that of the subacute and chronic intoxications which induce intra-myelinic oedema in experimental animals (up to 16 months in the monkey). It should be mentioned, however, that some vacuoles could be seen as soon as 15 days in rodents (rats and mice) intoxicated with very high doses of the drug. Longer experiments in culture are in progress.

Nevertheless some conclusions can already be drawn. The low levels of GABA observed in vigabatrin-treated cultures were associated with changes of the same order of magnitude as those seen in cultures exposed to very high doses of GABA. At least in these experimental conditions, a high concentration of GABA is not toxic for the central nervous system. The same statement is applicable to vigabatrin.

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Figure 1 Light microscopy of myelinated rat cerebellar cultures.

A and B: Control culture, 32 days *in vitro*. Numerous myelinated fibres are seen (arrows) in A. At a higher magnification, a few irregularities of the diameter of myelin are seen. Sudan Black B stain for myelin A $\times 450$, B $\times 1125$.

C and D: Sister culture intoxicated for 14 days by $1000 \text{ nmol ml}^{-1}$ GABA. No patent changes of the myelinated fibres are seen at low magnification (C). At a higher magnification (D), a few irregularities of the myelin sheath, more numerous than in control cultures, were seen (arrow). C: $\times 450$, D: $\times 1125$.

E and F: Sister culture intoxicated for 14 days by 500 nmol ml^{-1} vigabatrin. No patent changes of the density of myelinated fibres are seen (E). A few focal enlargements of the myelin sheaths are visible (F). E, $\times 450$, F, $\times 1125$.

Figure 2 Electron microscopy of myelinated rat cerebellar cultures.

A and B: Control culture, 32 days *in vitro*. At a low magnification (A), three normal myelinated fibres are seen. Note the presence of filament-laden astrocytes (X). At a higher magnification (B), a few irregularities of the myelin sheaths are seen (arrow).

C and D: Sister culture intoxicated for 14 days with $1000 \text{ nmol ml}^{-1}$ GABA. No patent changes of the myelinated fibres are seen at a low magnification (C). The few abnormalities of the myelin sheaths which were found were either artifacts, as demonstrated in D (arrow), or secondary to some axonal changes.

E and F: Sister culture intoxicated for 14 days by 500 nmol ml^{-1} vigabatrin. No patent changes of the myelinated fibres are visible either at low (E) or at high (F) magnification.

A, C, E: $\times 6600$; B, D, F: $\times 66000$.

Table 2 Effect of 500 nmol ml⁻¹ vigabatrin and 1000 nmol ml⁻¹ GABA added for 14 days to myelinated cultures of rat cerebellum. Density and morphology of myelinated fibres (duplicate experiment)

	Controls (18)			Vigabatrin (18)			GABA (16)		
	Better	Identical	Worse	Better	Identical	Worse	Better	Identical	Worse
Density	8	8	2	3	11	6	2	14	2
Morphology	1	4	13	0	2	18	0	7	12

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