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Free-GABA levels in the cerebrospinal fluid of patients suffering from several neurological diseases
Its potential use for the diagnosis of diseases which course with inflammation and tissular necrosis

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Summary. Free GABA levels were measured in the cerebrospinal fluid (CSF) of 74 neurological patients suffering from cerebral cysticercosis (n = 9), Parkinson's disease (n = 5), multiple sclerosis (n = 6), epilepsy (n = 24), meningeal tuberculosis (n = 6), viral encephalitis (n = 3), cerebrovascular disease (n = 8) and several kinds of dystonia (n = 5). A statistical significant four-fold elevation in free GABA levels was found in patients with cerebral cysticercosis. A non statistical significant two-fold increase in free GABA levels was also encountered in the CSF of patients affected by cerebrovascular disease and viral encephalitis. No changes in CSF free GABA levels were found in patients suffering from any of the other disorders. It is suggested that free GABA levels may be elevated in the CSF of patients suffering from neurological diseases which course with inflammation and tissular necrosis such as cerebral cysticercosis. Much work is needed however to establishd whether CSF free GABA levels can be used as a diagnostic tool in at least some type of these patients.

Keywords: Amino acids – GABA, CSF – Cysticercosis – CSF – GABA – Cerebral necrosis.

Introduction

It is now well accepted that γ-aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the mammalian brain (Fonnum, 1987) GABA neurons are ubiquitous and their terminals, which account for nearly 30% of all nerve endings (Bloom and Iversen, 1971), innervate practically all brain regions (Pérez de la Mora et al, 1981; Mugnaini and Oertel, 1985). It is therefore not surprising the

participation of GABA mechanisms in a wide range of neural activities (Seiler and Lajtha, 1987). Furthermore the involvement of GABA neurons in the etiology and/or physiopathology of several neurological and psychiatric disorders has been suspected (Seiler and Lajtha, 1987). Since extracellular GABA can reach the cerebrospinal fluid (CSF) the levels of this amino acid have been measured in patients affected by a variety of neurological diseases in an attempt for establish whether CSF GABA levels could be used as a diagnostic index.

Thus, it has been reported by some groups that free GABA levels are decreased in the CSF of patients suffering from epilepsy (Manyam et al., 1980; Wood et al., 1979), cerebellar ataxia (Manyam et al., 1980), Parkinson's disease (Enna et al., 1977; Araki et al., 1986; Manyam and Tremblay, 1984), multiple sclerosis (Manyam and Tremblay, 1984) or Huntington's chorea (Enna et al., 1977; Manyam et al., 1979; Manyam and Tremblay, 1984) while some other workers have been unable to confirm this finding in patients with epilepsy (Crawford and Chadwick, 1987; Pitkänen et al., 1988), Parkinson's disease (Teychenne et al., 1980; Perschak et al., 1987; Bonnet et al., 1987) or Huntington's chorea (Perry et al., 1982); Bonnet et al., 1987). Similar conflicting results seem to exist in patients with stroke since both an increase (Welch and Meyer, 1980) and a trend for a decrease Manyam et al, 1980) in free CSF GABA levels have been found. In order to contribute to establish whether CSF free GABA levels can be used for diagnostic purposes in this study we have measured free GABA levels in the CSF of neurological patients suffering from Parkinson's disease, multiple sclerosis, epilepsy, cerebrovascular disease or several types of dystonia. Free GABA levels were also assayed for the first time in the CSF of patients having cerebral cysticercosis, meningeal tuberculosis or viral encephalitis.

Material and methods

Free GABA levels were analyzed in a double-blind fashion in the CSF of 74 patients. CSF samples were taken indiscriminately from patients of the Mexican National Institute of Neurology who were in their vast majority under a diagnostic stage. CSF samples were withdrawn by lumbar puncture between the 3th and 5th vertebral bodies in patients kept in a lateral decubitus position. The CSF fraction taken for GABA analysis was collected in cold tubes and corresponded always to the first 2–3 ml obtained. Care was taken to eliminate all haemorrhagic samples. CSF was immediately frozen in dry-ice and stored at −70°C until analysis. In all cases CSF was obtained from 8:00 to 9:00 A.M. from patients who had fasted overnight and were drug-free for at least 24 hrs. After the completion of the whole analytical work the results were matched with the corresponding clinical diagnosis (see the Results section for the clinical description of the patients).

GABA measurement

On the day of GABA analysis CSF samples were thawed in a water-ice bath and proteins were precipitated by 3–4 min immersion in boiling water and by spun them down at 1 500 × g/10 min. Free GABA levels were measured in an aliquot of the supernatant by the radioreceptor assay of Enna and Snyder (1976).

Synaptic membranes were obtained by homogenizing (45 sec) in a Sorvall Omni-Mixer batches of 10 rat brains in 9 vols of 0.32 M sucrose. The homogenate was centrifuged at 1 000 × g for 10 min and the supernatant re-centrifuged at 20 000 × g for 30 min to obtain the crude mitochondrial fraction (P2) which was osmotically shocked by resuspension and

homogenization in bidestilled water (20 vol/g). Non disrupted material was precipitated by centrifugation at 8 000 × g for 20 min. The supernatant and the loosely packed upper layer of the pellet were combined and centrifuged at 48 000 × g for 20 min. The pellet containing the synaptic membranes was washed twice by resuspension in the same vol of water and by centrifugation at 48 000 × g for 20 min. The final pellet was resuspended in 0.1N tris.HCl buffer pH 7.4 (20 vol/g) containing 0.5% Triton X-100 and was incubated for 30 min at 37°C. At the end of the incubation period the synaptic membranes were collected by centrifugation at 48 000 × g for 20 min and stored at −70°C for a minimun of 24 hrs. Unless otherwise stated all operations were carried out at 0–4°C.

On the day of the GABA assay synaptic membranes were thawed and resuspended in 0.1M tris HCl buffer pH 7.4 and treated with Triton X-100 as above. After the 48 000 × g centrifugation the pellet containing the synaptic membranes was resuspended in the same tris-buffer to give a protein concentration of 1.0–1.2 mg/ml. For the GABA assay 250 μl of the membrane suspension were pippeted into 15 ml centrifuge tubes containing, by triplicate, 210 μl CSF, 40 μl water and 7.9 nM [2–3 ^{3}H]-GABA 30.3 Ci/mmol; New England Nuclear, Boston Massachusetts, U.S.A. The tubes were incubated at 4°C for 10 min and thereafter the reaction was stopped by centrifugation at 48 000 × g for 10 min. The resulting pellets were rinsed superficially with 2.0 ml cold water and digested with 1.0 ml 1 N NaOH at 37°C for 24 hrs. The samples were neutralized with 1 N HCl and quantitatively transferred into vials containing 5.0 ml Tritosol (Fricke, 1975). Radioactivity was counted in a Beckman LS spectrometer. Non-specific [^{3}H]GABA binding was obtained by carrying out the assay as described above but in the presence of 3.0 mM cold GABA. Non-specific [^{3}H]GABA binding was substracted in all cases to obtain the specific [^{3}H]GABA binding. GABA levels were extrapolated from a standard curve prepared for each determination containing as displacer 15–150 pmol unlabeled GABA in water instead CSF. Under these conditions and by plotting % of displacement vs pmol unlabeled GABA on semi-log paper the standard curve was linear from 0.33 to 150 pmol GABA. 7.5 pmol was the ammount of unlabeled GABA required to inhibit the [^{3}H]GABA binding by 50%.

Statistics

Results were evaluated by using a one-way ANOVA analysis followed by a post-hoc test.

All the CSF samples used in this study were taken from patients which were previously informed and gave their consent.

Results

Table 1 shows the clinical profile of the patients from which CSF was drawn for this study. As it is shown epilepsy and viral encephalitis were the disorders with the highest and lowest number of patients respectively. A similar number of patients were present in all other diseases. The age of the patients was, with the exception of those affected by Parkinson's and cerebrovascular diseases, very similar to that of the control group. The control group was integrated by patients suffering from tensional headache, and migraine in its asymptomatic phase and in which, has been reported (Welch et al., 1975), that CSF GABA levels do not change. This type of patients has been used as controls in similar studies (Araki et al., 1986; Pitkänen et al., 1988).

From a clinical point of view, all the epileptic patients studied complained of repetitive generalized convulsive crisis and in which neither gross anatomical abnormalities nor antibodies against cisticerci were found by computed tomography (CT) scanning and ELISA respectively. The electroencephalographic (EEG) studies carried out between the convulsive crisis showed the presence of

Table 1. Clinical profile of the population of patients studied

Disease	Number of patients	Male/Female ratio	Age (years)*	Range	Treatment
Control**	8	4/4	30 ± 3.9	20–47	Aspirine
Parkinson's	5	4/1	60.4 ± 4.3	51–76	Levodopa Biperiden
Dystonia	5	3/2	41.6 ± 7.4	24–58	Biperiden Clonazepan
Multiple sclerosis	6	2/4	31.8 ± 3.8	14–38	Prednisone
Epilepsy	24	12/12	22.5 ± 1.6	14–44	Carbamazepine Phenytoin Valproic acid
Cerebral cysticercosis	9	6/3	32 ± 3.2	22–48	Mebendazole Albendazole Phenytoin Carbamazepine
Meningeal tuberculosis	6	2/4	41.2 ± 9.5	17–76	Isoniazide Phenytoin
Viral encephalitis	3	0/3	42 ± 8.8	29–59	Prednisone Aspirine
Cerebrovascular disease	8	2/6	53 ± 3.7	38–71	Prednisone Aspirine

* Figures are means ± SEM

** Patients with either tensional headache or migraine in its asymptomatic phase were gathered under this group.

a theta rythm in one third of them. Parkinson's disease was diagnosed in all the patients of this group by the presence of rigidity, tremor and bradykinesia. Walking disturbances and affective alterations were also present in some of them. The diagnosis of multiple sclerosis was established by the presence in most of the cases of recurring episodes of either paresthesias, hemiparesis, nystagmus and/or visual impairment. Demyelinizing focus where observed by CT scanning in 4 patients of this group. Within the cerebrovascular disease group, 5 patients had CT evidence of cerebral infarction; two presented sudden focal neurological manifestations (quadriplegia, visual alterations) and one had clinical data compatible with the presence of vertebral insufficiency. The group of dystonic patients was integrated by individuals having persistent postural alterations. Patients with cysticercosis had developed (7 out of 9) generalized convulsive crisis and in most cases complained of headache. Brain CT scans practiced to 7 patients demonstrated the presence of cystic lesions within the cerebral tissue in 6 of these patients, and the existence of hydrocephalus in another different patient. Antibodies against the cisticerci were demonstrated in the serum of 3 patients with CT evidence of cysts in one with hidrocephalus and in two patients in which the CT scanning was not practiced but in who both headache and convulsive episodes were present. No specific antibodies were demonstrated in three patients with CT evidence of cysts.

Table 2. Free-GABA levels in the CSF of patients suffering from several neurological diseases

Disease	GABA levels (pmol/ml)*	Range
Control (8)	208 ± 41	72–387
Parkinson's disease (5)	446 ± 155	75–972
Dystonia (5)	274 ± 117	55–673
Multiple sclerosis (6)	245 ± 64	61–534
Epilepsy (24)	215 ± 31	49–613
Cerebral cysticercosis (9)	977 ± 341**	58–2804
Meningeal tuberculosis (6)	224 ± 78	75–599
Viral encephalitis (3)	536 + 258	134–1018
Cerebrovascular disease (8)	535 + 258	72–867

* Figures are means + SEM. For the clinical profile of patients see Table 1 and Results. Number of patients in parenthesis. Results were evaluated by a One-way ANOVA analysis followed by Fisher's test. Global F and probability values were 3.38 and 0.003 respectively** $p < 0.01$ when compared to the control group.

The diagnosis of meningeal tuberculosis was made in patients who have had a "clinical history" of systemic tuberculosis and presented a meningeal syndrome accompanied or not of fever. Lethargy, headache and fever were present in all patients with viral encephalitis. In addition, generalized convulsive crisis were present in one of them.

From a biochemical point a view, the CSF free GABA levels in the control group, were found in the 200 nM range (Table 2). Similar levels were observed in the majority of the cases with the exception of patients suffering of cerebral cysticercosis (Table 2), which showed 4 – fold higher free GABA levels. It is noteworthy to state that although free GABA levels whithin this group differed widely from one patient to another they were higher than 345 pmol/ml in 6 out of 9 patients. Patients suffering of viral encephalitis, Parkinson's and cerebrovascular diseases showed also increased CSF free GABA levels, although statistically significant levels were not reached (Table 2).

In order to ascertain whether mebendazole, (methyl-5-benzoylbenzimidazole-2-carbamate; Janssen Pharmaceutica, México) a widely used antihelmintic drug, may interfere with the radioceptor method used in this work to measure GABA, free GABA levels were measured in two different CSFs supplemented with different ammounts of this drug. As indicated in Table 3, mebendazole was able to displace [^{3}H]GABA from its receptor in a dose dependent fashion increasing artifactually the CSF free GABA levels. The potency of mebendazole as displacer was about 40% of that of unlabeled GABA and the degree of the interference depended of the concentration of mebendazole in the assay and the actual CSF free GABA levels. As an average control CSF free GABA levels were doubled by 240 pmol/ml mebendazole and increased between 2.5 to 3.0 fold by 360 pmol/ml mebendazole (Table 3).

Table 3. Degree of mebendazole interference on the measurement of CSF free GABA levels*

Mebendazole (pmol/ml CSF)	GABA levels (pmol/ml) Measured as "GABA"	Accounted for the presence of mebendazole
0	125	None
	72	
120	178	53
	119	47
240	221	96
	166	94
360	296	171
	223	151

* Free GABA levels were assayed by triplicate as described under material and methods in the CSF of two different patients. A stock solution of mebendazole containing 50 μg/ml was serially diluted with water to give working solutions from which a 20 μl aliquot was pipetted into the respective incubation mixtures in substitution of the same volume of water.

Discussion

GABA is present within the CSF in a free and a conjugated form. The conjugated GABA is not an homogeneous entity but includes GABA-containing peptides such as homocarnosine and 2-pirrolidinone which results from the ciclization of the free GABA form (Haeggele et al., 1987). Although the relationship between both forms of GABA is unknown some studies have shown that whereas free GABA levels changes under a variety of pathological conditions the conjugated GABA levels tend to remain stable (Manyam and Tremblay, 1984). In this work, CSF free GABA was measured in a double-blind way in a serie of 74 patients suffering from several neurological diseases (see Table 1) in order to contribute to establish whether its CSF levels could be used as a diagnostic criterium.

In order to prevent differences due to the cephalo-caudal concentration gradient which has been described for CSF GABA (Grove et al., 1982) CSF samples were always withdrawn from the same vertebral space and the fraction used for the GABA assay comprised the first 2–3 ml of the puncture. To avoid the transformation of conjugated GABA to free GABA, CSF samples were frozen in dry ice immediately after their obtention and stored at −70°C. Proteins were eliminated shortly before the GABA assay. Under these conditions the constancy of free GABA levels is warranted (Grossman et al., 1980; Ferraro et al, 1983). As mentioned above, the control group comprised patients affected by tensional headache and migraine in its asymptomatic phase in which CSF free GABA levels are normal (Welch et al., 1975). The age of these subjects matches very well the age of the patients of most groups (Table 1). The free GABA levels found in our control group are identical among others (Schechter and Sjoerdsma (1990) to those reported by Wood et al. (1979), Manyam et al. (1979) and Böhlen

et al. (1978) and sligthly higher to the values given by Ferraro et al. (1983) and Wood et al. (1978) for normal volunteers and neurologically healthy patients.

The main finding reported in this work is an increase in free GABA levels in the CSF of patients with cerebral cysticercosis (Table 2). It is not entirely clear the source of this extra free GABA but since it has been reported that GABA neurons are ubiquitous within the mammalian central nervous system (Pérez de la Mora et al., 1981; Mugnaini and Oertel, 1985) and GABA levels are high in the human brain (Fahn, 1976) it is conceivable that it may arise of leakage from the necrotic lesions produced by the implantation and growing of cisticerci within the cerebral tissue.

It is important to state that no specific antibodies were found in the sera of those patients who showed the lowest CSF free GABA levels (58 and 109 pmol/ml). In contrast patients with antibodies against the cisticerci and with clinical features of the malignant form of cerebral cysticercosis such as convulsions, headache, hydrocephalus and papilloedema showed the highest levels of this amino acid (2386 and 2804 pmol/ml). This is in agreement with Corona et al (1986) and Zini et al (1990) who have found in this form of the disease high levels of serum antibodies, and an excellent correlation between their presence and the existence of cysts. The absence of serum antibodies in 3 patients with CT evidence of cysts it is not surprising in view that the lack of such correlation is common in patients with the benign form of the cerebral cysticercosis (Corona et al, 1986; Zini et al, 1990).

It has been reported that imidazolacetic acid (Enna and Snyder, 1976), a compound structurally related to the widely used antihelmintic benzimidazoles drugs, shows a similar potency to unlabeled GABA for displace [^{3}H]GABA from synaptic membranes. In this study it was found that mebendazole a benzimidazole derivative taken by several of our patients, was indeed able to displace [^{3}H]GABA and to increase artifactually the CSF free GABA levels (Table 3). There is no information in the literature concerning the CSF or the plasma levels of any benzimidazol drug at the doses used (200–300 mg twice daily for three days) for the treatment of taeniasis (Keystone and Murdach, 1979). Mebendazole levels have been however measured in the plasma of patients with hydatid disease in which this drug is used at higher doses (40 mg/kg body weight) and for longer periods of time, and as an average it has been reported a plasmatic mebendazole concentration of 85 pmol/ml (Luder et al., 1985; Wilson et al., 1978). For the sake of discussion we will consider this concentration of mebendazole as the concentration of any benzimidazole drug in the CSF of patients treated with this type of drugs.

The possibility that the high free GABA levels in the CSF of patients with cerebral cysticercosis were due to a displacement of [^{3}H]GABA from its receptors by the presence in their CSF of any benzimidazole drug and/or its metabolites seems however unlikely because although three patients within this group were under mebendazole or albendazole treatment they completed their treatment between one or two weeks before the CSF sampling. Furthermore, even assuming that all the patients within the group of cerebral cysticercosis were receiving mebendazole or a similar drug by time of the CSF collection it is difficult to explain the 4-fold increase in their CSF free GABA levels solely as a

result of [^{3}H]GABA displacement since 120 pmol/ml mebendazole failed to double the CSF free GABA levels and 360 pmol/ml, allowing for the presence of 300 pmol/ml of structurally related metabolites, increased only between 2.5 to 3.0 fold the control CSF free GABA levels (Table 3).

High CSF free GABA levels have been reported in the stroke (Welsch and Meyer, 1980) and in the acute hypoxic encephalopathy (Manyam et al., 1980). Our results show also high CSF free GABA levels in patients with cerebrovascular disease, which due to the wide individual variation did no reach statistical significance. (Table 2). It is tempting however to speculate that as in the case of the cerebral cysticercosis, the increased CSF free GABA levels observed in these patients may result also of its leakage from the necrotic lesions produced by the cerebral infarcts. It is clear however that much more work is needed before this issue can be settle down with certainty.

There is considerable controversy in the literature concerning the CSF free GABA levels in epilepsy and Parkinson's disease since a decrease in CSF free GABA levels has been reported by several groups (Enna et al., 1977; Wood et al., 1979; Manyam et al., 1980; Araki et al., 1986) while no changes have been found by others (Teychenne et al., 1980; Crawford and Chadwick, 1987; Perschak et al., 1987; Bonnet et al., 1987; Pitkänen et al., 1988). The lack of statistical significant variations in the free GABA levels in the CSF of our epileptic patients may support the results obtained by the last group of workers. On the other hand, the non significant increase in the CSF free GABA levels found in Parkinson's disease owing to the small size of our sample can not support reliably any possible alternative.

In contrast to Manyam and Tremblay (1984) we did not found changes in free GABA levels in the CSF of patients with multiple sclerosis. The reason for this discrepancy is not clear but differences concerning the clinical state of the patients and/or the existence of some subtle methodological differences could be involved. A non significant increase and no changes in CSF free GABA levels were observed in patients suffering from viral encephalitis and meningeal tuberculosis respectively. However, our results may be considered as preliminary since there is no previous information in the literature concerning the CSF free GABA levels in these diseases, and the number of patients we have evaluated is small. In conclusion our results indicate that in some neurological disorders which seem to course with inflammation and/or tissular necrosis such as cerebral cysticercosis the CSF free GABA levels could be increased. However, since the CSF free GABA levels were not elevated in all the patients affected by these type of disorders we feel that much more work is needed to show under which clinical conditions the CSF free GABA levels are increased and in consequence in which kind of patients the CSF free GABA levels could be used as a diagnostic tool.

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