DIAZEPAM BINDING INHIBITOR IN BRAIN AND IN CEREBROSPINAL FLUID OF HUMAN

P. FERRERO (a), P. BENNA (a), F. VISCHIA (a), L. TARENZI (a), B. BERGAMASCO (b) and L. BERGAMINI (a)

(a) I and (b) III Neurological Clinic, University of Turin, Italy

Summary. – Human and rat brain contain an anxiogenic protein, termed Diazepam Binding Inhibitor (DBI), which displaces ligands bound to the B-Carboline/Benzodiazepine recognition sites. Using specific antibodies against the human purified protein, we investigated its distribution in the human brain. DBI is highly localized in hypothalamus, cerebellum and amygdala. Smaller concentrations were observed in cortex, basal ganglia and pituitary. The lowest content was measured in corpus callosum. The rank of order of DBI content in human brain is similar to that of rat brain. Moreover since DBI-like immunoreactive material was detected in the cerebrospinal fluid (CSF), it was of interest to establish whether there were differences in CSF content of DBI in patients suffering from neuropsychiatric disorders. The DBI immunoreactivity content in human lumbar spinal fluid was found to be increased in patients with Parkinson’s disease. In contrast no changes were observed in patients with disorders affecting spinal cord, multiple sclerosis and Alzheimer’s disease. However in the Parkinsonian group only the patients with an associate depressive disturbance showed higher concentrations of DBI when compared with control, while no differences were observed in the patients without depression. Thus the possibility that the increased CSF DBI observed in Parkinson’s may represent an impairment associated with depression itself is suggested.

Introduction

The most important inhibitory neurotransmitter in the central nervous system, γ-aminobutyric acid (GABA), exerts its effects mainly through a GABA\_ receptor that gates a picrotoxine sensitive chloride channel in the postsynaptic membrane of the target neurons [1-3]. These receptors are physically associated with regulatory sites, the benzodiazepine receptors (BDZR), through which ligands of different chemical classes can allosterically modulate the GABA\_ receptor function [4-8]. Occupancy of these binding sites by anxyolitic BDZ increases the probability of Cl\textsuperscript- channel opening mediated by GABA, in contrast occupancy of these binding sites by anxiogenic B-carbolines (BC) derivatives reduces GABA effects [9]. This opposite action is not surprising because the sites occupied by BDZ are not strictly
homologous with those occupied by BC and the existence of two conformational states of the BDZ/BCR has been recently proposed [10, 11].

Given the existence of endogenous ligands for the opioid receptor, there has been a similar interest in identifying endogenous ligands for the BDZ/BC recognition sites [12]. One of these putative endogenous modulator, a 11-kDA polypeptide, has been recently isolated from rat and human brain [13-15]. This polypeptide was termed Diazepam Binding Inhibitor (DBI) because it competitively displaces 3H-BDZ and 3H-BC from their recognition sites in both synaptic membranes from brain and in primary cultures of rat cerebellar neurons [15, 16]. When injected intracerebroventricularly in rats, DBI causes a proconflict action and when tested in patch-clamped mouse spinal cord neurons, DBI shortens the duration of chloride channel opening elicited by GABA [15-17]. Thus DBI has a pharmacological profile of a naturally occurring anxiogenic and, accordingly, it was also observed that DBI preferentially displaces 3H-BC over 3H-BDZ [14, 15]. Immunohistochemical studies [18] have revealed an uneven distribution of DBI-like immunoreactivity throughout the rat brain, the highest concentrations being found in the hypothalamus, cerebellum and limbic areas. In some regions (hippocampus, cerebellum) DBI has been shown to coexist with GABA, but such localization is not present in every neurons where DBI is located [19]. Tryptic digestion of DBI yields an octadecanecuropeptide which shares some of the biological properties of DBI [20, 21]. Therefore it is tempting to speculate that DBI might processed into small active peptides important for the allosteric modulation of GABA receptors.

In our previous studies [22] we found evidence that a DBI-like peptide, with comparable biological properties to rat DBI, is present in brain of human and recently, the isolation of a cDNA clone has provided the complete sequences of this protein [23]. Human DBI is highly homologous to rat DBI and still contains an ODN-like sequence endowed with its biological activity [22, 23]. A specific antiserum against the human purified protein could be used to measure its distribution in brain and to detect its presence in spinal fluid [24, 25]. Measurements of CSF DBI are of potential interest since they may reflect alterations occurring at this peptide level in human pathological conditions. In order to evaluate pathological changes in CSF DBI contents it is necessary to establish the reliability of its measurements. In the present study we used radioimmunoassay in conjunction with gel chromatography to investigate the occurrence and the molecular nature of DBI-like immunoreactivity (DBI-LI) in the lumbar CSF of control subjects and in patients with neurological disorders. Moreover we also investigated its regional distribution in human brain.

Materials and methods

Subjects. – CSF DBI was obtained by lumbar puncture from consenting hospitalized patients undergoing routine evaluation for neurological problems at our department. One initial cohort (Table 1) included 25 normal controls and 30 patients with various neurological disorders age and sex matched. The controls were selected from patients undergoing to mielography who were subsequently determined to be free from neurological pathologies. The pathological sample consisted of patients with spinal cord diseases, multiple sclerosis and a miscellaneous of neurological disorders (i.e. vascular and toxic). In a subsequent series CSF was obtained from a well characterized group of patients with different type dementia and with Parkinson’s disease (Fig. 3). This population, ranging from 50 to 65 years included 16 age matched controls (56.6 ± 3.1 yrs), 20 patients with dementia of Alzheimer’s type (DAT) (56.8 ± 1.6 yrs), 9 patients with “other” forms of dementia (60.1 ± 4.0 yrs) and 24 patients with Parkinson’s disease (58.8 ± 1.2 yrs). Diagnosis of dementia were made according to DSM III criteria [26]. In all groups a threshold for the diagnosis of dementia was established as a Mini Mental Status Examination (MMSE) [27] score of less than 24. Hachinsky Ischemic Scale (HIS) [28] as well as a large number of laboratory and instrumental examinations (including EEG and CT scanning) were used to assist the clinical diagnosis of different type of dementia. Depression was scored on Hamilton rating scale, moreover the Beck Depressive Inventory [29, 30] was obtained for the Parkinsonian patients. The clinical disability of Parkinsonian patients was assessed on both the Columbia and Webster rating scales and their motor impairment on Hohen and Yahr scales [31, 32]. All the controls resulted free from depression and scored more then 28 on MMSE. Both the demented groups were comparable for overall severity of dementia on MMSE scores and were free from depression. The Parkinsonian patients had a mild to moderate-disease severity [32] and resulted absent of dementia; 14 of the examined patients were mildly to severely depressed as scored on BDI (mean-SE:24.1.67).

| Table 1. – CSF DBI-LI Concentration in controls and in patients with various neurological disorders |
|---------------|--------|-------------|-----------------|-----------------|
| Patients      | N.     | Sex F/M     | Age years       | CSF DBI-LI (pmol/ml) |
| Controls      | 25     | 15/10       | 54.4 ± 3.2      | 1.31 ± 0.05      |
| Spinal cord diseases | 11    | 6/5         | 53.7 ± 4.9      | 1.32 ± 0.06      |
| Multiple sclerosis   | 9     | 5/4         | 47.1 ± 3.5      | 1.29 ± 0.07      |
| Others         | 10     | 4/6         | 54.9 ± 4.9      | 1.32 ± 0.07      |
| Total          | 55     | 30/25       | 53.8 ± 2.3      | 1.31 ± 0.06      |
Lumbar Puncture. — All lumbar punctures were performed in the morning, during bed rest after an overnight fast. The 4th to 6th ml were collected and immediately frozen at —30°C until the time of assay. Since in some experiments we assessed the presence of a CSF-DBI gradient, 4 serial spinal fluid aliquots (4ml each) were withdrawn from control subjects. Aliquots of these CSF samples were also used to examine the reliability of the CSF-DBI assays under varying time, temperature, incubation and extraction conditions.

Purification of human DBI. — Human brain were obtained at post mortem from patients (n=4) without history of neurological or psychiatric diseases. The brains were immediately dissected and then stored at 70°C until extracted in boiling 1N acetic acid (1ml per 100 mg wet weight tissue) for 15 min. After centrifugation the supernatants were applied to Sep-pak C18 mini columns and then eluted with 60% acetonitrile and then used for radioimmunoassays procedure.

Standard human DBI was purified accordingly to the previous determined procedure [13, 14] with minor modification. Briefly DBI was extracted from cerebellum (approx. 20 g) with hot (90°C) acetic acid and then purified through Sep-pak C18 mini columns. The acetonitrile eluates were redisolved in 0.1N acetic acid and then subjected to ammonium sulfate fractionation. The supernatants to a Sep-pak C18 and the eluate, after drying, applied to a μBondapack C18 rev-ph HPLC column eluted with a linear gradient of 0.1 TFA in acetonitrile. Under these conditions DBI is eluted, well separated from other contaminating peptide at 40% TFA/acet. The purity of DBI is tested on 15% SDS-PAGE gel electrophoresis. Starting with approx 20 g of cerebellum we recover approx. 20 mg DBI.

Radioimmunoassay. — DBI concentrations in different brain regions and in CSF were measured by radioimmunoassay (RIA) as previously described [18-20] using a specific antiserum raised in rabbits after repeated injections of purified DBI in Freund’s adjuvant. As a tracer standard DBI (10 μg) was labeled with 125I by the Bolton and Hunter technique and purified by gel-chromatography on Bio-Gel P2 column equilibrated with 1N acetic acid containing 0.25% gelatin. Optimal binding has been determined at an antiserum dilution of 1/10000. For routine analysis hDBI, acid tissues extracts or 100μl of dried spinal fluid were incubated at 4°C (for at least 12 hours) with the antiserum and the tracer in 0.25 ml 0.05M Na2HPO4 buff (PH 7.4) containing 0.2M NaCl, 5% BSA and 0.2% calf thymus hystone. Separation of free and bound was carried out by the addition of 0.75 mg of Protein A in 0.3 ml of 50 mM of TRIS-HCl buff (PH 8) containing 2mM MgCl2.

Immunaffinity chromatography. — Purified anti human IgG [33] were reacted with aliquots (2nl) of CSF or with standard DBI in 2ml of 0.01M citrate buffer (PH 7.4) containing 0.02% BSA for 12 hours. After the incubation the reaction mixtures were applied to a Protein A Seph. CL AB immunaffinity column equilibrated with 0.1M citrate buffer (PH 6.3). The first 4ml, corresponding to DBI-free CSF were collected. The bound CSF-DBI was then eluted from the column with 0.1M citrate buffer (PH 3.3) and 1ml fractions collected. The DBI immunoreactive fraction were subjected to HPLC fractionation.

Results

DBI distribution in human brain. — The distribution of DBI-LI in human brain is shown in Table 2. DBI could be detected in all brain regions investigated. Massive amounts were present in hypothalamus, with relatively large amounts in cerebellum, periaqueductal grey, amygdala, hippocampus, septal nuclei and hippocampus. Smaller concentrations were detected in cortex (predominantly in the sensory motor, Brodmann area 4), basal ganglia (caudate and putamen) and in thalamus (anteromedial and anterolateral). The lowest contents were detected in corpus callosum and in the white matter in gener. Extracts of five of human brain (hypothalamus, cerebellum, frontal cortex, caudate and corpus callosum) were fractionated on HPLC. DBI-LI in all the examined areas eluted in a single peak in identical position to standard human DBI. The DBI distribution is closely similar to that obtained

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<th>Table 2. Distribution of DBI-LI human brain</th>
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from extracts of fresh rat brain [18] and thus differs from that of any other peptide so far mapped. In addition DBI concentrations exceed even those of somatostine and NPY, thus it seems the most abundant of human neuropeptides. DBI differ in its regional distribution within the cortex, lower levels being found in sensory motor cortex and in occipital cortex and relatively moderate levels in limbic cortex and frontal pole (A 38 and A10 of Brodmann). This may be relevant to the high DBI-LI in both limbic regions (amigdala and hippocampus) and septal nuclei and in view of the participation of DBI in the control of emotions.

Validation of spinal fluid DBI measurements. – In order to assess whether CSF by itself interferes with DBI-LI levels measured in our assay two sets of experiments were carried out. In a first CSF was applied to an immunoaffinity column to extract the DBI-LI. The DBI free CSF was then used in the assay in place of samples or added to standard DBI displacement curve. As shown in Fig. 1 non specific interferences were excluded since the $^{125}$I DBI curves prepared either in the assay buffer or in presence of the DBI free CSF were superimposable. Secondly we found that serial dilutions of fresh CSF (up to 0.3 ml) displaced the tracer with identical slope to that obtained with standard DBI. The identity of the CSF immunoreactive material with DBI was established by fractionation on HPLC of both crude CSF samples and the DBI-LI eluted from the immunoaffinity. In both cases a single peak at the identical retention time of DBI was detected.

![Graph](image)

**Fig. 1.** Binding of $^{125}$I DBI to antisera as a function of dilutions of human CSF and standard DBI. Addition of immunoaffinity extracted DBI-free CSF had no effect on binding.

Serial dilutions of CSF (lower insert) paralleled the displacement of standard DBI (○○) indicates the standard DBI curve, while (○○) represents DBI standards in presence of the immunoaffinity extracted DBI-free CSF; (▲▲) represents serial dilutions of dried crude CSF.

DBI appears to be relatively stable in the CSF since several changes in the experimental conditions (including temperature, time of incubation, sample acidification or boiling) did not affect consistently its CSF concentration. Only repeated thawing (up to 5 times) produced a gradual loss of activity. We also observed that there was no evidence of a CSF DBI gradient since the concentrations of DBI in successive fractions (up to 16 ml) withdrawn from 4 different subjects were similar.

**CSF DBI in neurological diseases.** – CSF DBI-LI concentrations were assayed in a group of 25 control subjects and in 30 patients, 11 with spinal cord disorders, 9 with multiple sclerosis and 10 with a variety of neurological pathologies. The CSF concentration in the entire series ranged from 0.7 to 2.1 pmol/ml with a mean concentration of 1.31 ± 0.06 pmol/ml. The concentrations of DBI-LI in the pathological samples were not different from those of controls nor when the patients were subdivided into more homogeneous groups in relation to their pathologies (Table 1).

As shown in Fig. 3 CSF DBI-LI content increases significantly with age. The curve for the data fits the equation **Y=0.17X + 0.35** (r=0.75, p < 0.001). Over 65 years span from age 16 to 79, the average CSF DBI-LI increased by more than 70%. Moreover the levels of DBI-LI were significantly lower in females than in males. This sex relation seems dependent from age since the differences were significant only in the females younger than 55 (data not shown).
Patients with Alzheimer's (CSF-DBI-LI: 1.46 ± 0.07 pmol/ml) (Fig. 4) exhibited a slight and non significant increase of DBI-LI as compared to the controls (1.35 ± 0.06 pmol/ml). No significant correlation was observed with the mental impairment rated on MMSE. In contrast patients with Parkinson's were found to have highest DBI levels (1.62 ± 0.08 pmol/ml, p < 0.05). However when these patients were subdivided in two subgroups in relation to the presence of an associate depressive symptomatology, CSF DBI-LI was significantly increased only in the depressed patients (1.82 ± 0.06 pmol/ml, p < 0.01). Moreover a positive correlation was observed between the increase of CSF DBI-LI and the degree of depression rated on BDI scores.

Discussion

In the present report the existence of DBI in lumbar spinal fluid of human was demonstrated by a sensitive and specific radioimmunoassay. The immunoreactive material detected in the CSF has characteristics identical to those of authentic DBI since after HPLC a single peak of immunoreactivity, with retention time identical to that of DBI, was detected. Furthermore in the RIA there was no evidence of some specific interferences.

Measurements of DBI in neurologically normal patients indicates that DBI is present in CSF in high concentration and this finding was not surprisingly since massive amounts of DBI in brain could be detected. The observation that DBI increase with age seems of particularly interests since there are indications [34, 35] that changes at GABA/BZD levels may occur with aging. Hence the changes in DBI-LI theoretically may reflect a parallel fluctuation of putative endogenous ligand for the BZD receptors that could be primarily involved in the age related changes occurring at the GABAergic receptor level. The relationship of CSF-DBI with age is additionally complicated by the observation that women under 55 showed lower levels of DBI than males. This suggests that DBI levels in CSF varies in relation to certain hormonal mechanisms.

In our initial examination obtained from a small sample of patients suffering with disorders of spinal cords or with multiple sclerosis we measured a normal CSF DBI content as well in a clinically well characterized population of patients with dementia, including Alzheimer's diseases. The observation that the CSF of depressed Parkinsonian patients contained higher DBI level is of considerable interest. The increase appears to be specifically linked to the presence of concomitant depressive disturbances and unrelated to motor disabilities. Moreover the CSF DBI content in Parkinsonian without depression was practically superimposable with that of controls. Depression is a common disturbance in Parkinson's and it is of interest that DBI is highly localized in brain regions (for example hypothalamus, amigdala and other limbic areas) that are believed to be altered in depression [36, 37]. Moreover recent data on the pharmacological action of antidepressant and GABA agonists drugs suggest that GABAergic mechanisms may be involved in depressive disorders [38, 39]. Therefore it seems possible that the elevated concentrations of DBI in spinal fluid of the de-
pressed Parkinsonian patients underline an abnormal activity of CNS DBI systems. Whether these abnormalities are associated with Parkinson’s or whether they are conversely associated with the state of being depressed it is an important issue that remain to be elucidated.

REFERENCES


