Diazepam binding inhibitor gene expression: Location in brain and peripheral tissues of rat

(hybridization histochemistry/diazepam binding inhibitor mRNA brain location)

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ABSTRACT Diazepam binding inhibitor (DBI), an endogenous 10-kDa polypeptide was isolated from rat and human brain by monitoring displacement of radioactive diazepam bound to specific recognition sites in brain synaptic and mitochondrial membranes. The cellular location of DBI mRNA was studied in rat brain and selected peripheral tissues by in situ hybridization histochemistry with a 35S-labeled single-stranded complementary RNA probe. DBI mRNA was heterogeneously distributed in rat brain, with particularly high levels in the area postrema, the cerebellar cortex, and ependyma of the third ventricle. Intermediate levels were found in the olfactory bulb, pontine nuclei, inferior colliculi, arcuate nucleus, and pineal gland. Relatively low but significant levels of silver grains were observed overlying many mesencephalic and telencephalic areas that have previously been shown to contain numerous DBI-immunoreactive neurons and a high density of central benzodiazepine receptors. In situ hybridizations also revealed high levels of DBI mRNA in the posterior lobe of the pituitary gland, liver, and germinal center of the white pulp of spleen, all tissues that are rich in peripheral benzodiazepine binding sites. The tissue-specific pattern of DBI gene expression described here could be exploited to further understand the physiological function of DBI in the brain and periphery.

Diazepam binding inhibitor (DBI) (1), also called endozepine (2), is an endogenous 10-kDa polypeptide that was initially purified from mammalian brain based on its ability to displace specific ligands from benzodiazepine recognition sites on γ-aminobutyric acid (GABA
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Abbreviations: DBI, diazepam binding inhibitor; GABA, γ-aminobutyric acid; ODN, octadecaneuropeptide; cRNA, complementary RNA; LI, like immunoreactivity.

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clone by cytidine 111 (see figure 3 of ref. 5). The 3' nucleotide (part of the Rsa I site) is thymidine-5'-552. The orientation of the insert provides that in vitro transcription by T7 polymerase produces sense RNA and transcription by T3 polymerase produces antisense RNA. The single-stranded RNA transcripts were labeled with uridine 5'-[35S]triphosphate (5000 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) to a final specific activity of >106 cpm/μg.

**Tissue Treatment.** Adult male Sprague–Dawley rats (150–250 g) were housed in a quiet room under controlled lighting (12 hr on/12 hr off) with free access to food and water. The rats were anesthetized by injection of sodium pentobarbital (5 mg per 100 g of body weight) and the systemic circulation was rinsed by cardiac perfusion with 20 ml of 0.9% saline at room temperature. Animals were then perfused through the aorta with ∼200 ml of ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 20 min. After perfusion, the rats were decapitated and their brains, pituitaries, and livers were dissected and cryoprotected by immersion in 15% sucrose in phosphate-buffered saline for 60 min at 4°C. The tissues were then embedded in an M-1 matrix (Lipshaw Manufacturing, Detroit) and rapidly frozen in liquid nitrogen. Sections (10–15 μm) were cut with a Hacker cryostat microtome, thaw-mounted onto gelatin and poly-L-lysine-coated glass microscope slides, and stored at ~70°C until time of use.

**In Situ Hybridization Procedure.** The sections were thawed at room temperature and treated with proteinase K (Sigma; 5 μg/ml) in diethyl pyrocarbonate (DEPC)-treated deionized distilled H2O for 10 min at room temperature to partially deproteinize the tissue. The sections were rinsed in 2× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7). The tissue sections were then covered with a prehybridization solution [50% formamide/0.6 M NaCl/10 mM Tris HCl, pH 7.5/0.02% Ficoll/0.02% polyvinylpyrrolidone/0.1% bovine serum albumin/1 mM EDTA/salmon sperm DNA (50 μg/ml)/yeast total RNA (500 μg/ml)/yeast transfer RNA (50 μg/ml)] and prehybridized for 60–120 min at 50°C. After prehybridization, the buffer was removed, and the tissue sections were covered with hybridization solution [50% formamide/0.6 M NaCl/10 mM Tris HCl, pH 7.5/0.02% Ficoll/0.02% polyvinylpyrrolidone/0.1% bovine serum albumin/1 mM EDTA/salmon sperm DNA (10 μg/ml)/yeast total RNA (50 μg/ml)/yeast transfer RNA (50 μg/ml)] containing 2.5 × 106 cpm/ml of 35S-labeled DBI sense or antisense single-stranded RNA probes that had been heated and denatured at 100°C for 10–15 min at 65°C. The sections were hybridized for 16–18 hr at 50°C in boxes humidified with 50% formamide and 4× SSC.

After hybridization, the sections were washed in 3 liters of 2× SSC at 45°C for 30 min and subjected to RNase A digestion (30 μg/ml in 500 mM NaCl/10 mM Tris HCl, pH 7.5) for 60 min at 37°C. The sections were then washed in 3 liters of 2× SSC at 45°C for 30–60 min and subjected to a final high-stringency wash in 4 liters of 0.1× SSC/0.05% sodium pyrophosphate/14 mM 2-mercaptoethanol for 3 hr at 50°C, followed by an overnight wash at room temperature in the same buffer. The sections were then dehydrated in graded alcohols containing 0.3 M ammonium acetate and dried in a vacuum desiccator. For visualization of the hybrids, the slides were dipped in Kodak NTB2 nuclear track emulsion (Eastman Kodak) and exposed for the indicated times at 4°C in light-tight boxes, with desiccant. Development was carried out in Kodak D-19 developer as described by the manufacturer. After fixation, the slides were were washed for 30 min in distilled H2O, followed by staining with hematoxylin and eosin. The sections were analyzed in a Leitz light microscope equipped with polarized light epiluminescence and photographed with Kodak Ektachrome 160 tungsten film.

To test the specificity of hybridization in each brain area, identically treated serial sections were hybridized with the 35S-labeled antisense DBI probe and with the 35S-labeled sense DBI probe (control) or with a pro-opiomelanocortin probe.

**RESULTS**

In situ hybridization revealed that DBI mRNA was heterogeneously distributed in rat brain, pituitary, and spleen. In liver, the DBI mRNA was uniformly distributed throughout the tissue. Two kinds of controls were used to establish the specificity of the in situ hybridization data obtained in these studies. First, each section that was hybridized with an antisense 35S-labeled DBI complementary RNA (cRNA) probe was paired with an identically treated section that was hybridized with the sense-strand 35S-labeled DBI control probe. The sense strand used resulted in uniform randomly distributed background levels of silver grains. Hybridization of serial sections with 35S-labeled pro-opiomelanocortin cRNA failed to specifically label areas that were labeled by the 35S-labeled DBI probe, such as the area postrema.

Particularly high levels of DBI mRNA signal were observed in cerebellum, ependyma of third ventricle, and the area postrema, while a less dense signal was noted in the pontine nuclei, inferior colliculi, arcuate nucleus of the hypothalamus, pineal gland, and olfactory bulb, and low but higher than background levels of signal were seen in all brain areas when compared to identical sections hybridized with sense-strand control probe (Fig. 1).

At the cellular level, hybridization of brainstem sections with antisense 35S-labeled DBI cRNA revealed a dense autoradiographic labeling over the cells in the area postrema (Fig. 2A) and a less dense labeling in dorsal motor nucleus. When serial tissue sections were treated identically but

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**Fig. 1.** X-ray film autoradiograms of the distribution of DBI mRNA in situ hybridization in rat brain parasagittal sections. (A) Sections hybridized with 32P-labeled antisense probe show intense labeling in area postrema (AP), cerebellum (CB), and ependyma of third ventricle (E), and moderate labeling in nucleus arcuate (Arc), inferior colliculus (IC), olfactory bulb (OB), nucleus pontine (Pn), and corpus pineal (Pi). (B) Consecutive sections hybridized with sense probe (control).
hybridized with the sense $^{35}$S-labeled DBI control probe, only background levels of silver grains were observed (Fig. 2B). Hybridization of other brainstem areas with antisense $^{35}$S-labeled DBI cRNA revealed a high density of silver grains overlying the cells in the midline of lower brainstem, roughly corresponding to the raphe pallidus (Fig. 2C), as well as cells situated ventrolaterally to the inferior olive complex (Fig. 2D). Other scattered cells along the basal surface of the brainstem were labeled with the antisense $^{35}$S-labeled DBI cRNA probe. A lower density of silver grains was observed overlying the cells in the pontine nuclei. After hybridization with the $^{35}$S-labeled DBI sense-strand control probe, no specific labeling was observed in these areas.

Hybridization of coronal sections of the rat cerebellum with antisense $^{35}$S-labeled DBI cRNA revealed a dense band of silver grains at the junction between the granular and molecular layers of the cerebellar cortex, possibly overlying the Bergmann glial cells or Golgi type 2 neurons (Fig. 3A). Purkinje cells were not labeled. Also a high density of silver grains in the molecular layer, possibly overlying the basket and stellate neurons or glial cells of the cerebellar cortex was detected. Only a few silver grains were observed over the granular layer and white matter of the cerebellar cortex (Fig. 3A). When cerebellar tissue sections were hybridized with the control probe, only background levels of silver grains were observed (Fig. 3B).

Many mesencephalic and telencephalic areas such as cortex, hippocampus, and some thalamic nuclei revealed a low but above background density of silver grains when compared to sense RNA controls after DBI cRNA hybridization (Fig. 3C). Some nonneuronal structures around the third ventricle (ependyma, tanyocytes, and the subfornical organ) revealed a high density of silver grains after DBI mRNA hybridization (Fig. 3D). When serial sections of these areas were hybridized with the control probe, no specific labeling was observed.

Hybridization of the rat pituitary with antisense $^{35}$S-labeled DBI cRNA revealed a high level of silver grains overlying the posterior lobe (Fig. 4A), with only low levels of silver grains overlying the anterior and intermediate lobes.

Hybridization of liver sections with antisense $^{35}$S-labeled DBI cRNA revealed a uniform autoradiographic labeling overlying all cells of this tissue (Fig. 4B). Hybridization of spleen sections with antisense $^{35}$S-labeled DBI cRNA revealed specific labeling overlying the germinal center of the white pulp, with lower levels of silver grains overlying the surrounding red pulp (data not shown).

**DISCUSSION**

We have investigated the distribution of DBI mRNA in the rat brain and selected peripheral tissues by a sensitive and specific *in situ* hybridization method. Our results indicate that DBI mRNA is heterogeneously distributed in the rat brain, pituitary, and spleen. Particularly high levels of DBI mRNA were observed in the area postrema, the cerebellar cortex and ependyma of the third ventricle. However, after 2- to 4-week exposures with high specific activity $^{35}$S-labeled DBI cRNA probes, much lower levels of silver grains were observed overlying many mesencephalic and telencephalic areas such as cortex, hippocampus, and hypothalamic areas. These areas have previously been shown to contain numerous DBI immunoreactive neurons and DBI-L1 (14, 21, **). It is possible that the DBI-ODN in the cells of these areas has a slow turnover, which could be maintained by a low content of DBI mRNA. However, these areas are believed to be the sites effective in the anxiolytic and anticonvulsant actions of the benzodiazepines (1, 3, 14). In fact, the highest densities of central benzodiazepine recognition sites are found in the cerebral...
cortex and limbic structures, including the hippocampus (22, 23).

Previously, DBI mRNA and DBI immunoreactive polypeptides have been detected in numerous peripheral tissues including testis, heart, kidney, liver, lung, intestine, and adrenals (2, 5, 7, 10, 11). The present in situ hybridization studies confirm that DBI mRNA is located in liver and spleen cells. Peripheral benzodiazepine receptors have been identified based on their high affinity for 3H-labeled RO-54864, a benzodiazepine devoid of anxiolytic activity (24, 25). Peripheral benzodiazepine receptors have been detected in heart, lung, kidney, adrenals, pituitary, testis, liver, spleen, and in selected areas of the brain (18–20). The peripheral-type benzodiazepine receptor in rat adrenal gland has been localized to the mitochondrial outer membrane by the subcellular fractionation studies of Anholt et al. (25). The physiological function of the mitochondrial recognition site of peripheral-type benzodiazepines is unclear. However, numerous metabolic effects of peripheral-type benzodiazepines have been reported. These include (i) an enhanced melanogenesis in melanoma cells, (ii) an induction of hemoglobin synthesis in Friend erythroleukemia cells, (iii) an inhibition of prolifer-

Fig. 3. Dark-field photomicrographs of DBI mRNA localization. (A) Coronal section of rat cerebellum hybridized with antisense probe shows a high level of labeling overlying the inner third of the molecular layer (m) and a low level of labeling overlying the granular cell layer (g). (B) Similar section stained with sense (control) probe. (C) In the hippocampus a low density of silver grains was distributed throughout hippocampal formation; DG, dentate gyrus. (D) In the hypothalamic area the highest density of silver grains was observed in the ependyma and tanycytes of the third ventricle (3V), the nucleus arcuate (Arc), and the median eminence (ME). When serial sections were hybridized with a sense strand (control) probe, no specific labeling was observed in these areas, indicating that the antisense labeling was not a result of an edge artifact. (Bars = 20 μm).
these two different tissues (R.T.F. and J.B., unpublished observations). Finally, when a rat genomic Southern blot was probed with a 5' untranslated cDNA probe, only one major band was detected (5). Thus, it is likely that the majority of hybridizing sequences detected in rat and human DNA blot analyses are pseudogenes.

The recent observation by Alho and co-workers (17, ** that ODN-L1 is exclusively found in neurons, while DBI-L1 is present in some neurons, glia, and numerous peripheral tissues suggests that DBI may be processed differently in neurons compared to other cells. Consistent with this observation, Guidotti et al. (4, 30) have recently proposed that, in neurons, DBI is processed into ODN-like peptides, which are stored in synaptic vesicles (4, 17). According to this model, upon release, ODN may interact allosterically with the modulatory centers of various GABA<sub>A</sub> receptor subtypes, resulting in proconflict responses. Guidotti et al. (30) also propose that in glial cells, and cells in peripheral tissues, DBI may not be compartmentalized in subcellular particles and, in fact, it cannot be released from these cells by depolarization with veratridine or high K<sup>+</sup> (16). Thus, nonneuronal DBI may regulate intracellular functions.

In conclusion, we have shown that the DBI gene is expressed heterogeneously in the rat brain, pituitary, and spleen but homogeneously in the liver. The pattern of distribution of DBI mRNA described here leads to a consideration of alternative DBI function(s) unrelated to the allosteric modulation of GABA<sub>A</sub> receptors. The observation that the DBI polypeptide, or proteolytic fragments of DBI such as ODN and triakontatetraneuropeptide, injected intraventricularly, elicit two types of proconflict activities (4) inhibited by flumazenil or PK11195, respectively, indicates that, in brain, DBI may be the precursor of smaller peptides functioning as specific allosteric modulators of at least two GABA<sub>A</sub> receptor subtypes. However, further studies are necessary to establish the physiological function of DBI in the brain and nonneural tissue.