Journal of Neuroscience Research 64:493–500 (2001)

Up-Regulation of the Peripheral-Type Benzodiazepine Receptor Expression and [^{3}H]PK11195 Binding in Gerbil Hippocampus After Transient Forebrain Ischemia

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In mammalian CNS, the peripheral-type benzodiazepine receptor (PTBR) is localized on the outer mitochondrial membrane within the astrocytes and microglia. The main function of PTBR is to transport cholesterol across the mitochondrial membrane to the site of neurosteroid biosynthesis. The present study evaluated the changes in the PTBR density, gene expression and immunoreactivity in gerbil hippocampus as a function of reperfusion time after transient forebrain ischemia. Between 3 to 7 days of reperfusion, there was a significant increase in the maximal binding site density (B_{max}) of the PTBR antagonist [^{3}H]PK11195 (by 94–156%; $P < 0.01$) and PTBR mRNA levels (by 1.8- to 2.9-fold; $P < 0.01$). At 7 days of reperfusion, in the hippocampal CA1 (the brain region manifesting selective neuronal death), PTBR immunoreactivity increased significantly. Increased PTBR expression after transient forebrain ischemia may lead to increased neurosteroid biosynthesis, and thus may play a role in the ischemic pathophysiology. J. Neurosci. Res. 64:493–500, 2001.

Key words: forebrain ischemia; immunohistochemistry; mRNA; RT-PCR; mitochondrial benzodiazepine receptor; PK11195 binding

Transient forebrain ischemia induced in gerbil results in a significant delayed neuronal death, reactive astrogliosis and microglial activation in the hippocampal CA1 region between 3 and 7 days of reperfusion (Kirino and Sano, 1984; Petito et al., 1990; Gehrmann et al., 1992).

In CNS, activated astrocytes and microglia/macrophages are known to harbor the peripheral-type benzodiazepine receptor (PTBR) (Zisterer and Williams, 1997). The PTBR is structurally, functionally and pharmacologically distinct from the 'central-type' benzodiazepine receptor, that is a constituent of the ionotropic $GABA_A$ receptor complex localized on the neuronal plasma membrane. PTBR is mainly localized on the outer mitochondrial membrane. Structurally, PTBR exists as a hetero-oligomeric complex of 3 proteins viz., an 18 kDa isoquinoline carboxamide-binding protein, a 32 kDa voltage-dependent anion channel and a 30 kDa adenine nucleotide carrier (Kelly-Hershkovitz et al., 1998). The 18 kDa protein carries both antagonist (binds to the isoquinoline carboxamide PK11195) and agonist (binds to the benzodiazepine RO5-4864) binding domains (Joseph-Liauzun et al., 1997). The major function of PTBR seems to be the translocation of cholesterol across mitochondrial membranes to cytochrome-P450sec, that cleaves cholesterol to form the neurosteroid pregnenolone (Papadopoulous, 1998).

Our recent studies showed significant elevation in the PTBR expression in rat hippocampus and thalamus, two brain regions manifesting secondary neuronal death after traumatic brain injury (Rao et al., 2000b). Previous studies showed increased binding site densities of PTBR antagonist [^{3}H]PK11195 in rat models of cerebral ischemia (Benavides et al., 1990, Myers et al., 1991a,b; Stephenson

Contract grant sponsor: American Heart Association National; Contract grant number: 9950086N; Contract grant sponsor: American Heart Association Wisconsin Affiliate; Contract grant number: 9806376X; Contract grant sponsor: University of Wisconsin-Madison Medical School; Contract grant number: 161-9587; Contract grant sponsor: University of Wisconsin-Madison Start-Up; Contract grant sponsor: National Institute of Health; Contract grant numbers: NS28000 and NS31220.

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Received 17 April 2000; Revised 22 August 2000; Accepted 30 September 2000

et al., 1995). The purpose of the present study is to understand whether an increase in the affinity of the ligand to the existing binding sites or up-regulation of the PTBR gene expression is responsible for the ischemia-induced increased [^{3}H]PK11195 binding. Changes in [^{3}H]PK11195 binding, PTBR mRNA and protein expression were analyzed in gerbil hippocampus as a function of reperfusion time after transient forebrain ischemia. [^{3}H]PK11195 binding kinetics were evaluated by Scatchard analysis. PTBR mRNA expression was estimated by RT-PCR using PTBR 18 kDa subunit specific primers. PTBR protein expression was evaluated by immunohistochemistry using a polyclonal antibody raised against a recombinant fusion protein of 18 kDa PTBR subunit.

METHODS AND MATERIALS

Transient Ischemia

Transient forebrain ischemia was induced in 26 adult, male gerbils (body weight, 55–75 g) under halothane anesthesia as described earlier (Rao et al., 2000a). Briefly, both the common carotid arteries were occluded for 10 min followed by reperfusion. Body temperature was maintained at 37–38°C during the ischemic surgery. Seven gerbils were sacrificed at each reperfusion period (1 day, 3 days and 7 days), the hippocampus was quickly dissected out bilaterally, minced, mixed, and snap frozen in liquid nitrogen, and stored at −80°C. On Day 7 of reperfusion, the remaining 5 gerbils were sacrificed, the brains were snap frozen by immersing in isopentane cooled to −30°C and stored at −80°C. Sham-operated gerbils served as control.

[^{3}H]PK11195 Binding

[^{3}H]PK11195 filtration binding assays were performed as described earlier (Rao et al., 1997). Brain tissue was homogenized in 20 vol of ice-cold 50 mM Tris-HCl buffer (pH 7.4). Membranes were prepared and washed by repeated centrifugation (at 40,000 × *g* for 20 min at 4°C), freeze-thawed twice, and resuspended in 10 vol of fresh buffer. Protein content was estimated as described earlier (Lowry et al., 1951). Binding assays were performed by incubating the membrane preparations (40–50 μg protein equivalent) at 4°C for 2 hr in a final volume of 250 μl of fresh buffer containing 0.25 to 32 nM [^{3}H]PK11195 (specific activity, 86.9 Ci/mmol; NEN Life Science Products, Boston, MA). The assay was terminated by the addition of 2 ml of ice-cold buffer followed by vacuum filtration through GF/B glass microfiber filters (pretreated with 0.3% polyethylenimine). The filters were washed twice, dried, and radioactivity was determined by liquid scintillation spectrometry. Specific binding was defined as the difference between total and nonspecific binding (determined by inclusion of 20 μM PK11195) measured in parallel incubations. Data from saturation experiments was analyzed by iterative curve-fitting procedure to a single rectangular hyperbola and Scatchard analysis was performed by first-order nonlinear regression to estimate the equilibrium dissociation constant (K_D) and the maximal binding site density (B_{max}) (McPherson, 1994).

RT-PCR

PTBR and cyclophilin (used as a housekeeping gene) mRNA levels were estimated by RT-PCR as described earlier (Rao et al., 1999, 2000b). Total RNA extracted using QuickPrep total RNA extraction kit (Pharmacia Biotech, Piscataway, NJ) was treated with RNase-free DNase I to eliminate the putative contaminating DNA. RNA (0.5 μg) from each sample was reverse transcribed and PCR-amplified using Pharmacia Ready-To-Go RT-PCR beads (containing 10 mM Tris-HCl, pH 9, 60 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTPs, M-MULV reverse transcriptase, 2 U of Taq DNA polymerase, RNase inhibitor and BSA), 0.10 μCi/ml [α-^{32}P]dCTP (specific activity, 3,000 Ci/mmol) and 1 μM each of the gene-specific sense and antisense primers in a total volume of 50 μl. Reverse transcription (15 min at 42°C) was followed by inactivation (5 min at 95°C) and PCR amplification (95°C, 30 sec; 62°C, 30 sec; 72°C, 60 sec) for 22 cycles for PTBR and 13 cycles for cyclophilin (the amplification efficiency was observed to be linear between 16–26 cycles for PTBR and 8–16 cycles for cyclophilin ensuring that all the experiments were performed within the linear exponential phase of amplification). Aliquots of the amplification products were electrophoresed on polyacrylamide gels, stained with ethidium bromide, dried, autoradiographed and the Cerenkov radiation in each excised band was quantified in a scintillation counter. The oligonucleotide primers used for PCR were designed using the PRIMER program (Genetics Computer Group, University of Wisconsin-Madison, WI), based on the previously published PTBR sequence (Sprengel et al., 1989). The sequences of the forward and reverse primers were 5′-CCA TGC TCA ACT ACT ATG TAT GGC-3′ and 5′-GTA CAA CTG TCC CCG CAT G-3′ for PTBR and 5′-TGA GCA CTG GGG AGA AAG-3′ and 5′-AGG GGA ATG AGG AAA ATA-3′ for cyclophilin (Rao et al., 2000b).

Immunohistochemistry

The brains frozen at −80°C were thawed up to −15°C, sectioned in a cryostat (20 μm thick) and placed on superfrost glass slides. The slides were washed in chilled 70% ethanol (10 min) followed by 0.1 M phosphate-buffered saline (PBS; 3 × 5 min) and incubated in 3% H_2O_2 in 50% methanol (5 min) to quench the endogenous peroxidase activity. The slides were washed in 0.1 M PBS (3 × 5 min) and incubated in 10% fetal bovine serum in PBS (FBS) for 1 hr. The slides were then incubated overnight in PTBR antibody (1:500 diluted in FBS), washed in PBS (3 × 5 min) and incubated in FBS for 30 min. After a 1 hr incubation in biotinylated goat-anti-rabbit antibody (1:200, Vector Labs, Burlingame, CA), the sections were washed in PBS (3 × 5 min) and incubated in FBS for 30 min. Conjugation with the avidin-biotin complex (1:100, Vector Labs) was followed by visualization using 3,3′-diaminobenzidine tetrahydrochloride-hydrogen peroxide (Sigma, St. Louis, MO). Sections were dehydrated, cleared and mounted in Permount. Sections incubated without the primary antibodies served as negative control. The PTBR antibody used in this study (R & D Systems, Minneapolis, MN) was generated against purified, recombinant PTBR, that is a fusion protein with a molecular

weight of 20.4 kDa. The specificity of the PTBR antibody was validated by Western blot analysis using the purified PTBR protein as a positive control. Brain sections adjacent to those used for PTBR immunostaining were fixed for 10 min in 4% paraformaldehyde and 0.2% glutaraldehyde and stained with 1% cresyl violet (pH 6.3) for 10 min (Rao et al., 1999).

RESULTS

Transient Forebrain Ischemia Up-Regulated Hippocampal [^{3}H]PK11195 Binding

Scatchard analysis revealed that in gerbil hippocampus, the [^{3}H]PK11195 specific binding was saturable and to a single class of high-affinity sites with a B_{max} of 0.24 ± 0.06 pmol/mg protein and a K_d of 2.12 ± 0.29 nM. PK11195 (20 μm) displaced ~87% of the total [^{3}H]PK11195 binding. After transient forebrain ischemia, the B_{max} values for [^{3}H]PK11195 specific binding were significantly higher at 3 days (by ~94%, $P < 0.01$) and 7 days (by ~156%, $P < 0.01$) of reperfusion, compared to the sham-operated control (Fig. 1, Table I). There were no statistically significant changes in the K_d values of [^{3}H]PK11195 specific binding at any reperfusion period (Fig. 1, Table I).

Transient Forebrain Ischemia Up-Regulated the PTBR mRNA Expression in Hippocampus

PTBR mRNA amplification significantly elevated (by ~1.8- to 2.9-fold; $P < 0.05$) in the hippocampus, between 3–7 days of reperfusion after a 10 min transient forebrain ischemia, in comparison to sham-operated control (Fig. 2). Whereas, mRNA amplification of the housekeeping gene cyclophilin was unaltered at any reperfusion period (Fig. 2).

Transient Forebrain Ischemia Increased PTBR Immunoreactivity in Hippocampus

Cresyl violet staining showed significant neuronal death in the CA1 region of the hippocampus after transient forebrain ischemia. The average CA1 neuronal count of the sham-operated gerbils was 291 ± 42 (/mm length; mean ± SD). After transient forebrain ischemia, there was a progressive decrease in neuronal number in the hippocampal CA1 region between 3–7 days of reperfusion (with a maximal decrease of ~86% at 7 days), compared with the sham-operated control (Fig. 3). Sham-operated gerbil hippocampus showed very little PTBR immunoreactivity. PTBR immunoreactive cells were very few at 3 days of reperfusion, but abundant at 7 days of reperfusion in the hippocampal CA1 region (Fig. 4).

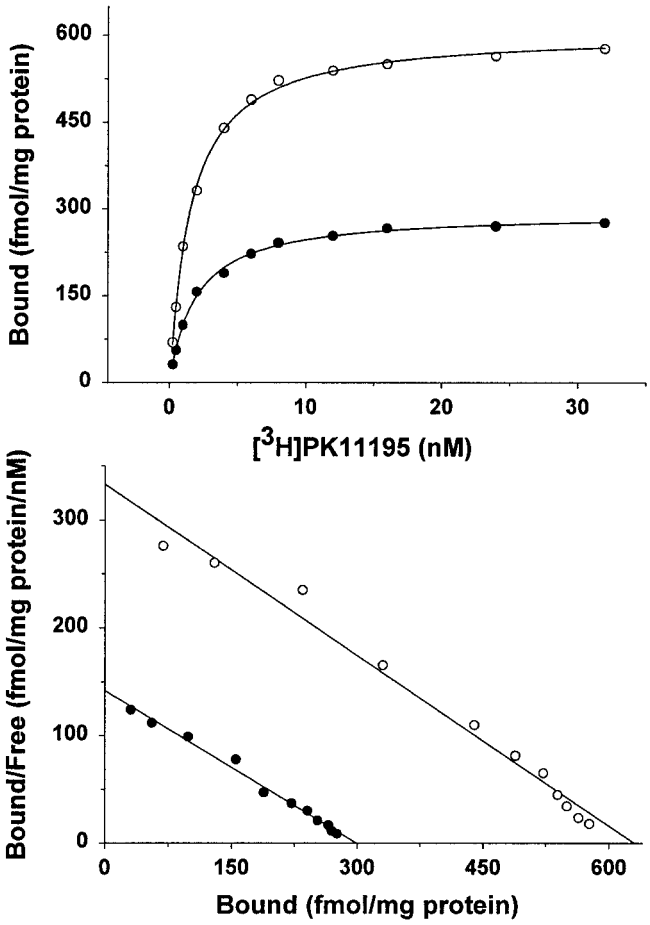

Fig. 1. Representative saturation isotherms (**upper panel**) and the corresponding Scatchard plots (**lower panel**) of specific [^{3}H]PK11195 binding in the hippocampus of gerbils after transient forebrain ischemia and 7 days of reperfusion (open circles) or sham-operation (filled circles). Data points are mean values of triplicate determinations with less than 10% SEM. Scatchard analysis was performed on each saturation curve by least square linear regression analysis. The mean ± SD of the B_{max} and K_d values calculated from six individual curves are presented in Table I.

DISCUSSION

Several neurological disorders are associated with altered PTBR density. Cerebral PTBR density decreases in post-traumatic stress disorder, major depression and generalized anxiety disorder, as an adaptive response for preventing chronic overproduction of glucocorticoids in the hyperarousal states (Gavish and Weizman, 1997). On the other hand, PTBR density increases in multiple sclerosis (Vowinckel et al., 1997), traumatic brain injury (Rao et al., 2000b), sciatic nerve injury (Lacor et al., 1996), motor neuron axotomy (Gehlert et al., 1997), focal cerebral ischemia (Benavides et al., 1990; Myers et al., 1991a,b), global cerebral ischemia (Stephenson et al., 1995; Conway et al., 1998), hepatic encephalopathy (Rao et al., 1994), congenital hyperammonemia (Rao et al., 1993), Alzheimer disease (McGeer et al., 1988), thiamine deficiency encephalopathy (Leong et al., 1996) and epilepsy (Ducis et al., 1990).

By virtue of being the mitochondrial cholesterol transporter, PTBR rate-limits the neurosteroidogenesis in CNS (Papadopoulos, 1998). Neurosteroids promote the CNS regeneration by increasing the survival, differentia-

TABLE I. Kinetics of [^{3}H]PK11195 Binding in Gerbil Hippocampus Following Transient Forebrain Ischemia*

	K_d (nM)	B_{max} (pmol/mg protein)
Sham	1.64 ± 0.45	0.18 ± 0.04
Reperfusion (1 day)	1.73 ± 0.52	0.23 ± 0.06
Reperfusion (3 days)	1.45 ± 0.39	0.35 ± 0.07[a]
Reperfusion (7 days)	1.68 ± 0.51	0.46 ± 0.12[a]

*The reperfusion times given are after a 10 min transient forebrain ischemia. Each value is mean ±SD (n of 6 per group) of triplicate determinations. Kinetic constants (B_{max} and K_d) were calculated by least square linear regression analysis from individual Scatchard plots generated from the data of saturation isotherms ran from 0.25–32 nM of [^{3}H]PK11195. The non-specific binding estimated in the presence of excess PK11195 was 10–16% in all cases.

[a]$P < 0.01$ compared with the sham-operated control by one-way ANOVA followed by Dunnet's multiple comparisons posttest.

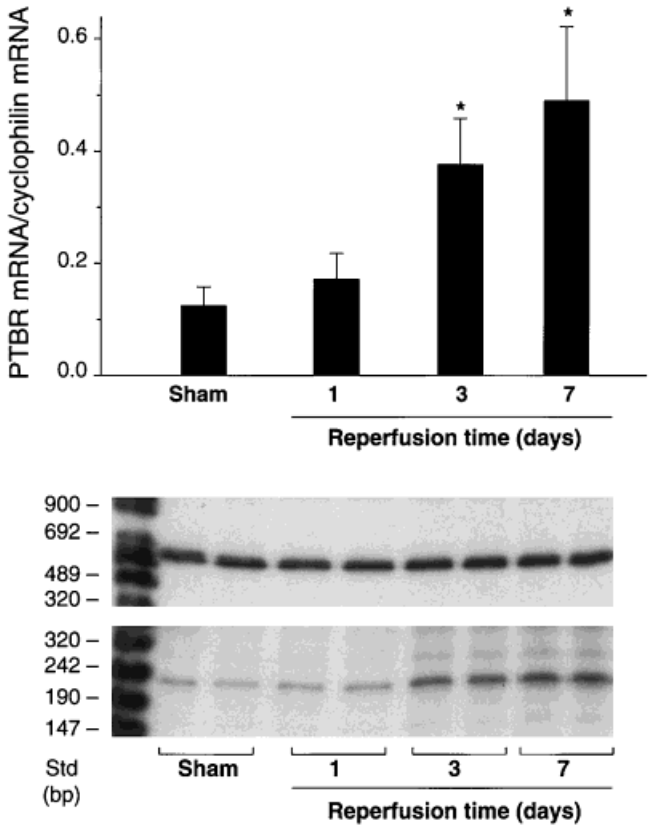

Fig. 2. RT-PCR analysis of PTBR mRNA expression in the hippocampus of gerbils at different period of reperfusion (1–7 days) after transient forebrain ischemia and sham-operation. Total RNA was reverse transcribed and PCR amplified in the presence of [α-^{32}P]dCTP and the PCR products were separated on polyacrylamide gels, stained with ethidium bromide, dried and autoradiographed. The RT-PCR yielded a ~234 bp product for PTBR and a ~521 bp product for cyclophilin. The figure shows representative gels showing PTBR (bottom gel) and cyclophilin (top gel) mRNA expression in two sets of animals. Similar results were obtained in six sets of animals analyzed in duplicate. The radioactivity in the excised PTBR and cyclophilin bands was determined by scintillation spectrometry.

tion and plasticity of both neurons and glia (Garcia-Segura et al., 1996; Schumacher et al., 1996). The cytoprotective nature of the neurosteroids can be attributed to their capacity to modulate the GABA-A receptor function resulting in an inhibition of chloride ion influx (Waters et al., 1997; Zhu et al., 1997). Pregnenolone, the most abundant neurosteroid in brain, promotes the recovery after spinal cord injury (Guth et al., 1994). Progesterone induces the formation of new myelin sheaths in the lesioned mouse sciatic nerve (Desarnaud et al., 1998) and reduces the neuronal damage after focal cerebral ischemia (Jiang et al., 1996). Neurosteroids also have anti-seizure effects (Frye et al., 1998), and reduces the memory impairment and learning deficits (Urani et al., 1998).

Transient forebrain ischemia leads to increased expression of the apoptosis-effector gene Bax promoting the neuronal death by disrupting the more favorable bcl-2 interaction (Antonawich et al., 1998). Transgenic mice with increased bcl-2 expression show significant reduction in the apoptotic neuronal damage after forebrain ischemia (Kitagawa et al., 1998). The PTBR antagonist PK11195 reverses the bcl-2 mediated cytoprotection, and thus may facilitate the induction of apoptosis (Hirsch et al., 1998). The biochemical mechanisms responsible for the induction of PTBR after neuronal insults are not yet understood clearly. Transient forebrain ischemia leads to microglial activation and macrophage infiltration and increased production of tumor necrosis factor (TNF) and the interleukin (IL) family of peptides that mediate the local inflammatory response (Sairanen et al., 1997; Uno et al., 1997). The PTBR agonist RO5-4864 inhibits the oxidative metabolism of macrophages and microglia and the production of TNF and IL 1 and 6 (Zavala et al., 1990). Furthermore, intracerebral injection of TNF and IL-1 up-regulate PTBR expression (Bourdiol et al., 1991). These studies suggest a link between increased PTBR expression and the inflammatory response after transient forebrain ischemia.

Available evidence suggests the usefulness of PTBR expression as a marker for neuronal injury. [^{11}C]-PK11195 was successfully used to visualize the excitotoxic lesions and to evaluate the cell types predominant in the site of injury in the brains of patients after ischemia (Ramsay et al., 1992), tumors (Black et al., 1990), Alzheimer disease (Groom et al., 1995) and multiple sclerosis (Vowinckel et al., 1997). It was also suggested to be a potential ligand for use in animal models of Wernicke-Korsakoff syndrome (Leong et al., 1996), hepatic encephalopathy (Rao et al., 1994), heavy metal toxicity (Kuhlmann and Guilarte, 2000) and experimental autoimmune encephalomyelitis (Vowinckel et al., 1997). A recent study used [^{123}I]iodo-PK11195 in single-photon emission tomography to visualize the inflammation in both humans and animals (Dierckx et al., 1999).

Both activated astrocytes and microglia express PTBR. The contribution of these two cell types toward

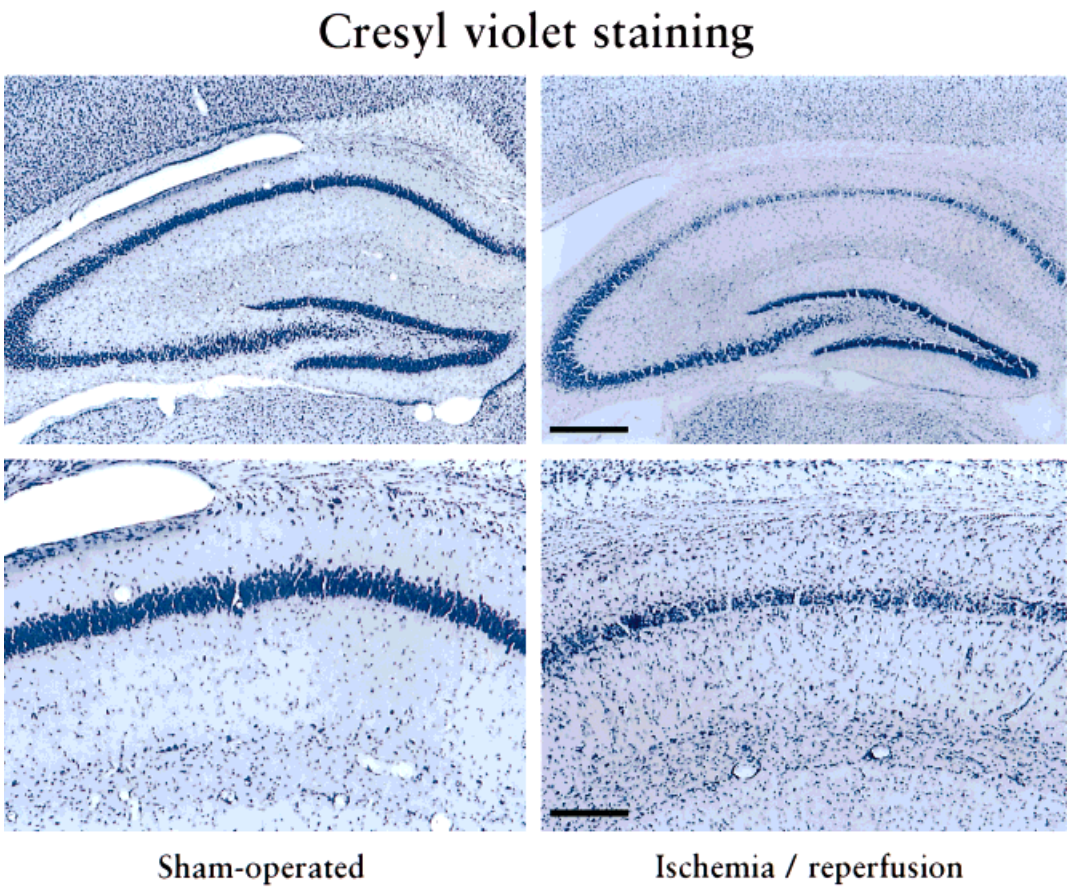

Fig. 3. Cresyl violet staining in the hippocampus of gerbils subjected to sham-operation (left panels) and transient forebrain ischemia and reperfusion (right panels) for 7 days. Significant neuronal loss in the ischemic gerbil can be seen. The pictures are from a representative set of animals. Similar result was observed in 5 sets of gerbils. Scale bar = 250 μm for top panels; 100 μm for bottom panels.

increased PTBR expression, however, depends on the type of insult to the CNS. In motor neuron axotomy and thiamine-deficiency encephalopathy, increased PTBR density was observed to be co-localized with both astrocytes and microglia (Leong et al., 1996; Gehlert et al., 1997; Todd and Butterworth, 1999). Whereas, in multiple sclerosis, increased PTBR expression was reported to be mainly associated with activated microglia rather than astrocytes (Vowinckel et al., 1997). Using the middle cerebral artery occlusion model of focal ischemia, Benavides et al. (1990) showed increased PTBR density in the astrocyte-like cells in the periphery of the lesion and macrophage-like cells in the core of the lesion. Whereas, Myers et al. (1991b) showed that increased PTBR density is mainly associated with the macrophages in the ischemic core after photochemically-induced focal ischemia. A study using a rat 4-vessel occlusion model suggested that activated microglia are mostly responsible for increased PTBR expression after global ischemia (Stephenson et al., 1995). A recent study from our laboratory suggested activated microglia/macrophages as the major site of increased PTBR density after traumatic brain injury (Rao et al., 2000b). All these three studies (Myers et al., 1991b; Stephenson et al., 1995; Rao et al., 2000b) correlated the PTBR to the two cell types by defining astrocytes and microglia by immunohistochemistry and PTBR density by autoradiography. To date, only one study examined the cellular localization of PTBR up-regulation using double labeling (Kuhlmann and Guilarte, 2000). Using a microglial-specific marker (*Griffonia simplicifolia* isolectin B4) or a astrocyte-specific marker (glial fibrillary acidic protein) together with PTBR antibody, they showed that both astrocytes and microglia are capable of expressing high levels of PTBR after trimethyltin induced hippocampal lesions (Kuhlmann and Guilarte, 2000). As transient forebrain ischemia in gerbils results in intense astrogliosis and microglial/macrophage infiltration, increased PTBR expression may be localized in both astrocytes and microglia/macrophages.

Results of the present study showed that increased number of binding sites (B_{max}) rather than increased affinity of the ligand to the binding sites is responsible for

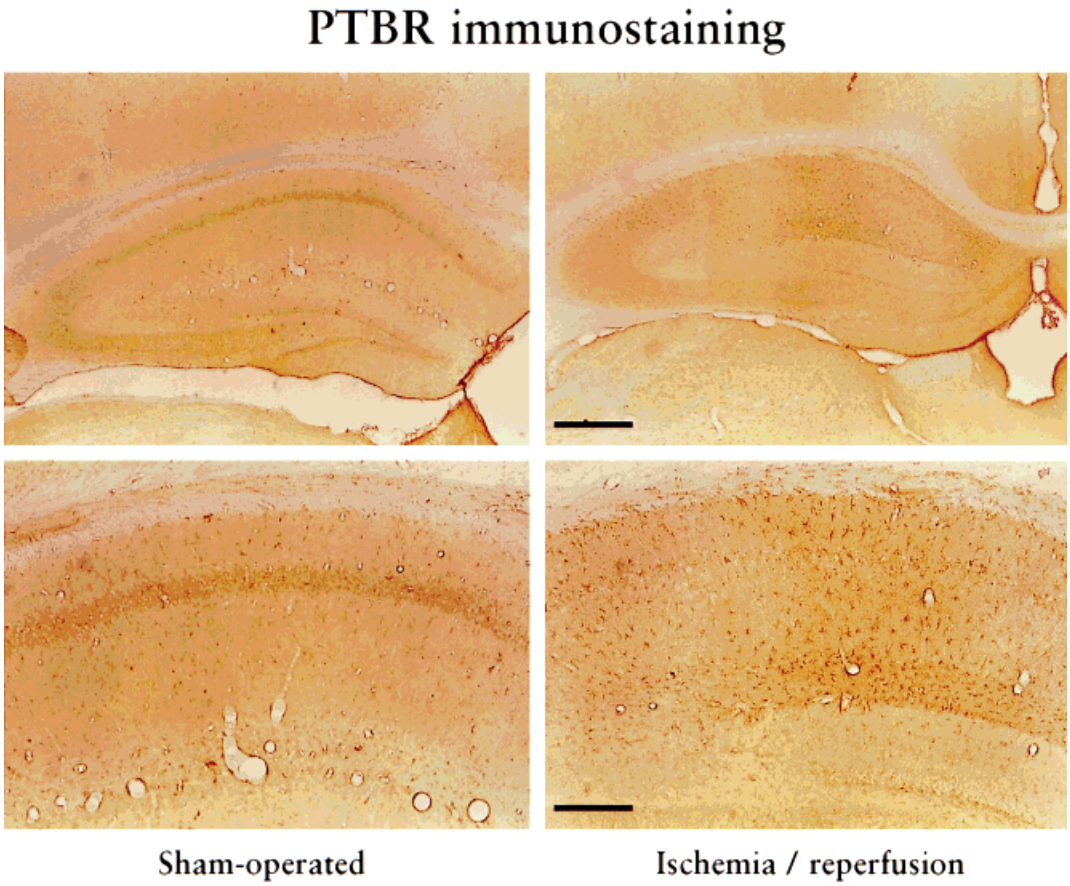

Fig. 4. PTBR immunoreactivity in the hippocampus of gerbils subjected to sham-operation (left panels) and transient forebrain ischemia and reperfusion (right panels) for 7 days. The pictures are from a representative set of animals. PTBR immunoreactivity increased in the CA1 region of the ischemic gerbil. Similar result was observed in 5 sets of gerbils. Scale bar = 250 μm for top panels; 100 μm for bottom panels.

increased [^{3}H]PK11195 binding after ischemia. Furthermore, increased PTBR density is associated with increased PTBR mRNA and protein expression. To the best of our knowledge, this is the first study showing increased PTBR mRNA expression responsible for increased [^{3}H]PK11195 binding. It is a difficult task to understand the significance of the changes in the expression of a protein like PTBR with a well-defined cellular and subcellular localization, but without a clearly defined function in CNS. PTBR agonists enhance the mitochondrial processing of manganese superoxide dismutase suggesting the therapeutic potential of PTBR in controlling mitochondrial import of proteins (Wright et al., 1999). A recent study also proposed a novel photodynamic therapy for tumor suppression using porphyrins, that binds to PTBR with high affinity (Verma et al., 1998). A better understanding of the changes in the PTBR expression and function after ischemia may lead to the development of novel neuroprotective therapies using the drugs, that modulate PTBR function.

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