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Rat Brain Thioltransferase: Regional Distribution, Immunological Characterization, and Localization by Fluorescent In Situ Hybridization

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Abstract: Thioltransferase (TTase) is a member of the family of thiol-disulfide oxidoreductases that are involved in the maintenance of sulfhydryl homeostasis in cells by catalyzing thiol–disulfide interchange reactions. One of the major consequences of oxidative stress in brain is the formation of protein-glutathione mixed disulfides (through oxidation of protein thiols), which can be reversed by TTase during the recovery of brain from oxidative stress. We therefore examined the presence of TTase in brain regions from rat. In the rat, TTase activity in the whole brain was comparable with the corresponding activity in liver, but significantly higher in hippocampus. The enzyme activity was significantly lower in striatum and cerebellum compared with activity in whole brain. Rat brain TTase shared immunological similarity with the human red blood cell enzyme, but not with the pig liver enzyme. The constitutive expression of the mRNA to TTase was demonstrable by northern blotting. Localization of the TTase mRNA in rat brain by fluorescent in situ hybridization showed the presence of high amounts of mRNA in the olfactory bulb, cortex, and hippocampus and its predominant localization in the neurons. TTase mRNA was also present in Purkinje cells in the cerebellum, in giant reticular neurons in the midbrain, and in the striatal and thalamic neurons. This study demonstrates the constitutive presence of a functional TTase system in brain and delineates the regional and cellular localization of the enzyme in rat brain. **Key Words:** Thiol-disulfide oxidoreductase—Thioltransferase—Glutaredoxin—Oxidative stress—Brain—Glutathione.
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Brain is highly vulnerable to oxidative injury due to its high oxygen tension, relatively low levels of antioxidant protective mechanisms, and elevated levels of polyunsaturated fatty acids. The more recent discoveries on the role of oxidative injury in the pathogenesis of several neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease (Marttila and Rinne, 1989; Schapira, 1996) have stimulated the need to examine the protective mechanisms that exist in brain.

One of the major consequences of oxidative injury in brain, such as that occurring during reperfusion after cerebral ischemia or in vitro treatment of isolated brain mitochondria with *tert*-butyl hydroperoxide, is the loss of the cellular antioxidant thiol GSH. The GSH lost is essentially recoverable as protein-glutathione mixed disulfides leading to loss of protein thiols (Ravindranath and Reed, 1990; Shivakumar et al., 1995). The loss of protein thiols is reflected in the inhibition of activities of enzymes that are known to have thiol groups in their active sites, such as sodium potassium ATPase (Shivakumar et al., 1995). After the recovery of brain from oxidative stress, GSH levels are restored and protein thiols are regenerated, reestablishing the thiol homeostasis in cells. Although glutathionylation of proteins is seen in cells of nonneural origin (Schuppe et al., 1992; Rokutan et al., 1994), it is more pronounced in brain because extrusion of GSSG does not seem to occur in brain (Ravindranath and Reed, 1990) as observed in other tissues such as lung and liver (Reed, 1990).

Thiol-disulfide oxidoreductases are a class of enzymes that are primarily involved in the catalysis of thiol–disulfide interchange reactions. The enzyme systems include thioltransferase (TTase) (glutaredoxin, EC 1.8.4.2; Mannervik and Axelsson, 1980; Wells et al., 1993), thioredoxin, and protein-disulfide isomerase (Holmgren, 1989). Whereas thioredoxin and protein-disulfide isomerase have broad sub-

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Abbreviations used: DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; RBC, red blood cells; TTase, thioltransferase.

strate specificity (Mieyal et al., 1995), TTase specifically reduces mixed disulfides that contain glutathione with greater efficiency than does thioredoxin (Axelsson and Mannervik, 1980; Gravina and Mieyal, 1993).

TTase is a low molecular mass protein (~12 kDa) essentially localized in the cytosol. The hepatic enzyme has been extensively characterized from rat, rabbit, and pig (Hatekayama et al., 1985; Gan and Wells, 1986, 1987*a*,*b*). TTase has also been purified from human red blood cells (RBCs) (Mieyal et al., 1991). Studies using the purified enzyme have demonstrated the substrate specificity of TTase and its ability to reduce certain protein-glutathione mixed disulfides more efficiently than thioredoxin (Gravina and Mieyal, 1993).

There is a paucity of information on brain TTase. More recently, the enzyme has been cloned using a cDNA library prepared from pooled cortices of several human brains and expressed in *E. coli* (Chrestensen et al., 1995). The human brain TTase nucleotide sequence shares 100% homology to the known human RBC enzyme (Papov et al., 1994). However, the actual presence of the functional enzyme in brain, its regional distribution, and its characterization are yet to be demonstrated. In the present study, we have examined the presence of a functional TTase system and its localization in brain regions of rat.

MATERIALS AND METHODS

Chemicals

Glutathione reductase, glutathione, dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), and NADPH were purchased from Sigma Chemical, U.S.A. Cysteinyl glutathione disulfide was purchased from Toronto Research Chemicals, Canada. Northern blot analysis was performed using the digoxigenin labeling kit from Boehringer Mannheim, Germany. Fluorescent in situ hybridization experiments were performed using the Tyramide signal-amplification kit from NEN Life Sciences Products, U.S.A.

Animals

Male Wistar rats (3–4 months, 225–250 g) were obtained from the Central Animal Research Facility of the Institute. Animals had access to pelleted diet and water ad libitum.

Preparation of cytosol

Rats were anesthetized with ether and perfused transcardially with ice-cold normal saline before decapitation. The brain regions (cortex, hippocampus, striatum, midbrain, cerebellum, and thalamus) were dissected out (Glowinsky and Iversen, 1966). Brain regions from 15 rats were pooled and homogenized in 10 ml of potassium phosphate buffer (0.137 *M*, pH 7.6). The homogenate was centrifuged at 100,000 *g* for 1 h. The supernatant was used for the estimation of TTase activity and immunoblot analysis. The supernatant was aliquoted and stored at −70°C. Cytosol was also prepared from whole brain and liver of rat.

Synthesis of cysteine *S*-sulfate

Cysteine *S*-sulfate, the substrate used for estimation of TTase activity, was synthesized according to the method of Segel and Johnson (1963) by aerating stoichiometric amounts of cysteine and sodium sulfite in aqueous ammonia containing trace amounts of cupric chloride. It was recrystallized two to three times from aqueous ethanol.

Assay of TTase

Activity of TTase was estimated spectrophotometrically at 340 nm, using cysteine-*S*-sulfate as substrate (Gan and Wells, 1986). The reaction mixture consisted of 200–300 μg of cytosolic protein from brain or liver, 50 μl of 5 m*M* GSH, 50 μl of 25 m*M* cysteine *S*-sulfate, and 50 μl of GSH reductase solution containing 24 U/ml, and the volume was made up to 450 μl with potassium phosphate buffer (0.137 *M*, pH 7.6). The reaction was initiated by adding 50 μl of 3.5 m*M* NADPH solution. The decrease in the absorbance at 340 nm was recorded for 5 min. Blanks were run simultaneously without addition of cytosol to measure nonenzymatic reaction. The net enzymatic rate was obtained by subtraction of nonenzymatic rate from total rate. The activity was calculated using the molar extinction coefficient of NADPH, which is 6,220 $M^{-1} \cdot cm^{-1}$.

TTase activity was also measured using cysteinyl glutathione disulfide as substrate. The reaction mixture consisted of 100 μg of cytosolic protein from brain or liver in 0.11 *M* phosphate buffer containing 0.5 m*M* GSH, 2 U/ml glutathione reductase, and 0.35 m*M* NADPH. The volume was made up to 0.45 ml with 0.11 *M* potassium phosphate buffer (pH 7.4). Reaction was initiated by the addition of the substrate cysteine glutathione disulfide (100 μ*M*, final concentration). The decrease in absorbance of NADPH at 340 n*M* was measured for 3 min. Blanks containing no cytosolic protein, no substrate, or no GSH reductase were also run simultaneously.

The enzyme activity was expressed as nanomoles of NADPH oxidized per minute per milligram of protein. Protein was estimated in the cytosol by a dye-binding method (Bradford, 1976).

Studies on effect of pH and temperature on the activity of rat brain cytosol

Potassium phosphate buffer (0.137 *M*) of various pH values (4.5, 5.5, 6.5, and 7.6), and Tris-HCl buffers (0.137 *M*) of different pH values (8.0 and 8.5), were prepared. The activity of TTase was estimated as described above using assay buffers of different pH values.

The cytosol prepared from whole brains of rats was aliquoted and heated in a water bath until the contents reached the particular temperature (37, 45, 60, and 80°C). The cytosol was incubated at that temperature for 1 min and immediately transferred to ice before estimation of enzyme activity. Cytosol kept at 4°C was used as control. Cytosol was also prepared from whole brain of rats by homogenizing the tissue in potassium phosphate buffer (0.137 *M*, pH 7.6) containing 0.1 m*M* DDT or 0.1 m*M* PMSF or both, and the thermal stability of the enzyme in each of these buffers was determined by incubating rat brain cytosol at various temperatures as described before.

Estimation of K_m and V_{max} for rat brain and liver TTase

The effect of protein concentration on the activity of TTase in rat brain was determined by measuring the enzyme activity with various concentrations of cytosolic protein. The activity of TTase in rat was also measured for varying periods of time ranging from 0.5 to 5 min. Blank reactions were also run simultaneously. The activity of TTase in the cytosol from brain and liver was estimated using various concentrations of either substrate, namely, cysteine *S*-sulfate (0.1–3 m*M*) or cysteinyl glutathione disulfide (50–600 μ*M*). The K_m and V_{max} values were calculated from an Eadie–Hofstee plot.

Immunoblot analyses

Cytosolic protein from brain regions of rat were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Laemmli and Favre, 1973). Proteins were transferred from the gel to nitrocellulose paper (Towbin et al., 1979) and incubated with the antibody to the human RBC enzyme (Mieyal et al., 1991) or pig liver TTase (Gan and Wells, 1986), followed by incubation with anti-rabbit IgG labeled with alkaline phosphatase. The immunostained bands were detected using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as chromogens.

Northern blotting

The total RNA from rat whole brain and liver was extracted as described by Chomczynski (1993). Total RNA was separated electrophoretically and transferred onto positively charged nylon membrane by capillary transfer (Kevil et al., 1997) followed by UV cross-linking and hybridized with the digoxigenin-labeled antisense riboprobe prepared using the cDNA to human brain TTase (Chrestensen et al., 1995). The sense, unlabeled RNA probe was synthesized using T3 polymerase and subjected to northern blotting as above. The membrane was hybridized overnight with digoxigenin-labeled antisense riboprobe at 55°C, washed, incubated with antibody to digoxigenin conjugated with alkaline phosphatase, and the bands were visualized using chromogenic substrate for alkaline phosphatase.

Fluorescent in situ hybridization

Male Wistar rats were anesthetized and perfused transcardially with normal saline followed by buffered formalin (200 ml/rat) before the removal of brain. The tissue was processed for paraffin embedding, and serial sections (8–10 μm thick) were cut in the coronal plane. Sections were dewaxed, hydrated in graded ethanol, acetylated, and treated with proteinase K. The sections were then rinsed in phosphate-buffered saline and dehydrated using graded ethanol. Digoxigenin-labeled sense and antisense cRNA probes were synthesized from cDNA to human brain TTase. Sections were hybridized overnight at 55°C with the sense or antisense probes. After hybridization, the sections were washed, incubated with blocking reagent 0.5% (wt/vol, NEN Life Sciences), and incubated with antibody to digoxigenin conjugated to horseradish peroxidase. After washing, the sections were incubated with biotinylated tyramide followed by fluorescein isothiocyanate-labeled streptavadin. Finally, the sections were washed, counterstained with Evans Blue, dried, and mounted before examination under fluorescence microscope.

RESULTS

TTase activity in rat brain regions

TTase activity was measurable in the cytosol prepared from all regions of the rat brain that were examined, such as the cortex, hippocampus, striatum, midbrain, cerebellum, and thalamus. The activity in the whole brain was not significantly different from that seen in the liver. However, differences were seen among the different regions in the brain; the highest activity was detected in the hippocampus and lowest activity in the striatum and cerebellum (Fig. 1). TTase activity in the hippocampus was significantly higher than that in the striatum, cerebellum, and liver.

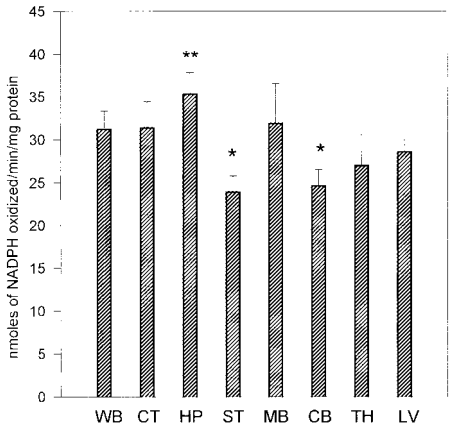

FIG. 1. Activity of TTase in cytosol of rat brain regions, whole brain, and liver. Activity of TTase was estimated in the cytosol prepared from rat brain regions, whole brain, and liver, using cysteine *S*-sulfate as substrate. Activity is expressed as nanomoles of NADPH oxidized per minute per milligram of protein. CT, cortex; HP, hippocampus; ST, striatum; MB, midbrain; CB, cerebellum; TH, thalamus; WB, whole brain; LV, liver. Data are expressed as mean ± SEM values (n = 8 animals). The significantly different values are indicated by asterisks ($p < 0.05$). *Significantly different from whole brain. **Significantly different from liver.

The K_m and V_{max} of TTase were determined in the cytosol prepared from the whole brain using cysteine *S*-sulfate or cysteine glutathione disulfide as substrates. The K_m and V_{max} values for the whole brain (Fig. 2), calculated from the Eadie–Hofstee plot, were 175 μM and 29.5 nmol of NADPH oxidized/min/mg, using cysteine S-sulfate as substrate. In the liver, using cysteine S-sulfate as substrate, the K_m and V_{max} values were estimated to be 43 μM and 37.5 nmol of NADPH oxidized/min/mg (Table 1).

TTase activity in whole brain, using cysteine glutathione disulfide as substrate, was 26.1 nmol of NADPH oxidized/min/mg of protein, which was comparable with the activity seen with cysteine *S*-sulfate (Fig. 1). The TTase activity was linear over a range of protein concentrations (800 μg/ml of cytsolic protein). The activity was linear for >5 min, and the rate-limiting factor was the availability of NADPH with either of the substrates tested (Fig. 3). The K_m and V_{max} values of TTase in whole brain, using cysteine glutathione disulfide as substrate, were 65.8 μM and 48.77 nmol of NADPH oxidized/min/mg of protein, whereas in the liver the corresponding values were 36.3 μM and 29.5 nmol of NADPH oxidized/min/mg of protein (Table 1).

Effect of pH and temperature on TTase activity in rat brain

The effect of pH on the activity of TTase was studied in the cytosolic fractions from whole brain by using the buffers of various pH values over a range of 4.5–8.5 (4.5, 5.5, 6.5, 7.0, 7.6, and 8.5). The activity of the enzyme increased with increasing pH and was maximal at pH 8.5, the highest pH tested (data not shown).

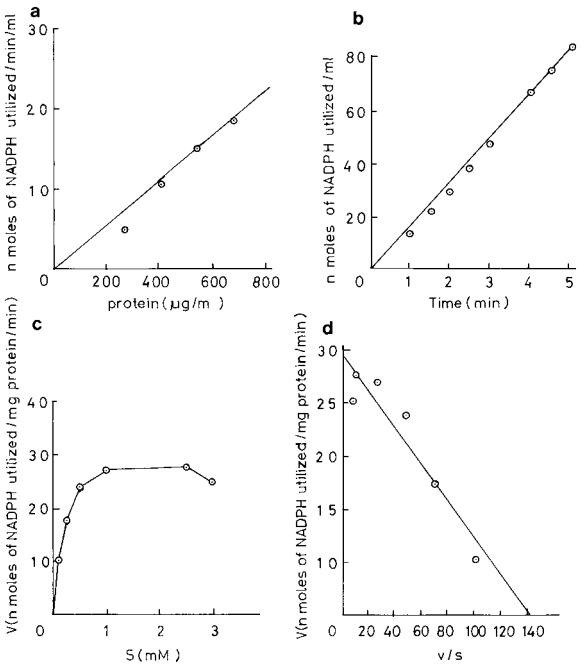

FIG. 2. TTase activity in rat brain cytosol, using cysteine *S*-sulfate as substrate. Effect of varying concentration of protein (**a**), time (**b**), and concentration of substrate (**c**) on TTase activity in rat brain cytosol and Eadie–Hoftsee plot (c) is represented (**d**). K_m and V_{max} values, calculated from d, are 175 μM and 29.5 nmol of NADPH oxidized/min/mg of protein, respectively. Enzyme assay was performed as described in Materials and Methods; values shown are averages of three independent assays.

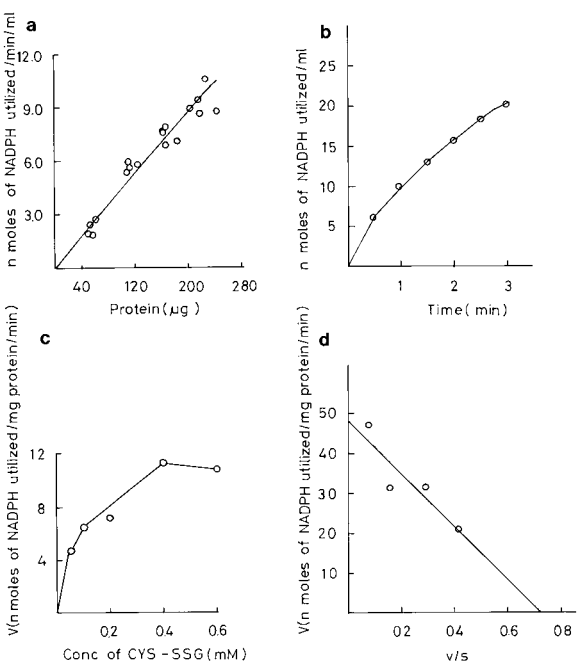

FIG. 3. TTase activity in rat brain cytosol, using cysteine glutathione disulfide as substrate. Effect of varying concentration of protein (**a**), time (**b**), and concentration of substrate (**c**) on TTase activity in rat brain cytosol and Eadie–Hoftsee plot (c) is represented (**d**). K_m and V_{max} values, calculated from d, are 66 μM and 48.8 nmol of NADPH oxidized/min/mg of protein. Enzyme assay was performed as described in Materials and Methods; values shown are averages of three independent assays.

The thermal stability of the enzyme in the absence of the thiol reductant (DTT) or protease inhibitor (PMSF) was determined by incubating rat brain cytosol at various temperatures before the estimation of the activity. There was a gradual loss in the activity of the enzyme with increasing temperature; 26% of the activity was lost after incubation of rat brain cytosol at 45°C for 1 min and most of the activity was lost at 65°C (Fig. 4). In the presence of DTT and PMSF, there was no loss in the activity of the enzyme even at 80°C. In the presence of PMSF alone, there was a gradual loss in the activity with increasing temperature. However, there was no such temperature-dependent loss in enzyme activity when the activity was estimated in presence of DTT alone, indicating that thiol oxidation and not proteolytic cleavage was responsible for the loss in TTase activity.

TABLE 1. *Kinetic properties of rat brain and liver TTase-catalyzed reactions using two different substrates*

	Cysteine *S*-sulfate		Cysteinyl glutathione disulfide	
	K_m	V_{max}	K_m	V_{max}
Brain	175.0	29.5	65.8	48.8
Liver	43	37.5	36.3	29.5

Rat brain and liver cytosolic protein was used for assay of enzyme activities. K_m and V_{max} values are expressed as micromolar and nanomoles of NADPH oxidized per minute per milligram of protein, respectively. Experimental conditions are described in the legends to Figs. 2 and 3.

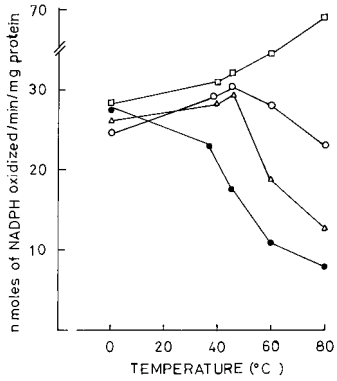

FIG. 4. Effect of temperature on the activity of rat brain TTase. Rat brain cytosol was incubated at varying temperatures in buffer alone (●----●), buffer containing DTT (○----○), buffer containing PMSF (△----△), or buffer containing both DTT and PMSF (□----□) for 1 min before the estimation of enzyme activity, using 2.5 m*M* cysteine *S*-sulfate. Activity of the enzyme using cysteine *S*-sulfate as substrate is expressed as nanomoles of NADPH oxidized per minute per milligram of protein. Values shown are averages of duplicate analysis.

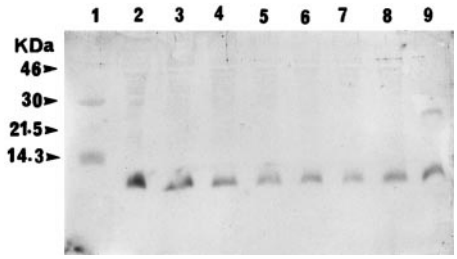

FIG. 5. Immunoblot analysis of cytosol prepared from rat brain regions, immunostained with antibody to human RBC TTase. Each lane was loaded with 30 μg of cytosolic protein from various regions of brain or whole brain. The wells were loaded as follows: Lane 1, marker proteins; lane 2, cortex; lane 3, hippocampus; lane 4, striatum; lane 5, midbrain; lane 6, cerebellum; lane 7, thalamus; lane 8, whole brain; lane 9, human RBC TTase.

Immunological studies

The immunological cross-reactivity of rat brain TTase with the enzyme from human RBCs was discernible in immunoblotting experiments. The constitutive presence of the enzyme in various regions of rat brain could be demonstrated by immunoblotting, using the antibodies raised against human RBC TTase, wherein the presence of a single band of ~11.5 kDa was seen in all regions of the brain that were examined (Fig. 5). The immunoreactive protein comigrated with the recombinant human brain TTase. Similar experiments performed with antibody to pig liver TTase (Gan and Wells, 1986) did not reveal any immunoreactive bands (data not shown).

Localization of TTase by fluorescent in situ hybridization

Northern blot analysis of total RNA from rat brain cortex demonstrated the expression of TTase mRNA in rat brain. The molecular mass of the transcript was ~300 bp (Fig. 6). The molecular mass of the unlabeled cRNA (Fig. 6, lane 1) synthesized using the linearized plasmid containing the cDNA to human brain TTase was of larger molecular mass (420 bp), as it contained the multiple cloning site of the vector. Fluorescent in situ hybridization (FISH) studies demonstrated the presence of TTase mRNA predominantly in neuronal cells in rat brain regions. High levels of TTase mRNA were seen in the olfactory bulb (Fig. 7A), cerebral cortex (Fig. 7B), and hippocampus (Fig. 8A). In the olfactory bulb, the neurons in the glomeruli (Fig. 7A) and anterior olfactory nucleus (Fig. 7A, inset) were intensely labeled. The neuronal cells in the cerebral cortex showed intense cytosolic staining indicating the presence of the TTase mRNA (Fig. 7C), whereas the sections hybridized with the sense probe showed no fluorescence (Fig. 7D). In the cerebellum, the Purkinje cells were intensely fluorescent, but the granule cell layer was less intensely stained (Fig. 7E). In the midbrain, the reticular neurons were selectively labeled, indicating the predominant presence of TTase mRNA in these cell populations (Fig. 7G). Maximal staining was seen in hippocampus, in the pyramidal neurons of CA1, CA2, CA3, and CA4, the granule cell layer in the dentate gyrus (Fig. 8A), and in Ammon's horn (Fig. 8F). The endothelial cells lining the blood vessels were intensely labeled (Fig. 8E). The habenular nucleus (Fig. 8C), the fornix, and nucleus triangularis septi (Fig. 8E) were also labeled. There was only sparse labeling of neurons in the striatum, whereas the anterior thalamic nuclei was intensely fluorescent (Fig. 8G).

DISCUSSION

TTase, which belongs to the class of thiol-disulfide oxidoreductases, is an enzyme that is specifically involved in the reduction of protein-glutathione mixed disulfides to protein thiols in the presence of GSH, using reducing equivalents of NADPH. As the formation of protein-glutathione mixed disulfide is one of the major consequences of oxidative stress in the brain, TTase would potentially play an important role in the recovery of brain from oxidative stress when protein thiol homeostasis in the brain is restored. The present study is one of the first attempts to examine the presence of the functional enzyme in the brain and to study its regional distribution. The enzyme activity in rat brain was comparable with that seen in liver. Observations made in the present study, using cysteinyl glutathione disulfide as substrate, indicate that the brain TTase has lower affinity (K_m = 65.8 μM) and higher activity (48.77 nmol of NADPH oxidized/min/mg of protein) compared with liver (36.3 μM and 29.5 nmol of NADPH oxidized/min/mg of protein, respectively; Table 1). In earlier studies using partially purified rat liver cytosol, the K_m has been calculated as 50 μM, using cysteinyl glutathione as substrate (Axelsson et al., 1978).

In view of the fact that GSH levels (an essential cofactor for TTase) in the brain are typically one-third of those in liver (Ravindranath et al., 1989), it is interesting that the TTase activity in brain is detectable in significant amounts. The presence of this enzyme in high amounts in brain points to the probable role of this enzyme in the reduction of protein-glutathione mixed disulfides, especially those that are formed in high amounts during oxidative stress in brain (Shivakumar and Ravindranath, 1992, 1993; Shivakumar et al., 1995). The enzyme is not homogeneously distributed in brain, as demonstrated by

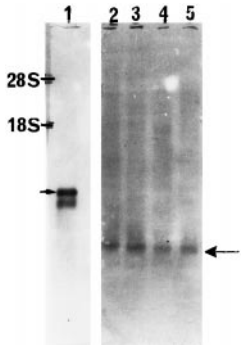

FIG. 6. Northern blot analysis of brain RNA, using the cDNA to human brain TTase. The sense unlabeled cRNA was synthesized using the cDNA to TTase and loaded in lane 1. Lanes 2 and 3 were loaded with total RNA from rat brain cortex (15 and 30 μg, respectively), and lanes 4 and 5 were loaded with total RNA from liver (15 and 30 μg, respectively).

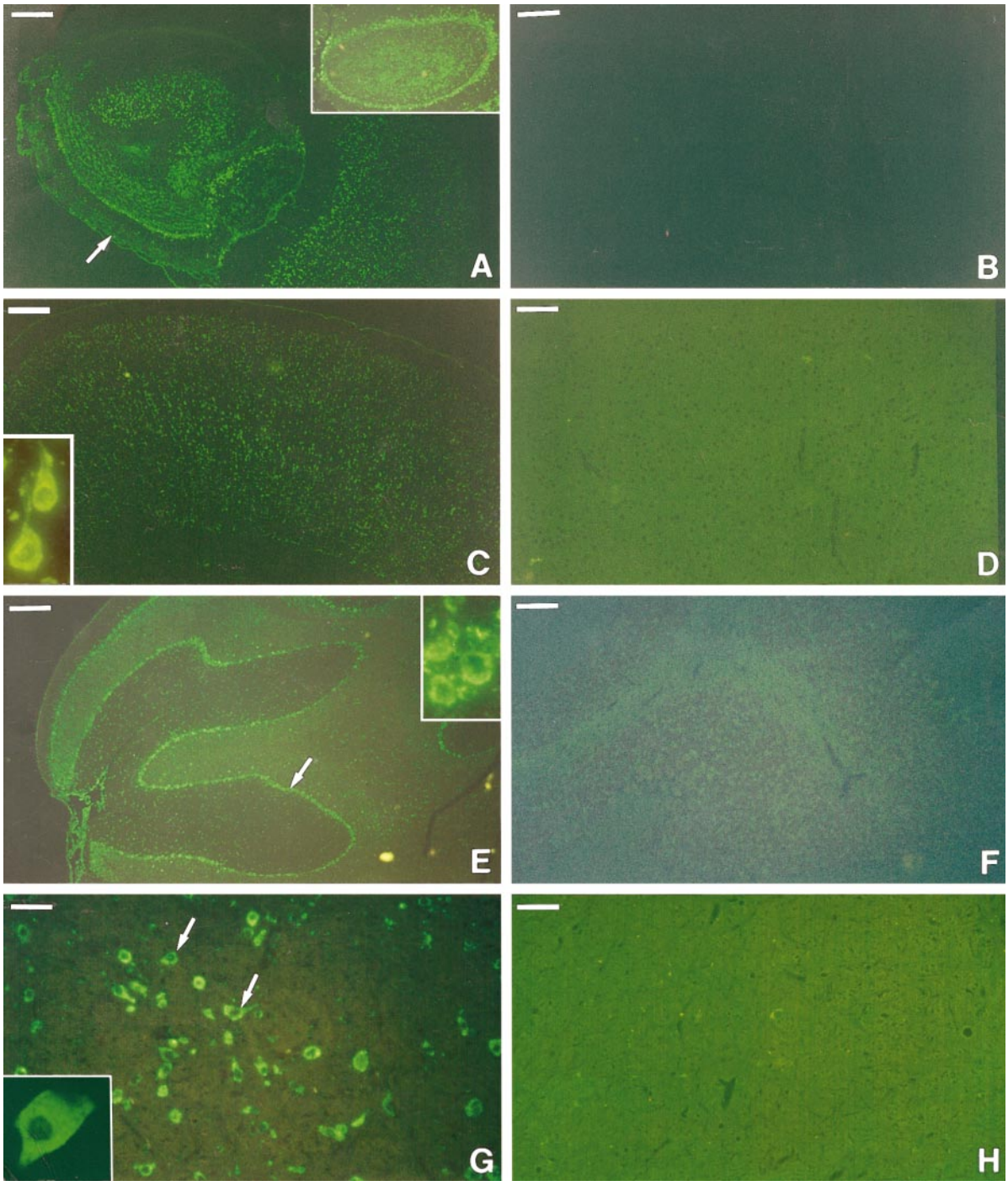

FIG. 7. Localization of TTase in rat brain by fluorescent in situ hybridization, using the cDNA to human brain TTase. **A:** In situ hybridization of coronal sections from rat brain showing the intense labeling of the neurons in the olfactory bulb, which express TTase mRNA. The neurons of the anterior olfactory nucleus (**inset**) are intensely fluorescent. The neurons of the glomeruli (arrow) are also fluorescent. **B:** Control section hybridized with the sense probe. Bar = 200 μm. **C:** The presence of TTase mRNA in the neurons of cerebral cortex. Note the differential fluorescence in the laminar architecture of the cortex. **Inset:** Higher magnification of the cortical neurons, showing exclusive staining in the cytoplasm. **D:** Control section hybridized with the sense probe. Bar = 200 μm. **E:** Fluorescent labeling of the Purkinje cells (arrow) in the rat cerebellum. **Inset:** Higher magnification of the Purkinje neurons containing the TTase mRNA. **F:** Control section of rat cerebellum hybridized with the sense probe and counterstained with Evans Blue. Bar = 200 μm (E) and 100 μm (F). **G:** The reticular neurons (arrow) in the midbrain expressed the TTase mRNA. **Inset:** Higher magnification of a giant reticular neuron. **H:** Control section depicting the midbrain. Bar = 100 μm.

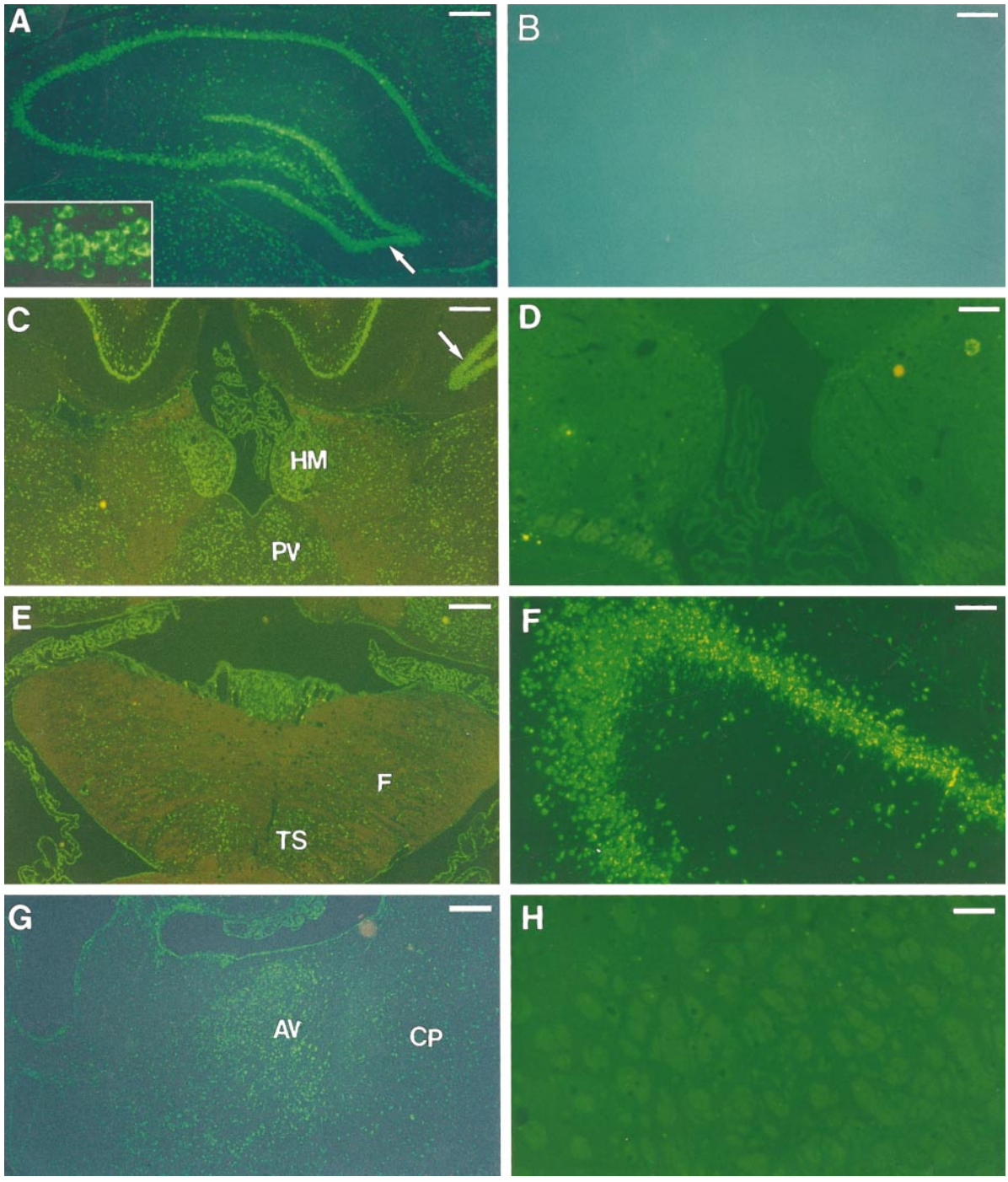

FIG. 8. Localization of TTase mRNA in rat brain, using fluorescent in situ hybridization. **A:** Intense fluorescence was seen in the pyramidal cell layer of the hippocampus. The granule cell layer of the dentate gyrus (arrow) was also intensely fluorescent. **Inset:** Higher magnification of the CA3 neurons of the hippocampus. **B:** Control section hybridized with the sense probe. Bar = 200 μm. **C:** The habenular nucleus (HM) and the paraventricular nucleus of the thalamus (PV) expressed the TTase mRNA. The neurons of the dentate gyrus (arrow) are also labeled. The corresponding control section is depicted in (**D**). Bar = 200 μm (C) and 100 μm (D). **E:** The blood vessels in the ventricles were intensely fluorescent, indicating the presence of TTase mRNA. The nucleus triangularis septi (TS) and the fornix (F) were also labeled. Bar = 200 μm. **F:** The neurons in Ammon's horn were intensely fluorescent, indicating the presence of TTase mRNA. Bar = 100 μm. **G:** The anteroventral nucleus of the thalamus (AV) is strongly fluorescent, and the neurons in the caudate putamen (CP) are sparsely stained. **H:** Control section stained with the sense probe. Bar = 200 μm (G) and 100 μm (H).

the variable activity seen in different regions. In the rat brain, highest activities were seen in the hippocampus followed by the cortex and midbrain, whereas low levels of the enzyme were present in the striatum and cerebellum, which was reflected in the fluorescent in situ hybridization studies. Indeed, the hippocampus responds to oxidative stress by substantial loss in GSH levels and formation of protein-glutathione mixed disulfide. Further, it is in this region that the maximum rebound of the GSH synthesis is observed and the protein–thiol hemostasis is restored after moderate oxidative stress (Shivakumar et al., 1995). The variability in the regional distribution of the enzymes may be particularly significant, as the brain is highly heterogeneous anatomically, functionally, and biochemically. Brain regions such as the hippocampus, which have high activity of TTase, could potentially be more adept at recovering from the damage caused by oxidative stress.

Studies on the pH dependence of the enzyme revealed that TTase showed maximum activity at pH 8.5, which is similar to the rat liver TTase (Gan and Wells, 1986). One of the most dramatic differences between the liver and brain TTase (both rat and human) was observed in the thermostability experiments. Rat brain TTase activity decreased by 26% when the cytosol was heated to 45°C, and, when the temperature was raised to 60°C, 86% of the activity was lost (Fig. 2). This is in contrast to the observations made with TTase from other sources (such as rat and pig liver, human RBC, or *E. coli*) where the enzyme is known to be entirely stable up to 60°C. However, rat brain TTase was remarkably sensitive to even small increases in temperature (Figs. 2 and 7). The presence of DTT (a thiol-reducing agent) in the homogenization buffer completely protected the enzyme activity from the temperature-induced loss, indicating that oxidation of thiol groups of the enzyme could be responsible for loss of activity at higher temperatures, whereas presence of only PMSF in homogenization buffer did not protect from the heat-induced loss.

Immunoblot analysis of rat brain cytosol, using the antibody raised against purified human RBC TTase, revealed that immunological similarity exists between the rat and human brain enzymes. The constitutive expression of TTase in rat brain was further confirmed by northern blot analysis. These studies revealed the presence of a band corresponding to the mRNA of TTase. The molecular size of mRNA corresponding to TTase was 300 bp, which is similar to that of TTase cDNA cloned from human brain.

The predominant localization of the enzyme in neuronal cells, as demonstrated in this study, suggests a potential role of the enzyme in the recovery of neuronal cells from oxidative stress, such as that which occurs under certain clinical conditions. Recent research on the biochemical mechanism of several neurodegenerative disorders has implicated oxidative stress in both pathogenesis and progression of disease (Schapira, 1996). The presence of TTase in specific brain regions and its predominant neuronal localization, demonstrated for the first time in this study, suggests an important role of some of the factors involved in recovery of the brain from oxidative injury. The endogenous thiol GSH is predominantly localized in the glial cells in the brain; in fact, very little staining of the neurons is seen during histochemical evaluation of GSH (Philbert et al., 1991). The differential localization of GSH and TTase in the glial and neuronal cells, respectively, is indeed surprising, as GSH is necessary for TTase activity. The presence of a sodium-dependent GSH transporter has been identified in brain microvessels (Kannan et al., 1996); however, it remains to be seen if such a transporter also exists in the neuronal cells for transport of GSH from the glial cells.

TTase enzyme plays a major role in the maintenance of the thiol/disulfide ratio in the cell (Mannervik and Axelsson, 1980). Most studies performed to date have focussed mainly on characterization and purification of the enzyme from various sources. The regulation and function of TTase normally, as well as during oxidative stress, have not been clarified in any system so far (Mieyal et al., 1995). Although the present study has demonstrated for the first time the presence of a functional enzyme in brain, it is important to clarify the physiological role of TTase in the brain. In view of the increased formation of protein-glutathione mixed disulfides in the brain during oxidative stress, understanding the function of TTase in the brain during oxidative stress assumes greater importance.

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