Glutathione S-Transferase Isoenzymes in Rat Brain Neurons and Glia

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The glutathione S-transferases (GSTs) constitute a family of cytosolic isoenzymes and a structurally unrelated microsomal enzyme that is involved in the detoxication of electrophilic xenobiotics. These enzymes also participate in the intracellular binding and transport of a broad range of lipophilic compounds including bilirubin, and hormones such as the glucocorticoids and thyroid hormones. The present investigation demonstrates that GSTs are present in neurons of the brainstem, forebrain, and cerebellum. An isoenzyme-specific distribution of GSTs was found in cytoplasm, nuclei, and nucleoli.

The regional and cellular distribution of cytosolic GSTs in the brain was studied by immunohistochemistry, spectrophotometric enzyme assay, and reverse-phase HPLC. Polyclonal antibody against microsomal GST was strongly reactive with Purkinje cells throughout the cerebellar cortex, and with neurons in the brainstem and hippocampus. Nuclei of Purkinje cells and of neurons in the brainstem, hippocampus, and cerebral cortex were immunopositive for α -class GST 1-1 (Y_aY_a), whereas α -class GST 2-2 (Y_cY_c) antibody was consistently immunoreactive with the nucleolus, but not with the nucleus or soma. All α -class GST antibodies studied were reactive, to various degrees, with astrocytes and choroid plexus; however, ependymal cells of the subventricular zones were immunonegative. α -class GST 8-8 (Y_kY_k) immunoreactivity was specifically localized to endothelial cells and/or astrocytic end feet associated with blood vessels.

Reverse-phase HPLC indicated that there were also substantial regional differences in the pattern of α -, μ -, and π -class GST subunit expression. For example, the thalamus/hypothalamus had the highest GST activity and the greatest concentration of total GST protein and μ -class GST subunit 6 (Y_{b3}), whereas the brainstem had the greatest concentration of π -class GST subunit (Y_p). This regional variation in GST expression may be reflective of regional differences in cell populations. In cerebellar cortex, the concentration of μ -class

GST subunit 4 (Y_{b2}) was greatest in the flocculus and lowest in the vermis. This is of clinical interest because the pattern of expression of μ -class GST subunit 4 (Y_{b2}) in the cerebellum coincides with the known regional susceptibility of this structure to degeneration after exposure to toxic or metabolic insults. The vermis is most susceptible to these insults, whereas the lateral lobes and flocculus are most resistant. The nuclear localization of α -class GSTs in neurons, the cytoplasmic localization of microsomal GST in neurons, and the association between concentration of μ -class GST subunit 4 (Y_{b2}) and resistance of neurons to toxic events in the cerebellar cortex suggest that GSTs may confer protection against exogenous and/or endogenous neurotoxic metabolites.

[Key words: glutathione S-transferase, neurons, Purkinje cells, Bergmann glia, cerebellum, immunohistochemical localization, HPLC]

Glutathione S-transferases (GSTs; EC 2.5.1.18) constitute a family of multifunctional enzymes that are involved in metabolic detoxication of a variety of electrophilic xenobiotics (Ketterer et al., 1988; Mannervik and Danielson, 1988; Vos and Van Bladeren, 1990). Cytosolic GSTs are present in most mammalian organs and exist as dimeric proteins consisting of two identical or closely related subunits from the same class (α , μ , π , θ ; Mannervik and Danielson, 1988; Meyer et al., 1991). Rat tissues contain at least 20 dimers made up of 15 different subunits (Tu et al., 1983; Ketterer et al., 1988; Mannervik and Danielson, 1988; Kispert et al., 1989; Hayes et al., 1990a,b; Hiratsuka et al., 1990; Tsuchida and Sato, 1990; Harris et al., 1991; Hayes et al., 1991; Meyer et al., 1991). Microsomal GST is a homotrimer consisting of a subunit that is structurally distinct from the cytosolic enzymes (Morgenstern and DePierre, 1985; Morgenstern et al., 1985). Cytosolic GST isoenzyme profiles are altered in preneoplastic foci, tumor tissue, and multidrug-resistant cell lines (Sato et al., 1985; Cowan et al., 1986; Moscow et al., 1989). Enzymatic activity is regulated at the transcriptional level by a variety of inducing compounds (Pickett et al., 1984; Ding et al., 1986; Lee et al., 1986; Benson et al., 1989; Di Simplicio et al., 1989; Rushmore et al., 1990). In contrast, no compounds have been shown to increase hepatic concentrations of microsomal GST (Morgenstern and DePierre, 1985; McLellan and Hayes, 1989; McLellan et al., 1991). Post-translational regulation of GSTs is also suggested by reports that GSTs are activated by active oxygen species (Murata et al., 1990), that α -class GSTs are substrates for protein kinase C (Pyerin et al., 1987; Taniguchi and Pyerin, 1989), and that μ -class GSTs are methylated by specific methyltransferases (Neal et al., 1988; Johnson et al., 1990, 1992a).

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GSTs have been shown to be involved in intracellular transport mechanisms and the binding of endogenous nonsubstrate ligands (Listowsky et al., 1988). These enzymes bind thyroid hormones L-3,3',5-triiodothyronine (T_3) and L-3,3',5,5'-tetraiodothyronine (T_4), which have been shown to control cerebellar development (Kornguth et al., 1967; Kornguth et al., 1979; Legrand, 1979; Silva and Rudas, 1990; Legrand and Clos, 1991). GSTs also bind bilirubin, which is toxic to the cerebellum (Schutta and Johnson, 1967; Sawasaki et al., 1976; Takagishi and Yamamura, 1989), and are postulated to be involved in cellular uptake and intracellular transport of bilirubin (Listowsky et al., 1988). Li et al. (1986) demonstrated the tissue-specific expression of a unique μ -class GST polyA RNA species in rat brain and the expression of α -class Y_c , but not Y_a , RNA in rat brain. In addition, a gene encoding the μ -class GST 6 (Y_{b3}) protein was found to be selectively expressed in rat brain (Abramovitz and Listowsky, 1987). At least two isoenzymes have been localized to glial cells in human, rat, and mouse brain (Senjo and Ishibashi, 1985; Senjo et al., 1986; Abramovitz et al., 1988; Cammer et al., 1989; Carder et al., 1990; Campbell et al., 1991; Tansey and Cammer, 1991a,b). In rats and mice, the μ -class isoenzymes were immunohistochemically localized in astrocytes, subventricular zone cells, ependymal cells, and tanocytes, whereas π -class GST was found in oligodendrocytes (Abramovitz et al., 1988; Cammer et al., 1989; Tansey and Cammer, 1991b) and associated with myelin (Tansey and Cammer, 1991a). In human brains, either α -class isoenzymes were not detectable or immunoreactivity was inconsistent (Carder et al., 1990; Campbell et al., 1991). Neurons in rat and mouse brain were not immunoreactive with either μ - or π -class GST antibodies (Abramovitz et al., 1988; Cammer et al., 1989; Tansey and Cammer, 1991a), and in humans, neurons were immunonegative for antibodies against α -, μ -, and π -class GSTs (Carder et al., 1990; Campbell et al., 1991). To date, the immunohistochemical distribution of cytosolic α -class GSTs in rodent brain and microsomal GST in rodent and human brain remains unknown. The present investigation was designed to: (1) identify, by immunohistochemical methods, the cells of the rat brain that were immunopositive for cytosolic GST isoenzymes of the α -, μ -, and π -class, and for microsomal GST; and (2) determine, by reverse-phase HPLC, the concentrations of cytosolic α -, μ -, and π -class GST subunits in different brain regions.

Materials and Methods

Materials. Bovine serum albumin (BSA) was purchased from Sigma Chemical Company. Polyclonal rabbit anti-rat GST antisera against α -class GST 1-1 (Y_aY_a), GST 2-2 (Y_cY_c), GST 8-8 (Y_kY_k); μ -class GST 3-3 ($Y_{b1}Y_{b1}$), GST 4-4 ($Y_{b2}Y_{b2}$); π -class GST 7-7 (Y_pY_p); and microsomal GST were purchased from Biotrin (Dublin, Ireland). Preimmune rabbit serum was also purchased from Biotrin, and normal rabbit sera were prepared in our laboratory. Goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) and normal goat serum (NGS) were from Vector Labs (Burlington, CA). *S*-hexylglutathione, glutathione (GSH), and epoxy-activated Sepharose 6B were purchased from Sigma Chemical Company and coupled as previously described (Mannervik and Guthenberg, 1981; Simons and Vander Jagt, 1981). Acetonitrile was from Burdick and Jackson Company, and trifluoroacetic acid (TFA) was from Aldrich Chemical Company. The System Gold HPLC System from Beckman Instruments was used for HPLC analysis. Male Wistar rats were purchased from Harlan Sprague-Dawley (Madison, WI). All other materials were products of Sigma Chemical Company.

Immunohistochemistry. Male rats of the Wistar strain, 2–3 months old, weighing 250–300 gm, were anesthetized with sodium pentobarbital and perfused through the left ventricle with 50–75 ml of 3% parafor-

maldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4). The paraformaldehyde-fixed brains were placed in 0.1 M PBS (pH 7.4) for 24 hr followed by 30% sucrose in 0.1 M PBS (pH 7.4), mounted in a block of OCT embedding medium, and stored at -80°C . Cryostat sections (10 μm) were cut in the coronal plane, collected on subbed slides, encircled with a PAP pen, dried at room temperature for 15 min, and washed in 10 mM PBS (pH 7.4). After three washes (10 min each) in PBS, sections were incubated in PBS containing 2% NGS and 5% BSA for 2 hr at room temperature to block nonspecific staining. The sections were then covered with a solution of rabbit anti-rat GST antiserum (1:2000) in 10 mM PBS (pH 7.4) and incubated overnight at 4°C . Sections were rinsed with 10 mM PBS (pH 7.4) and then incubated for 2 hr at room temperature in a solution containing goat anti-rabbit IgG conjugated to FITC (1:100). The sections were rinsed again, covered with Gel Mount, and coverslips applied. Vectastain ABC Reagent kits were also used to treat some sections following the protocol of Vector Labs.

Negative control experiments were carried out by substituting preimmune rabbit sera (PRS; 1:2000) and PBS for the solution containing the rabbit anti-rat GST. To control for the specificity of the rabbit anti-rat GST, the primary antibody was incubated initially with purified GST antigen and then placed on the sections. A previous study using μ -class GST antiserum gave an impression of localization of μ -class GST in neurons (Johnson et al., 1991, 1992b).

Antibody specificity and cross-reactivity. The antibodies purchased from Biotrin have been used extensively for immunohistochemistry (Cammer et al., 1989; Harrison et al., 1990a; Daly et al., 1991; Tansey and Cammer, 1991a,b). The specificity and cross-reactivity have been analyzed by Western blot and are well documented (Hayes and Mantle, 1986a; Kispert et al., 1989; McLellan and Hayes, 1989; Steinberg et al., 1989; Hayes et al., 1991; McLellan et al., 1991). The μ -class antibodies raised against GST 3-3 ($Y_{b1}Y_{b1}$) and GST 4-4 ($Y_{b2}Y_{b2}$) show strong immunological cross-reactivity with one another and with GST 6 (Y_{b3}) but not with GST subunits of the α - or π -classes. μ -Class antibody against GST 4-4 ($Y_{b2}Y_{b2}$) is more reactive against μ -class subunit 11 (Y_c) than against GST 3-3 ($Y_{b1}Y_{b1}$). In contrast, α -class GST 1-1 (Y_aY_a), GST 2-2 (Y_cY_c), and GST 8-8 (Y_kY_k) antisera show weak cross-reactivity with each other. The π -class GST 7-7 (Y_pY_p) and microsomal GST antibodies show no cross-reactivity with any other GSTs.

Isolation of GSTs. Male Wistar rats were killed and brains were removed. The cerebellum was dissected into flocculi, lateral hemispheres, and vermis. Brainstem, thalamus/hypothalamus, and cortex were dissected. In each experiment, brain regions from five animals were pooled and cytosolic fractions were prepared by ultracentrifugation (100,000 $\times g$) of tissues homogenized in 10 mM Tris-HCl, pH 7.8, containing 0.25 M sucrose, 10 mM EDTA, 2.0 mM EGTA, and 2.0 mM dithiothreitol (DTT). All purification steps were carried out at 4°C and cytosolic fractions were stored at -80°C . Protein concentrations were determined by the method of Bradford (1976), with bovine γ -globulin as standard. Cytosolic GST activity was measured by assaying the amount of 1-chloro-2,4-dinitrobenzene conjugated with GSH per minute per milligram of cytosolic protein (Habig and Jakoby, 1981). GSTs were isolated from cytosolic fractions by affinity chromatography as follows: equivalent amounts of cytosolic protein from the different brain regions were loaded on an *S*-hexylglutathione-Sepharose affinity column, and the material that did not bind was immediately loaded on a GSH-Sepharose affinity column according to the method of Hayes (1988). Both columns (0.7 cm $\times$ 1.0 cm) were equilibrated with 10 mM Tris-HCl, pH 7.8, containing 2.0 mM DTT (buffer A); the columns were then washed sequentially with buffer A, buffer A containing 0.2 M NaCl (buffer B), and finally eluted with 2.0 ml of buffer B containing 5 mM *S*-hexylglutathione.

Separation of GST subunits by HPLC. The small mass of tissue from the various brain regions and the low levels of GST with respect to total cytosolic protein (0.2–0.4%) made it necessary to increase the sensitivity of our previously published HPLC technique (Johnson et al., 1992a). This was accomplished by utilizing a narrow-bore Vydac C_{18} (2.1 mm $\times$ 25 cm) 300 Å reverse-phase column, which increased the sensitivity of analysis five- to seven-fold and allowed us to determine the concentration of GST subunits in a minimum tissue mass of 20–25 mg wet weight, with a limit of detection of any individual GST subunit of 2.0 pmol, or approximately 50 ng of subunit protein. Solvent A was 0.1% trifluoroacetic acid in deionized water and solvent B was 70% acetonitrile and 0.1% trifluoroacetic acid in deionized water. GST subunits were resolved using a linear gradient from 36.4% to 51.8% acetonitrile over 70 min. The flow rate was 0.25 ml/min and UV absorbance at

214 and 280 nm was monitored. There was no evidence of memory between samples; recovery of GSTs ranged from 90% to 100%. GST subunits were identified by SDS-PAGE and by comparison with previously published HPLC profiles (Hayes and Mantle 1986b; Ostlund Farrants et al., 1987; Ketterer et al., 1988; Kispert et al., 1989; Meyer et al., 1989; Hayes et al., 1990a; Hiratsuka et al., 1990; Johnson et al., 1990, 1992a). The amount of each GST subunit was determined from the peak height at 214 nm, by using the molar absorptivity (ϵ_{214}) for the individual subunits (Johnson et al., 1992a). The concentrations of individual subunits were expressed as picomoles of subunit protein per milligram of cytosolic protein. These values were summed to determine the concentration of total GST subunit protein or total μ -class GST subunit protein.

Statistical analysis. All groups of data were evaluated by analysis of variance and the individual means compared by the Fisher protected least significant difference test ($p < 0.05$).

Results

Nonspecific binding of preimmune and normal rabbit sera

Nonspecific binding of preimmune serum, especially to neuronal cell bodies (cytoplasmic and perinuclear), was noted at dilutions of preimmune rabbit serum of less than or equal to 1:1000, when Vectastain ABC Reagent kits were used to treat the sections. Similarly, the use of a secondary biotinylated goat anti-rabbit IgG followed by avidin conjugated to FITC resulted in a high degree of nonspecific neuronal staining. Analysis of five different normal rabbit sera (Vector Labs, and four individual rabbit sera prepared in our laboratory) showed the same type of nonspecific neuronal immunohistochemical staining. Purkinje cells of the cerebellar cortex were the most reactive with normal rabbit sera. In contrast, sections treated with goat anti-rabbit IgG (secondary antibody) conjugated to FITC markedly reduced nonspecific neuronal staining and overall background. Nonspecific staining was also reduced by further dilution of the antisera (1:2000) without loss of specific immunoreactivity. Based on these preliminary data, use of secondary biotinylated antibodies was abandoned and all subsequent investigations were performed using secondary antibody directly conjugated with FITC, and preimmune and primary rabbit antisera dilutions of 1:2000. Examples of preimmune sections are presented below (see Figs. 1A, 2A, 3C, 5A). These experimental conditions allowed us to determine unequivocally the immunohistochemical identification of GST-positive neurons and glia.

Immunohistochemical identification of GST-positive neurons

Throughout the brain, neurons and glial cells were identified as immunopositive to GST antibodies. Figures 1 and 2 illustrate examples of GST-positive neurons from sections through the brainstem and cerebellar cortex, respectively. Lipofuscin is present in most neurons and, under fluorescent microscopy, appears as orange-colored grains in the neuronal cytoplasm (Figs. 1–5). Polyclonal antibody against microsomal GST was strongly reactive with the cytoplasm of neurons of the brainstem (Fig. 1B) and Purkinje cells of the cerebellar cortex (Fig. 2B). Microsomal GST immunoreactivity was strongest in the cytosol, and in most cells both the perikaryon and the proximal segment of the main dendritic trunk were intensely labeled. This was particularly evident in Purkinje cells (Fig. 2B) and in neurons of the brainstem nuclear groups associated with the auditory, vestibular, facial, and trigeminal systems (Fig. 1B). Fine dendritic processes of Purkinje cells were also immunopositive for microsomal GST, and these fine processes permeated the molecular layer of the cerebellar cortex (Fig. 2B). Neurons of the hippocampus and proximal dendritic trunks of the hippocampal pyramidal cells were also immunopositive for microsomal GST (data not shown). Nuclei of Purkinje cells (Fig. 2C) and neurons in the brainstem (Fig. 1C) and hippocampus (data not shown) were immunopositive for α -class GST 1-1 ($Y_a Y_a$). A related α -class GST 2-2 ($Y_c Y_c$) antibody was consistently immunoreactive with the nucleolus, but with neither the nucleus nor soma of brainstem neurons and Purkinje cells (Figs. 1D, 2D).

Immunohistochemical localization of GST-positive non-neuronal cells

In contrast to the other α -class antibodies against GST 1-1 ($Y_a Y_a$) and GST 2-2 ($Y_c Y_c$), α -class GST 8-8 ($Y_k Y_k$) antibody showed no reactivity with either the nucleus or nucleolus of neurons, but was strongly immunoreactive with endothelial cells and/or astrocyte end-foot processes on blood vessels throughout the brain (Fig. 3A,B). All antibodies against α -class GSTs were reactive with astrocytes (Fig. 3A,B) and choroid plexus (Fig. 3D) to varying degrees; however, cells of the ependyma and the subventricular zones were immunonegative (Fig. 3D). The choroid plexus was most intensely reactive with GST 8-8 ($Y_k Y_k$) antibody (Fig. 3D). In addition, the choroid plexus, cells of the ependyma and subventricular zones, and pia mater were im-

Figure 1. Neuronal localization of GSTs in the brainstem. Cryosections (10 μ m) were incubated with A) preimmune rabbit serum (A), rabbit anti-rat microsomal GST antiserum (B), rabbit anti-rat α -class GST 1-1 ($Y_a Y_a$) antiserum (C), and rabbit anti-rat α -class GST 2-2 ($Y_c Y_c$) antiserum (D) (1:2000) overnight at 4°C. This was followed by incubation with goat anti-rabbit IgG conjugated to FITC (1:100) for 2 hr at room temperature. Arrows indicate immunopositive brainstem neurons. Scale bar, 20 μ m.

Figure 2. Neuronal localization of GSTs in the cerebellar cortex. Cryosections (10 μ m) were incubated with pre-immune rabbit serum (A), rabbit anti-rat microsomal GST antiserum (B), rabbit anti-rat α -class GST 1-1 ($Y_a Y_a$) antiserum (C), and rabbit anti-rat α -class GST 2-2 ($Y_c Y_c$) antiserum (D) as described in Figure 1. Arrows indicate immunopositive Purkinje cells that separate the molecular (m) and granular (g) layers. Scale bar, 20 μ m.

Figure 3. Immunohistochemical localization of α -class GST 8-8 ($Y_k Y_k$) in the cerebellar cortex and choroid plexus. Cryosections (10 μ m) through the cerebellar cortex (A, B) and the fourth ventricle (C, D) were incubated with rabbit anti-rat α -class GST 8-8 ($Y_k Y_k$) antiserum (A, B, D) and preimmune rabbit serum (C) as described in Figure 1. Long arrows (A, B) indicate immunopositive endothelial cells and/or astrocyte end-foot processes associated with blood vessels; short arrows (A, B) indicate immunopositive astrocytes in the granular layer (g) and cerebellar fiber tract (f). m, molecular layer. Scale bar, 20 μ m.

Figure 4. Immunohistochemical localization of μ -class GSTs in the cerebellar cortex, brainstem, ependymal cells, and choroid plexus. Cryosections (10 μ m) through the cerebellar cortex (A), brainstem (B), and the fourth ventricle (C, D) were incubated with rabbit anti-rat μ -class GST 4-4 ($Y_{b2} Y_{b2}$) antiserum (A, B, D) and rabbit anti-rat μ -class GST 3-3 ($Y_{b1} Y_{b1}$) antiserum (C) as described in Figure 1. Short arrows indicate immunopositive Bergmann radial glial cells (A), astrocytes (B), and ependymal cells (C, D). Long arrow indicates immunopositive choroid plexus (D). m, molecular layer; g, granule layer. Scale bar, 20 μ m.

Table 1. Cytosolic GST activity and total cytosolic GST subunit protein in different brain regions of male Wistar rats

	GST activity (nmol/min/mg protein)	Total GST protein (pmol/mg protein)
Cerebellum		
Flocculus	163 ± 4.88‡§#	53.3 ± 3.96‡§#
Lateral	177 ± 5.44#	56.6 ± 1.71§#
Vermis	196 ± 3.52*#	63.7 ± 1.94*§#
Brainstem	198 ± 10.3*#	73.0 ± 1.74¶
Thalamus/hypothalamus	300 ± 9.66¶	86.2 ± 2.97¶
Cortex	186 ± 8.75#	59.1 ± 2.32§#

GST activity was measured by assaying the conjugation of 1-chloro-2,4-dinitrobenzene with GSH. Values are the means ± SEM ($n = 3$) expressed as nmol of 1-chloro-2,4-dinitrobenzene conjugated/min/mg of cytosolic protein. The concentrations of individual subunits were determined by HPLC. These values were summed to determine the concentration of total GST subunit protein. Values are the means ± SEM ($n = 3$) expressed as pmol of GST subunit protein/mg of cytosolic protein.

* Significantly different from the corresponding value for flocculus ($p < 0.05$).

‡ Significantly different from the corresponding value for vermis ($p < 0.05$).

§ Significantly different from the corresponding value for brainstem ($p < 0.05$).

Significantly different from the corresponding value for thalamus/hypothalamus ($p < 0.05$).

¶ Significantly different from the corresponding values for all other brain regions ($p < 0.05$).

munopositive for microsomal GST (data not shown). The distribution of μ - and π -class-labeled glial cells was similar to that described by others (Senjo et al., 1986; Abramovitz et al., 1988; Cammer et al., 1989; Tansey and Cammer, 1991a,b). μ -Class GST-immunoreactive Bergmann radial glia were found in all regions of the cerebellar cortex (Fig. 4A). μ -Class GST 3-3 ($Y_{b1}Y_{b1}$) and 4-4 ($Y_{b2}Y_{b2}$) antibodies were reactive with astrocytes (Fig. 4B), pia mater, cells of the ependyma and subventricular zones (Fig. 4C,D), and choroid plexus (Fig. 4D). The patterns of immunoreactivity for μ -class GST 3-3 ($Y_{b1}Y_{b1}$) and 4-4 ($Y_{b2}Y_{b2}$) were very similar, with the exception of the choroid plexus,

which was consistently more immunopositive for GST 4-4 ($Y_{b2}Y_{b2}$) than GST 3-3 ($Y_{b1}Y_{b1}$) (Fig. 4C,D). π -Class GST 7-7 (Y_pY_p) was the only GST localized to oligodendrocytes (Fig. 5B–D). Furthermore, the choroid plexus was also immunopositive for π -class GST (Fig. 5D).

Regional distribution of GST activity and GST subunits

GSTs were isolated from different brain regions by affinity chromatography and the concentrations of individual subunits were determined by reverse-phase HPLC (Fig. 6). The regions examined included brainstem, cerebellar cortex, thalamus/hypothalamus, and three regions of the cerebellar cortex: the flocculus, lateral hemispheres, and vermis. Brain regions differed with respect to total cytosolic GST activity and profiles of GST subunits (Tables 1–3). The thalamus/hypothalamus had the highest GST activity and the greatest concentration of GST subunit protein (Table 1). The brainstem had a significantly higher concentration of total GST subunit protein than the three regions of the cerebellar cortex; the same, however, was not true for GST activity (Table 1). This was due to the lower protein concentration of μ -class GST subunits in brainstem compared to the cerebellar cortex (Tables 2, 3), and the greater concentration of π -class GST subunit 7 (Y_p) in brainstem compared to cerebellum (Tables 2, 3). The lower specific activity of GST 7-7 (Y_pY_p) compared to μ -class GST proteins (Ketterer et al., 1988; Mannervik and Danielson, 1988; Vos and Van Bladeren, 1990) accounts for this discrepancy between total GST protein concentration and enzyme activity. In cerebellar cortex, the concentration of α -class GSTs [subunit 2 (Y_{c1}) and subunit 8 (Y_k)] was threefold less than that in other brain regions (Tables 2, 3). Such regional variation in GST expression may be reflective of regional differences in cell populations. Cytosolic α -class GST subunits 1 (Y_{a1}) and 1' (Y_{a2}) were not detected in any of the brain regions examined (Tables 2, 3).

In cerebellar cortex, GST enzyme activity and the concentration of total GST subunit protein were significantly greater in

Table 2. Concentrations of cytosolic GST subunit protein in brain regions of the male Wistar rat

GST subunit	Flocculus	Lateral	Vermis	Brainstem	Thalamus/hypothalamus	Cortex
1 (Y_{a1} , α)	—	—	—	—	—	—
1' (Y_{a2} , α)	—	—	—	—	—	—
2 (Y_{c1} , α)	4.70 ± 0.69§¶	4.27 ± 0.04§¶	4.90 ± 0.63§¶	13.0 ± 0.45*†‡	13.2 ± 1.06*†‡	14.2 ± 0.22*†‡
3 (Y_{b1} , μ)	20.0 ± 1.15‡§¶	21.9 ± 0.96‡§¶	24.8 ± 0.75@	9.55 ± 0.13@	19.3 ± 1.13‡§¶	12.7 ± 0.31@
4 (Y_{b2} , μ)	1.10 ± 0.04‡§¶	0.93 ± 0.11‡¶	0.66 ± 0.05*†	0.74 ± 0.06*	0.86 ± 0.15¶	0.52 ± 0.09*†#
6 (Y_{b1} , μ)	5.65 ± 0.45§¶	6.38 ± 0.10§¶	6.33 ± 0.40§¶	8.55 ± 0.36*†‡#	17.2 ± 0.76@	10.4 ± 1.14*†‡#
7 (Y_p , π)	13.0 ± 1.21‡§¶	13.8 ± 0.85‡§#	16.2 ± 0.33*†§#	32.8 ± 0.84@	26.2 ± 0.14@	15.5 ± 0.89*§#
8 (Y_k , α)	0.96 ± 0.05§¶	0.85 ± 0.04§¶	0.81 ± 0.03§¶	1.30 ± 0.13*†‡¶	1.31 ± 0.08*†‡¶	0.38 ± 0.08@
9 (Y_{a2} , μ)	—	—	—	—	—	—
10 (Y_{l1} , α)	—	—	—	—	—	—
11 (Y_o , μ)	7.90 ± 0.57‡¶	8.45 ± 0.01‡§¶	10.1 ± 0.36@	6.98 ± 0.19*†‡¶	8.08 ± 0.40‡¶	5.46 ± 0.20@

The protein concentrations of GST subunits present in each brain region were calculated from the reverse-phase HPLC profiles of affinity-purified GST pools. Values represent the means ± SEM ($n = 3$) of pmol of GST subunit protein/mg of cytosolic protein. Undetectable GST indicated by —.

* Significantly different from the corresponding value for flocculus ($p < 0.05$).

† Significantly different from the corresponding value for lateral ($p < 0.05$).

‡ Significantly different from the corresponding value for vermis ($p < 0.05$).

§ Significantly different from the corresponding value for brainstem ($p < 0.05$).

Significantly different from the corresponding value for thalamus/hypothalamus ($p < 0.05$).

¶ Significantly different from the corresponding value for cortex ($p < 0.05$).

@ Significantly different from the corresponding values for all other brain regions ($p < 0.05$).

Table 3. Relative levels of cytosolic GST subunit protein in male Wistar rat brain

GST subunit	Flocculus	Lateral	Vermis	Brainstem	Thalamus/hypothalamus	Cortex
1 (Y_{a1} , α)	—	—	—	—	—	—
1' (Y_{a2} , α)	—	—	—	—	—	—
2 (Y_{c1} , α)	8.72 $\pm$ 0.69§#¶	7.55 $\pm$ 0.15§#¶	7.68 $\pm$ 0.88§#¶	17.8 $\pm$ 0.26@	15.3 $\pm$ 0.79@	24.0 $\pm$ 0.58@
3 (Y_{b1} , μ)	37.7 $\pm$ 0.92§#¶	38.7 $\pm$ 0.56§#¶	38.9 $\pm$ 0.74§#¶	13.1 $\pm$ 0.14@	22.3 $\pm$ 0.58*†‡§	21.5 $\pm$ 0.75*†‡§
4 (Y_{b2} , μ)	2.09 $\pm$ 0.15@	1.64 $\pm$ 0.20@	1.04 $\pm$ 0.07*†	1.02 $\pm$ 0.10*†	1.00 $\pm$ 0.16*†	0.90 $\pm$ 0.18*†
6 (Y_{b3} , μ)	10.6 $\pm$ 0.35#¶	11.3 $\pm$ 0.49#¶	9.92 $\pm$ 0.44#¶	11.7 $\pm$ 0.23#¶	20.0 $\pm$ 0.82@	17.6 $\pm$ 1.34@
7 (Y_{p1} , π)	24.3 $\pm$ 0.86§#¶	24.4 $\pm$ 0.79§#¶	25.4 $\pm$ 0.44§#	45.0 $\pm$ 0.58@	30.5 $\pm$ 1.17@	26.1 $\pm$ 0.66*†‡§
8 (Y_{k1} , α)	1.82 $\pm$ 0.05‡¶	1.51 $\pm$ 0.12¶	1.27 $\pm$ 0.01*§¶	1.79 $\pm$ 0.20‡¶	1.51 $\pm$ 0.06¶	0.63 $\pm$ 0.11@
9 (Y_{n2} , μ)	—	—	—	—	—	—
10 (Y_{l1} , α)	—	—	—	—	—	—
11 (Y_{o1} , μ)	14.8 $\pm$ 0.26‡§#¶	15.0 $\pm$ 0.44‡§#¶	15.8 $\pm$ 0.08@	9.56 $\pm$ 0.04*†‡	9.36 $\pm$ 0.14*†‡	9.24 $\pm$ 0.04*†‡

The percentage of GST subunit protein present in each brain region was derived from the reverse-phase HPLC profiles of affinity-purified GST pools. Values represent the means $\pm$ SEM ($n = 3$) of the percentage of subunit protein present with respect to the total GST subunit protein in a given brain region. Undetectable GST indicated by —.

* Significantly different from the corresponding value for flocculus ($p < 0.05$).

† Significantly different from the corresponding value for lateral ($p < 0.05$).

‡ Significantly different from the corresponding value for vermis ($p < 0.05$).

§ Significantly different from the corresponding value for brainstem ($p < 0.05$).

Significantly different from the corresponding value for thalamus/hypothalamus ($p < 0.05$).

¶ Significantly different from the corresponding value for cortex ($p < 0.05$).

@ Significantly different from the corresponding value for all other brain regions ($p < 0.05$).

the vermis than in the lateral lobes and flocculus (Table 1). Similarly, the concentration of total μ -class GST subunits [summation of concentrations of subunits 3 (Y_{b1}), 4 (Y_{b2}), 6 (Y_{b3}), and 11 (Y_{o1})] was greatest in the vermis (Table 2); the percentages of total μ -class GST subunit protein in regions of the cerebellar cortex, however, were not significantly different (Table 3). Analysis of the protein concentrations of the individual μ -class subunits demonstrated that the most significant difference in cerebellar regions occurred with respect to subunit 4 (Y_{b2}) (Tables 2, 3). There was a regional gradient in the pattern of μ -class GST subunit 4 (Y_{b2}) expression. The flocculus had the highest concentration (Table 2) and greatest relative levels (Table 3) of subunit 4 (Y_{b2}), and the vermis the lowest. The concentrations of the μ -class GST subunits 3 (Y_{b1}) and 11 (Y_{o1}) were significantly greater in the vermis than in either the flocculus or lateral lobes of the cerebellar cortex (Table 2). When expressed as percentage of total GST subunit protein, however, there was little regional variation in the levels of subunits 3 (Y_{b1}) and 11 (Y_{o1}) (Table 3).

Discussion

Our major findings are that (1) neurons throughout the brain contain GST; (2) microsomal GST is localized to neuronal soma; (3) α -class GSTs are localized to the nucleus and nucleolus of neurons, and are also found in neuroglia and endothelial cells and/or astrocyte end-foot processes associated with blood vessels; (4) there are regional differences in the pattern of α -, μ -, and π -class GST subunit expression; and (5) in the cerebellum, the flocculus has a higher concentration of μ -class GST subunit 4 (Y_{b2}) than the vermis.

Preliminary experiments resulted in the development of an immunohistochemical procedure that allowed us to identify, unequivocally, GST-positive neurons and glia in rat brain. Recently, an HPLC technique developed by Ostlund Farrants et al. (1987) has greatly improved the ability to identify and quantify GST proteins (Ketterer et al., 1988; Kispert et al., 1989; Meyer et al., 1989, 1991; Hayes et al., 1990a; Hiratsuka et al.,

1990; Johnson et al., 1990, 1992a). We have increased the resolution and sensitivity of this HPLC technique and reduced the limit of detection to approximately 50 ng of subunit protein. This improved HPLC technique has allowed us to characterize the GSTs in discrete brain regions.

Neither the α -class nor microsomal GSTs have been studied extensively by immunohistochemistry in rat brain. Recently, Tansey and Cammer (1991b) suggested that α -class GST 2-2 (Y_{c1}) is present in astrocytes, but they did not use other available α -class antisera. Our data are consistent with the previous reports that μ -class GSTs are present in astrocytes, Bergmann radial glia, and cells of the ependyma and the subventricular zones (Abramovitz et al., 1988; Cammer et al., 1989), and that π -class GST is localized to oligodendrocytes (Cammer et al., 1989; Tansey and Cammer, 1991a). We have also demonstrated that polyclonal antibody against microsomal GST was reactive with the cytoplasm of the perikaryon and proximal dendritic trunks of neurons in the brainstem, hippocampal neurons, and Purkinje cells of the cerebellar cortex. Nuclei of this same set of neurons were immunoreactive for α -class GST 1-1 (Y_{a1}), whereas the nucleolus, but not the nucleus or soma of these neurons, was immunoreactive with a related α -class GST 2-2 (Y_{c1}) antibody. The nuclear localization of GSTs has been shown in numerous tissues, including rat pancreas (π -class) (Daly et al., 1991), human liver (α -class) (Harrison et al., 1990b; Campbell et al., 1991), pia mater and choroid plexus of human brain (π -class) (Carder et al., 1990), human ovary (α -class) (Rahilly et al., 1991), human breast tissue (α -, μ -, and π -class) (Forrester et al., 1990; Cairns et al., 1992), and rat liver (μ -class) (Bennett et al., 1986). In contrast, specific immunohistochemical staining of the nucleolus with GST antibody has not been documented previously.

The function of nuclear and nucleolar GST(s) remains to be determined. Bennett et al. (1986) isolated a non-histone-binding protein (BA), which they determined was a mixture of μ -class GSTs 3-3 (Y_{b1}) and 4-4 (Y_{b2}). These GST isoenzymes

Figure 5. Immunohistochemical localization of π -class GST 7-7 (Y_pY_p) in the cerebellar cortex and choroid plexus. Cryosections ($10\ \mu\text{m}$) through fiber tracts of the cerebellar cortex (*A, B*), molecular (*m*) and granular (*g*) layers of the cerebellar cortex (*C*), and fourth ventricle (*D*) were incubated with rabbit anti-rat π -class GST 7-7 (Y_pY_p) antiserum (*B, C, D*) and preimmune rabbit serum (*A*) as described in Figure 1. Short arrows indicate immunopositive oligodendrocytes (*B, C, D*); long arrow indicates positive choroid plexus (*D*). Scale bar, $20\ \mu\text{m}$.

Figure 6. Reverse-phase HPLC of cytosolic GSTs isolated from cerebellar flocculi of male Wistar rat brain. Cytosolic GSTs were isolated by affinity chromatography from 4.0 mg of cytosolic protein and subjected to analysis by reverse-phase HPLC. A gradient from 52% solvent B (70% acetonitrile, 0.1% TFA) to 74% solvent B over 70 min (broken line) separated the GST subunits. The absorbance at 214 nm was monitored (solid line). GST subunits were numbered as previously described by Jakoby et al. (1984), Mannervik and Danielson (1988), Ketterer et al. (1988), and Hayes et al. (1990b).

were immunohistochemically colocalized with small nuclear ribonucleoproteins (snRNPs) to discrete nuclear regions in normal rat liver cells (Bennett et al., 1986). One hour after microinjection of purified and radiolabeled nuclear GSTs into cytoplasm of Walker 256 cells, over 85% of the labeled protein was associated with the nucleus (Bennett and Yeoman, 1987). These data suggest that GSTs may be involved in RNA processing and/or regulation of transcription (Bennett et al., 1986). In addition, since GSTs are present in the nucleolus as well as the nucleus, they could be involved in the production or regulation, or both, of ribosomes and snRNPs. GSTs have also been shown to modulate DNA adduct formation and may be involved in DNA repair after peroxide injury and in protection of DNA from oxidative damage (Ketterer et al., 1987; Liu et al., 1991). Finally, GSTs have been shown to take part in intracellular binding and transport of endogenous compounds including bilirubin, steroids, thyroid hormones, and neurotransmitters, as well as the removal of toxins and harmful metabolic byproducts (Abramovitz et al., 1988; Listowsky et al., 1988).

Thus, GSTs may control the uptake and release of such compounds in brain via a GSH-dependent binding to GST.

Philbert et al. (1991) have recently described the regional and cellular distribution of GSH in the nervous system. In general, neuronal soma throughout the CNS were negative for GSH histochemical staining. In the cerebellum, however, both granule cells and Purkinje cells were stained positively for GSH (Philbert et al., 1991). These investigators also showed that GSH was present throughout the CNS neuropil and noted that the choroid plexus was intensely stained for GSH. Although all the GST antisera used in our experiments were immunoreactive with choroid plexus to varying degrees, the reactivity of α -class GST 8-8 ($Y_k Y_k$) antibody was particularly intense. Furthermore, GST 8-8 ($Y_k Y_k$) antiserum showed specific immunoreactivity to endothelial cells and/or astrocyte end-foot processes associated with blood vessels throughout the brain. This colocalization of GST and GSH supports the proposal that GSTs may control the uptake and release of various compounds and the removal of toxins and harmful metabolic byproducts via a GSH-dependent binding to GST (Abramovitz et al., 1988; Listowsky et al., 1988). This may explain the decreased induction of glycerol-phosphate dehydrogenase by hydrocortisone administration in GSH-depleted rats (Tansey and Cammer, 1991b). Based on these data, it is proposed that one function of GSTs is transport of hydrocortisone between or within neurons and glia. Furthermore, GST 8-8 ($Y_k Y_k$) has remarkably high activity toward 4-hydroxyalkenals, which are prominent endogenous electrophiles produced as byproducts of metal-catalyzed decomposition of polyunsaturated fatty acyl hydroperoxides (Ketterer et al., 1988). Thus, the high level of α -class GST 8-8 ($Y_k Y_k$) expression associated with choroid plexus and blood vessels may also be a critical factor in neuroprotection.

Regional expression of μ -class GST subunit 4 (Y_{b2}) in the cerebellum coincides with the regions most resistant to degeneration after exposure to toxic or metabolic insults. The vermis is more susceptible to these insults than the lateral lobes and flocculus. These regional differences in susceptibility are well illustrated by the observations that both an extensive loss of Purkinje cells and glial proliferation occur in the anterior portion of the cerebellar vermis in the Wernicke-Korsakoff syndrome (Victor, 1976) and in nutritional cerebellar degeneration (Adams, 1976), whereas in the posterior region and flocculi these neurons are spared. Cerebellar dysgenesis in humans has also been associated with neonatal cystic kidney disease (Kornguth et al., 1977; Marin-Padilla, 1989), autism (Courchesne et al., 1988), trisomy 21 (Warkany et al., 1966), fragile X syndrome (Reiss et al., 1988), paraneoplastic cerebellar degeneration (Chen et al., 1990), and Joubert syndrome (Lambert et al., 1989). In nonhuman mammals, alcohol exposure *in utero* (Kornguth et al., 1979; Bonthius and West, 1991), hyperbilirubinemia (Schutta and Johnson, 1967; Sawasaki et al., 1976; Takagishi and Yamamura, 1989), methadone withdrawal (Zagon and McLaughlin, 1978), exposure to mutated diphtheria toxin (Riedel et al., 1990), hypothyroidism (Kornguth et al., 1967; Nicholson and Altman, 1972; Kornguth et al., 1979; Legrand, 1979; Silva and Rudas, 1990), and malnutrition (Neville and Chase, 1971; Patel et al., 1973; Yu et al., 1974; Sima and Persson, 1975; Gopinath et al., 1976; McConnell and Berry, 1978) all result in maldevelopment of the cerebellar cortex.

Cytosolic GST activity, total concentration of GST subunit protein, and the concentration of μ -class GST subunits were not demonstrably different between the regions of the cerebellar

cortex. This might suggest that all regions of the cerebellum would be equally susceptible to toxic agents. However, there is a region-specific loss of Purkinje cells due to toxic or metabolic insult, and this pattern of Purkinje cell loss was inversely correlated with the distribution of μ -class GST subunit 4 (Y_{b2}). The cytoplasmic localization of microsomal GST in neurons, the nuclear localization of α -class GSTs in neurons, and the relationship between the concentration of μ -class GST subunit 4 (Y_{b2}) and resistance of neurons to toxic events in the cerebellar cortex suggest that GSTs may confer protection against exogenous and/or endogenous neurotoxic metabolites. Preliminary work in our laboratory using the Gunn rat as a model for hyperbilirubinemia supports this suggestion (Johnson et al., *in press*).

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