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Effects of hyperbilirubinaemia on glutathione S-transferase isoenzymes in cerebellar cortex of the Gunn rat

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The glutathione S-transferases (GSTs) are a family of isoenzymes involved in the detoxication of a variety of electrophilic xenobiotics. The present investigation demonstrates that GST activity and the concentration of cytosolic GSTs in cerebellar cortex of Gunn rats were increased in hyperbilirubinaemic animals compared with non-jaundiced controls. Age-dependent and region-specific increases in GST isoenzymes were seen in three regions of the cerebellar cortex of jaundiced Gunn rats, whereas GST concentrations were not altered in the brainstem, thalamus/hypothalamus, cortex or liver. Cytosolic GST activity was increased 1.3-fold in the flocculus and lateral hemispheres of 20-day-old and 1.7-fold in the flocculus, lateral hemispheres and vermis of 60-day-old jaundiced (jj; homozygous) Gunn rats compared with non-jaundiced (Jj; heterozygous) Gunn rats. H.p.l.c. was used to determine the GST subunit protein concentrations in cytosolic fractions isolated from liver and brain regions of jaundiced and non-jaundiced animals. In all regions of the cerebellum from 20-day-old animals, the levels of Alpha-class GST subunits 2 (Y_{c1}; 3.0-fold) and 8 (Y_k; 2.0-fold) were increased in jaundiced rats. In 60-day-old animals, the concentrations of Alpha-class GST subunits 2 (Y_{c1}; 5.0-fold) and 8 (Y_k; 3.0-fold), Mu-class subunit 11 (Y_o; 2.5-fold) and Pi-class subunit 7 (Y_p; 2.0-fold) were increased in all regions of cerebellar cortex of jaundiced animals. In cerebellum of 10-, 20- and 60-day-old non-jaundiced and jaundiced Gunn rats, the flocculus had the highest concentration of Mu-class GST subunit 4 (Y_{b2}) and vermis the lowest; hyperbilirubinaemia increased the concentration of subunit 4 (Y_{b2}; 3- to 5-fold) in the flocculus and lateral hemispheres, but not the vermis, of 20- and 60-day-old rats. Intraperitoneal injection of sulphadimethoxine, a long-acting sulphonamide which displaces bilirubin from its albumin-binding sites and increases the bilirubin levels in tissues, further increased the already elevated concentrations of GST subunits in the lateral regions of cerebellar cortex of hyperbilirubinaemic rats. For example, the concentration of subunit 4 (Y_{b2}) was increased 2.2-fold (compared with non-jaundiced controls) in Gunn rats injected with saline and 7.4-fold in rats injected with 100 mg of sulphadimethoxine/kg body weight. In contrast, GSTs in the vermis of jaundiced animals were not affected by sulphadimethoxine injection. Sulphadimethoxine had no effect on GST concentrations in lateral regions and vermis of heterozygous (Jj) Gunn rats. These region-specific alterations in GST protein concentrations are inversely related to previously documented region-specific cerebellar hypoplasia in Gunn rats. Others have shown that Purkinje cells are severely affected in the cerebellar vermis, whereas neurons present in the lateral hemispheres and flocculi were less affected; these effects on the cerebellar cortex were exacerbated by sulphadimethoxine injection. Our data thus suggest that region-specific GST expression may confer protection against endogenous and/or exogenous neurotoxic agents.

INTRODUCTION

Glutathione S-transferases (GSTs; EC 2.5.1.18) constitute a family of multifunctional enzymes which share structural and functional homology and are involved in metabolic detoxication of a variety of electrophilic xenobiotics (Mannervik et al., 1985; Mannervik and Danielson, 1988; Ketterer et al., 1988; Vos and Van Bladeren, 1990). Cytosolic GSTs are dimeric proteins consisting of two identical or closely related subunits from the same class (Alpha, Mu, Pi or Theta; Mannervik and Danielson, 1988; Meyer et al., 1991). These enzymes are present in most mammalian organs, and rat tissues contain at least 20 dimers of 15 different subunits (Tu et al., 1983; Mannervik and Danielson, 1988; Ketterer et al., 1988; Kispert et al., 1989; Hayes et al., 1990a,b, 1991; Hiratsuka et al., 1990; Tsuchida and Sato, 1990; Meyer et al., 1991; Harris et al., 1991). GST isoenzyme profiles are altered in preneoplastic foci, tumour tissue and multidrug-resistant cell lines (Satoh et al., 1985; Cowan et al., 1986; Moscow et al., 1989). GST 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; Di Simplicio et al., 1989; Benson et al., 1989; Rushmore et al., 1990).

GSTs have been shown to be involved in intracellular transport mechanisms and the binding of endogenous non-substrate ligands (Listowsky et al., 1988). These enzymes bind hormones which have been shown to control cerebellar development: L-3,3′,5-triiodothyronine and L-3,3′,5,5′-tetraiodothyronine (thyroxine) (Kornguth et al., 1967, 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 the cellular uptake and intracellular transport of bilirubin (Listowsky et al., 1988). In the liver, bilirubin is bound to a cytoplasmic binding protein (ligandin) which prevents the reflux of bilirubin back into the sinusoidal space and is responsible for the transport of bilirubin from the sinusoidal membrane to the endoplasmic reticulum. Ligandin has been shown to be a GST(s) (Habig et al., 1974). Bilirubin is then

Abbreviations used: GST, glutathione S-transferase; ARE, antioxidant-responsive element.

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Table 1 Effect of hyperbilirubinaemia on cytosolic GST activity in different brain regions and liver of non-jaundiced (Jj) and jaundiced (jj) Gunn rats

GST activity was measured by assaying the conjugation of 1-chloro-2,4-dinitrobenzene with GSH and values are the means ± S.E.M. ($n = 3$) expressed as nmol of 1-chloro-2,4-dinitrobenzene conjugated/min per mg of cytosolic protein.

Rats	Flocculus	Lateral hemisphere	Vermis	Brainstem	Thalamus/ hypothalamus	Cortex	Liver
10 days old							
Jj	84.8 ± 6.30	94.0 ± 2.98	91.1 ± 4.51	113 ± 5.57	144 ± 4.14	82.1 ± 4.51	435 ± 13.3
jj	92.1 ± 3.50	86.1 ± 1.96	91.5 ± 4.65	103 ± 1.14	145 ± 10.2	69.0 ± 5.54	424 ± 27.5
Fold	–	–	–	–	–	–	–
20 days old							
Jj	83.1 ± 3.57	102 ± 3.38	117 ± 7.36	129 ± 6.63	181 ± 6.18	120 ± 2.91	654 ± 14.3
jj	106 ± 4.90*	137 ± 7.27*	125 ± 7.41	124 ± 3.19	182 ± 7.80	117 ± 5.10	643 ± 30.8
Fold	1.28	1.35	–	–	–	–	–
60 days old							
Jj	166 ± 2.92	177 ± 17.5	186 ± 15.0	179 ± 19.6	294 ± 12.9	189 ± 7.46	1430 ± 120
jj	276 ± 20.8*	307 ± 36.1*	313 ± 21.8*	187 ± 4.00	316 ± 36.8	181 ± 6.32	1250 ± 130
Fold	1.67	1.73	1.72	–	–	–	–

* Significantly different from the corresponding value for Jj animal of the same age ($P < 0.05$).

conjugated to glucuronide by bilirubin–UDP-glucuronosyltransferase, in the endoplasmic reticulum, and the bilirubin conjugate is transported into the canaliculus and subsequently excreted in the bile.

Hyperbilirubinaemia is a common occurrence in humans during the neonatal period (Amit et al., 1992). In addition, Crigler–Najjar syndrome type I is characterized by severe unconjugated hyperbilirubinaemia due to an inherited absence of bilirubin–UDP-glucuronosyltransferases (Crigler and Najjar, 1952; Roy-Chowdhury et al., 1990; Bosma et al., 1992). An animal model for unconjugated hyperbilirubinaemia has been described (Gunn, 1938). Gunn rats (JJ, Jj and jj) are a mutant strain of Wistar rats lacking bilirubin–UDP-glucuronosyltransferase activity due to a single-base frameshift deletion in the bilirubin–UDP-glucuronosyltransferase gene, resulting in a nonfunctional truncated gene product (Iyanagi et al., 1989; Roy-Chowdhury et al., 1991). In Gunn rats, hyperbilirubinaemia causes cerebellar hypoplasia associated with ataxia (Schutta and Johnson, 1967; Sawasaki et al., 1976). Cerebellar Purkinje cells are severely affected in central regions of the cerebellar vermis, whereas neurons present in the posterior vermis, lateral hemispheres and flocculus are less affected (Sawasaki et al., 1976; Takagishi and Yamamura, 1987, 1989). Glial cells are less vulnerable to the toxic actions of bilirubin; astrocytes in the Gunn rat undergo gliosis (Sawasaki et al., 1976; Takagishi and Yamamura, 1987, 1989). Recently, two distinct glutathione conjugates of the dimethyl ester of bilirubin were isolated from bile of Gunn rats infused intravenously with the dimethyl ester of bilirubin (Odell et al., 1991). Concurrently, Igarashi and coworkers (1992) identified a cytosolic liver Alpha-class GST subunit (Y_x) which is specifically induced in a strain of hyperbilirubinaemic rats (EHB) derived from the Sprague–Dawley rat.

Thus the purpose of the following investigation was to: (1) determine, by reversed phase h.p.l.c., the concentrations of cytosolic Alpha-, Mu- and Pi-class GST subunits in Gunn rat brain regions and liver; (2) evaluate the effects of hyperbilirubinaemia on the regulation of GST expression; (3) determine the effects of sulphadimethoxine, a long-acting sulphonamide which displaces bilirubin from its albumin-binding sites and increases bilirubin levels in tissues (Johnson et al., 1959; Schutta and Johnson, 1969; Rose and Wisniewski, 1979; Conlee and Shapiro, 1991), on GST expression in jaundiced and non-jaundiced Gunn rats.

MATERIALS AND METHODS

Materials

S-Hexylglutathione, GSH and epoxy-activated Sepharose 6B were purchased from Sigma Chemical Co. and coupled as previously described (Mannervik and Guthenberg, 1981; Simons and Vander Jagt, 1981). Acetonitrile was from Burdick and Jackson Co. and trifluoroacetic acid from Aldrich Chemical Co. System Gold from Beckman Instruments was used for h.p.l.c. analysis. Gunn rats were reared in our breeding colony. Sulphadimethoxine and all other materials were products of Sigma.

Isolation and assay of GSTs

Jaundiced (jj; homozygous) and non-jaundiced (Jj; heterozygous) Gunn rats were generated by breeding heterozygous females with homozygous males; jaundiced Gunn rat pups were identified by their yellow colour at 5–7 days of age. Male and female non-jaundiced (Jj) and jaundiced (jj) Gunn rats were killed by decapitation, and livers and brains were removed. Brains were dissected to give brainstem, thalamus/hypothalamus, cerebral cortex and cerebellar flocculi, lateral hemispheres and vermis. In each of three experiments, brain regions from six 10-day-old, four 20-day-old and four 60-day-old animals were pooled. To control for interlitter variability, animals in each pool were randomly selected from a minimum of six litters. Cytosolic fractions were prepared by ultracentrifugation (100000 *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. 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 the standard. Cytosolic GST activity was measured by assaying the rate of conjugation of 1-chloro-2,4-dinitrobenzene (Habig and Jakoby, 1981). GSTs were isolated from cytosolic fractions by affinity chromatography as follows: equivalent amounts of cytosolic protein from each brain region were loaded on to an *S*-hexylglutathione–Sepharose

affinity column and the material that did not bind was immediately loaded on to a GSH–Sepharose affinity column by the method of Hayes (1988). Both columns (0.7 cm × 1.0 cm) were equilibrated with 10 mM Tris/HCl, pH 7.8, containing 2.0 mM dithiothreitol (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.

Sulphadimethoxine injection

Three groups of male non-jaundiced (Jj) and jaundiced (jj) Gunn rats containing six to eight animals each were injected intraperitoneally with saline, 50 and 100 mg of sulphadimethoxine/kg body weight at 13 and 16 days of age. To control for interlitter variability, animals in each pool were randomly selected from a minimum of ten litters. Animals were killed at 20 days of age and brains were removed and the cerebellum was dissected into flocculi/lateral hemispheres and vermis. In each of three experiments, brain regions from two animals were pooled.

Separation of GST subunits by h.p.l.c.

The small mass of tissue from the various brain regions and the low levels of GST (0.2–0.4 % of total cytosolic protein) made it necessary to increase the sensitivity of our previously published h.p.l.c. technique (Johnson et al., 1992). This was accomplished by utilizing a narrow-bore Vydac C_{18} 30 nm reversed-phase column (2.1 mm × 25 cm), which increased the sensitivity of analysis by 5- to 7-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/0.1 % trifluoroacetic acid in deionized water. GST subunits were resolved by a 70 min linear gradient from 36.4 to 51.8 % acetonitrile. The flow rate was 0.25 ml/min and u.v. 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 h.p.l.c. profiles (Hayes and Mantle, 1986a; Hayes et al, 1990a; Ostlund Farrants et al., 1987; Ketterer et al., 1988; Kispert et al., 1989; Meyer et al., 1989; Hiratsuka et al., 1990; Johnson et al., 1990, 1992). The amount of each GST subunit was determined from its peak height at 214 nm, by using the molar absorptivity (ϵ_{214}) for the individual subunits (Johnson et al., 1992). The concentrations of individual subunits were expressed as pmol of subunit protein/mg of cytosolic protein. These values were summed to determine the concentration of total GST subunit protein or total Mu-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-squares difference test ($P < 0.05$). Student's t test was used for analysis of paired sets of data ($P < 0.05$).

RESULTS

Effect of hyperbilirubinaemia on GST activity

GST activities in cytosolic fractions from the liver and brain regions of jaundiced (jj; homozygous) and non-jaundiced (Jj; heterozygous) Gunn rats were determined spectrophotometrically (Table 1); no differences in GST activity between jaundiced and non-jaundiced rats were seen at 10 days of age in any brain region. In 20-day-old jaundiced Gunn rats, GST activity was significantly increased in the flocculus and lateral hemispheres of cerebellar cortex compared with non-jaundiced animals (Table 1). Hyperbilirubinaemia significantly increased GST activity in all three regions of cerebellum in 60-day-old

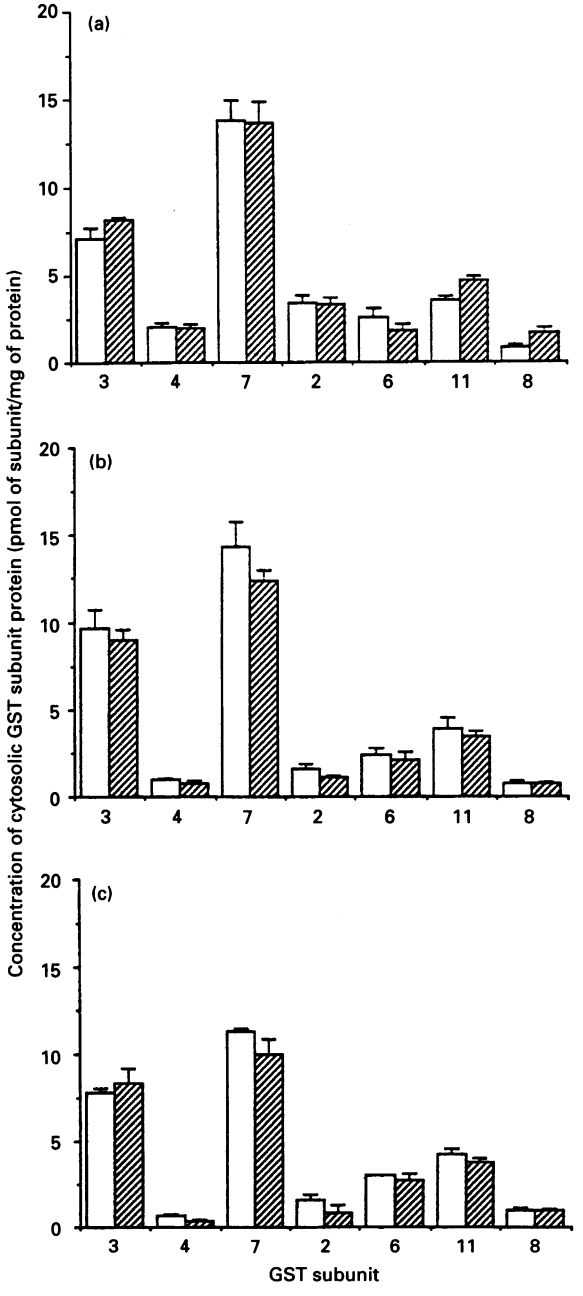

Figure 1 Concentrations of cytosolic GST subunit protein in regions of the cerebellar cortex of 10-day-old non-jaundiced (Jj) and jaundiced (jj) Gunn rats

Cytosolic GSTs were isolated from (**a**) flocculi, (**b**) lateral hemispheres and (**c**) vermis of cerebellar cortex of non-jaundiced (open bar) and jaundiced (hatched bar) Gunn rats by affinity chromatography and subjected to analysis by reversed-phase h.p.l.c. Values represent the means ± S.E.M. ($n = 3$) of pmol of GST subunit protein/mg of cytosolic protein. Mu-class subunits: 3 (Y_{b1}); 4 (Y_{b2}); 6 (Y_{b3}); 11 (Y_o). Alpha-class subunits: 2 (Y_{c1}); 8 (Y_k). Pi-class subunit: 7 (Y_p).

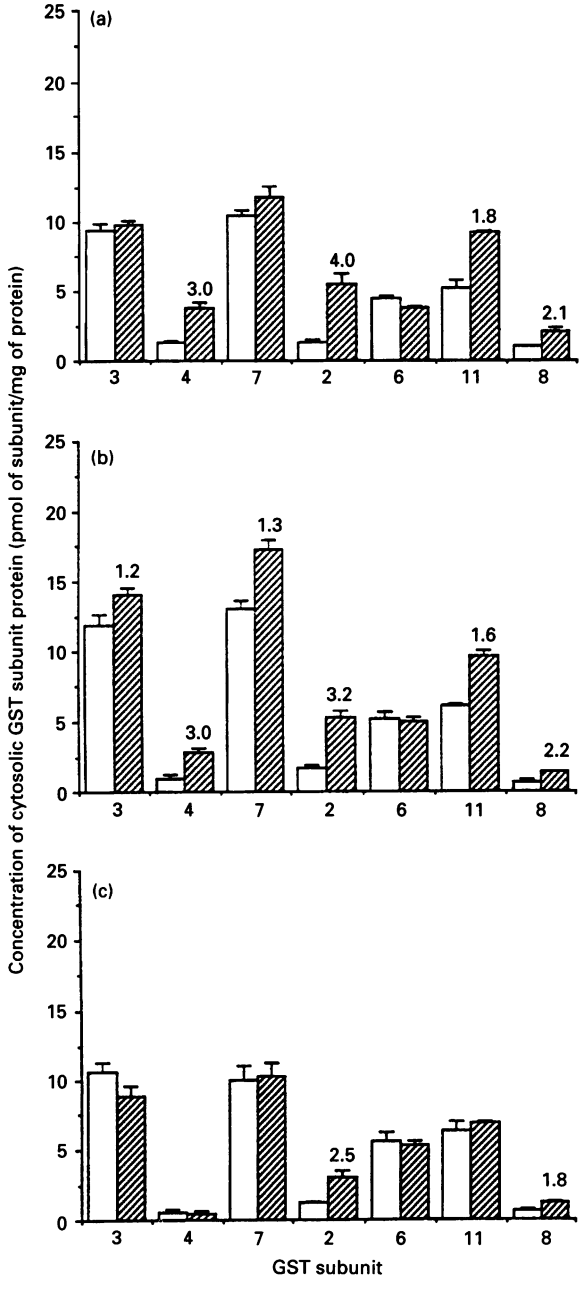

Figure 2 Concentrations of cytosolic GST subunit protein in regions of the cerebellar cortex of 20-day-old non-jaundiced (Jj) and jaundiced (jj) Gunn rats

Cytosolic GSTs were isolated from (**a**) flocculi, (**b**) lateral hemispheres and (**c**) vermis of cerebellar cortex of non-jaundiced (open bar) and jaundiced (hatched bar) Gunn rats by affinity chromatography and subjected to analysis by reversed-phase h.p.l.c. Values represent the means ± S.E.M. ($n = 3$) of pmol of GST subunit protein/mg of cytosolic protein. Numbers above the bars indicate that the jaundiced value is significantly different from the corresponding value for non-jaundiced animals ($P < 0.05$) and are the fold increase in subunit concentration (jj/Jj). Mu-class subunits: 3 (Y_{b1}); 4 (Y_{b2}); 6 (Y_{b3}); 11 (Y_o). Alpha-class subunits: 2 (Y_{c1}); 8 (Y_k). Pi-class subunit: 7 (Y_p).

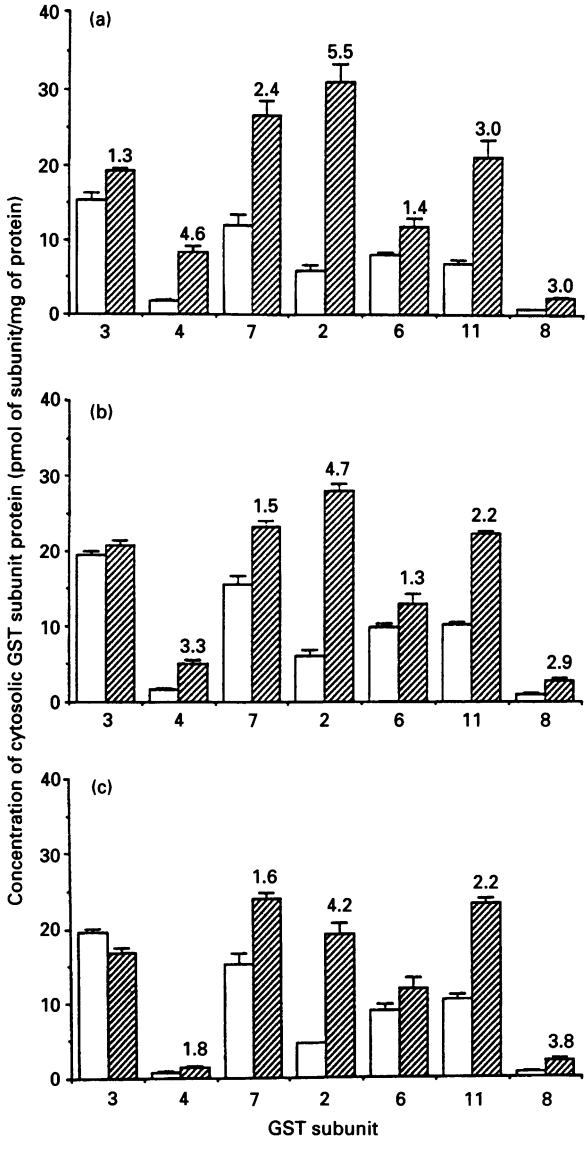

Figure 3 Concentrations of cytosolic GST subunit protein in regions of the cerebellar cortex of 60-day-old non-jaundiced (Jj) and jaundiced (jj) Gunn rats

Cytosolic GSTs were isolated from (**a**) flocculi, (**b**) lateral hemispheres and (**c**) vermis of cerebellar cortex of non-jaundiced (open bar) and jaundiced (hatched bar) Gunn rats by affinity chromatography and subjected to analysis by reversed-phase h.p.l.c. Values represent the means ± S.E.M. ($n = 3$) of pmol of GST subunit protein/mg of cytosolic protein. Numbers above the bars indicate that the jaundiced value is significantly different from the corresponding value for non-jaundiced animals ($P < 0.05$) and are the fold increase in subunit concentration (jj/Jj). Mu-class subunits: 3 (Y_{b1}); 4 (Y_{b2}); 6 (Y_{b3}); 11 (Y_o). Alpha-class subunits: 2 (Y_{c1}); 8 (Y_k). Pi-class subunit: 7 (Y_p).

animals. There were no effects of hyperbilirubinaemia on GST activity in the brainstem, cerebral cortex or thalamus/hypothalamus (Table 1). Cytosolic GST activity in the livers of 10-, 20- and 60-day-old Jj and jj Gunn rats were not significantly different (Table 1).

GST isoenzyme expression in Jj and jj Gunn rats

To determine which isoenzymes were responsible for the increase in cerebellar GST activity in homozygous Gunn rats, GSTs were isolated from cytosolic fractions of cerebellar flocculi, lateral hemispheres and vermis, and analysed by reversed-phase h.p.l.c. Individual GST subunit protein concentrations in these regions of the cerebellar cortex of 10-, 20- and 60-day-old animals are shown in Figures 1, 2 and 3 respectively. In cerebellum, there were increases in total GST subunit protein concentrations (Table 2) which correlated with the increased GST activity in jj Gunn rats. H.p.l.c. analysis in other brain regions and liver

Table 2 Effect of hyperbilirubinaemia on total cytosolic GST subunit protein in different brain regions and liver of non-jaundiced (Jj) and jaundiced (jj) Gunn rats

The concentrations of individual subunits were determined by h.p.l.c. These values were summed to determine the concentration of total GST subunit protein and values are the means ± S.E.M. ($n = 3$) expressed as pmol of GST subunit protein/mg of cytosolic protein.

Rats	Flocculus	Lateral hemisphere	Vermis	Brainstem	Thalamus/ hypothalamus	Cortex	Liver
10 days old							
Jj	33.3 ± 2.33	33.7 ± 3.63	29.5 ± 0.66	33.3 ± 4.55	39.7 ± 1.39	23.1 ± 0.97	204 ± 2.86
jj	35.3 ± 0.67	29.6 ± 2.14	27.0 ± 2.11	32.7 ± 1.83	39.0 ± 0.93	19.6 ± 1.39	218 ± 5.89
Fold	–	–	–	–	–	–	–
20 days old							
Jj	33.2 ± 0.47	39.6 ± 2.09	35.0 ± 2.79	53.7 ± 3.33	45.6 ± 7.16	29.6 ± 1.20	309 ± 15.8
jj	45.9 ± 1.59*	55.5 ± 2.46*	36.1 ± 2.49	51.3 ± 1.45	49.6 ± 2.72	29.7 ± 1.29	307 ± 22.6
Fold	1.38	1.40	–	–	–	–	–
60 days old							
Jj	50.5 ± 1.46	63.6 ± 1.85	59.9 ± 2.50	73.8 ± 8.30	94.8 ± 3.79	60.3 ± 1.33	638 ± 48.3
jj	120 ± 1.91*	115 ± 0.58*	97.9 ± 1.28*	90.8 ± 3.87	109 ± 8.19	58.6 ± 1.75	594 ± 55.1
Fold	2.38	1.81	1.63	–	–	–	–

* Significantly different from the corresponding value for Jj animal of the same age ($P < 0.05$).

Table 3 Distribution of Mu-class GST subunit 4 (Y_{b2}) in regions of the cerebellar cortex of non-jaundiced (Jj) and jaundiced (jj) Gunn rats

The protein concentrations of GST subunits present in each brain region were calculated from the reversed-phase h.p.l.c. profiles of affinity-purified GST pools. Values represent the means ± S.E.M. ($n = 3$) of pmol of GST subunit protein/mg of cytosolic protein.

		Subunit protein concentration (pmol of GST subunit protein/mg of cytosolic protein)					
	Rats...	10-day-old		20-day-old		60-day-old	
Condition	Region...	Flocculus	Vermis	Flocculus	Vermis	Flocculus	Vermis
Jj		2.06 ± 0.19*	0.67 ± 0.12	1.28 ± 0.12*	0.57 ± 0.14	1.81 ± 0.23*	0.76 ± 0.19
jj		1.93 ± 0.74*	0.40 ± 0.03	3.81 ± 0.37*†	0.47 ± 0.16	8.38 ± 0.74*†	1.41 ± 0.20†

* Significantly different from the corresponding value for vermis at the same age ($P < 0.05$).
† Significantly different from the corresponding Jj values ($P < 0.05$).

indicated that no significant change in GST subunit expression was associated with hyperbilirubinaemia (Table 2).

Individual GST subunit concentrations in cerebellum at 10 days of age were not significantly different between jaundiced and non-jaundiced rats (Figure 1). However, by 20 days, there were specific increases in the concentrations of Alpha-class subunits 2 (Y_{c1}) and 8 (Y_k) in all cerebellar regions of jj animals (Figures 2a, 2b and 2c), whereas Mu-class GST subunits 4 (Y_{b2}) and 11 (Y_o) were increased in flocculi and lateral hemispheres (Figures 2a and 2b), but not vermis in hyperbilirubinaemic Gunn rats (Figure 2c). The magnitude of increases in GST subunit concentrations was greater and more widespread in the chronically hyperbilirubinaemic Gunn rats at 60 days of age (Figure 3). However, the major effects were again seen with respect to Alpha-class subunits 2 (Y_{c1}) and 8 (Y_k), and Mu-class GST subunits 4 (Y_{b2}) and 11 (Y_o). In all regions of the 60-day-old cerebellar cortex, concentrations of subunits 2 (Y_{c1}), 8 (Y_k) and 11 (Y_o) were increased in hyperbilirubinaemia (Figures 3a, 3b and 3c), and the Mu-class GST subunit 4 (Y_{b2}) showed a concentration gradient, with flocculus (Figure 3a), demonstrating the largest and vermis (Figure 3c) the smallest increases. The distribution of this Mu-class subunit is presented in greater detail in Table 3. In cerebellum of 10-, 20- and 60-day-old non-jaundiced and jaundiced Gunn rats, the flocculus had the highest concentration of Mu-class GST subunit 4 (Y_{b2}) and vermis the lowest (Table 3). Furthermore, hyperbilirubinaemia increased the concentration of subunit 4 (Y_{b2}; 3-fold) in the flocculus, but not the vermis, of 20-day-old rats. In cerebellar cortex of 60-day-old jaundiced Gunn rats, the concentration of Mu-class GST subunit 4 (Y_{b2}) was increased 4.6-fold in the flocculi and less than 1.8-fold in the vermis (Table 3).

Sulphadimethoxine injection

We have demonstrated that there was no increase in GST protein concentration at 10 days of age and intermediate increases in specific subunits at 20 days of age, compared with 60-day-old Gunn rats (Figures 1, 2 and 3). Thus between 10 and 20 days of age, GST protein concentrations began to increase in the jaundiced Gunn rat.

Sulphadimethoxine is a long-acting sulphonamide which displaces bilirubin from its albumin-binding sites and increases bilirubin levels in tissues (Johnson et al., 1959; Schutta and Johnson, 1969; Rose and Wisniewski, 1979; Conlee and Shapiro, 1991). To determine whether sulphadimethoxine administration would augment the observed increases in GST protein concentrations and if there are region-specific effects on GST subunit concentrations associated with sulphadimethoxine injection,

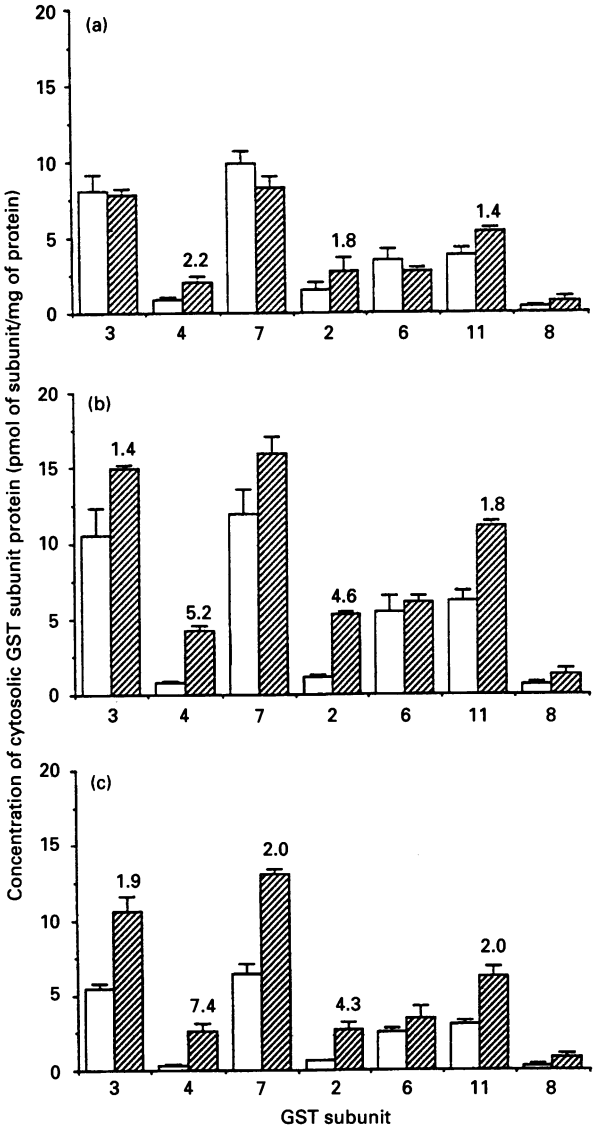

Figure 4 Effect of sulphadimethoxine on the concentrations of cytosolic GST subunit protein in lateral regions of the cerebellar cortex

Jaundiced and non-jaundiced rat pups were injected with saline or sulphadimethoxine on postnatal days 13 and 16, and were killed at 20 days of age. Cytosolic GSTs were isolated from flocculi/lateral hemispheres of cerebellar cortex of non-jaundiced (open bar) and jaundiced (hatched bar) Gunn rats by affinity chromatography and subjected to analysis by reversed-phase h.p.l.c. Animals were injected with (**a**) saline, (**b**) 50 mg of sulphadimethoxine/kg body weight or (**c**) 100 mg of sulphadimethoxine/kg body weight. Values represent the means ± S.E.M. ($n = 3$) of pmol of GST subunit protein/mg of cytosolic protein. Numbers above the bars indicate that the jaundiced value is significantly different from the corresponding value for non-jaundiced animals ($P < 0.05$) and are the fold increase in subunit concentration (jj/Jj). Mu-class subunits: 3 (Y_{b1}); 4 (Y_{b2}); 6 (Y_{b3}); 11 (Y_o). Alpha-class subunits: 2 (Y_{c1}); 8 (Y_k). Pi-class subunit: 7 (Y_p).

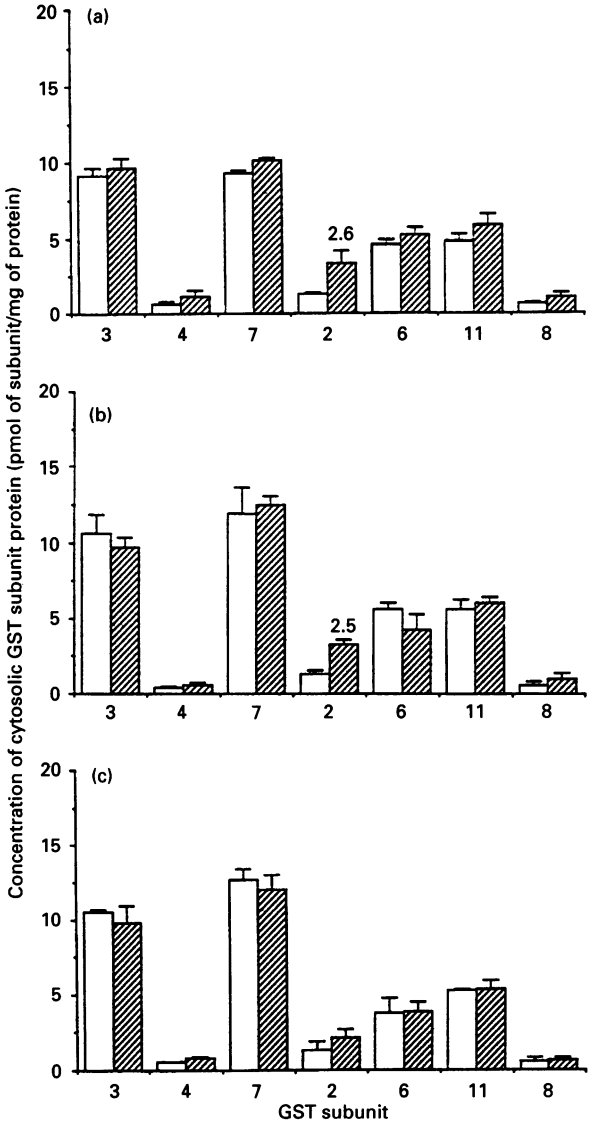

Figure 5 Effect of sulphadimethoxine on the concentrations of cytosolic GST subunit protein in vermis of the cerebellar cortex

Jaundiced and non-jaundiced rat pups were injected with saline or sulphadimethoxine on postnatal days 13 and 16, and were killed at 20 days of age. Cytosolic GSTs were isolated from vermis of cerebellar cortex of non-jaundiced (open bar) and jaundiced (hatched bar) Gunn rats by affinity chromatography and subjected to analysis by reversed-phase h.p.l.c. Animals were injected with (**a**) saline, (**b**) 50 mg of sulphadimethoxine/kg body weight or (**c**) 100 mg of sulphadimethoxine/kg body weight. Values represent the means ± S.E.M. ($n = 3$) of pmol of GST subunit protein/mg of cytosolic protein. Numbers above the bars indicate that the jaundiced value is significantly different from the corresponding value for non-jaundiced animals ($P < 0.05$) and are the fold increase in subunit concentration (jj/Jj). Mu-class subunits: 3 (Y_{b1}); 4 (Y_{b2}); 6 (Y_{b3}); 11 (Y_o). Alpha-class subunits: 2 (Y_{c1}); 8 (Y_k). Pi-class subunit: 7 (Y_p).

jaundiced and non-jaundiced Gunn rats were injected with saline, 50 and 100 mg of sulphadimethoxine/kg body weight at 13 and 16 days and killed at 20 days of age. There was no effect on weight gain due to sulphadimethoxine treatments; the percentage of original weight gained was not significantly different for any of the Jj to jj comparisons (results not shown). Since at 20 days of age the effects of hyperbilirubinaemia in the lateral hemispheres and flocculus were similar (Figures 1 and 2), these two cerebellar regions were pooled in the sulphadimethoxine experiment (Figure 4) and compared with vermis (Figure 5). The patterns of GST subunit expression in jaundiced and non-jaundiced animals injected with saline (Figures 4a and 5a) were similar to those seen in uninjected animals (Figure 2). In lateral regions of the cerebellar cortex, Mu-class subunits 4 (Y_{b2}) and 11 (Y_o) and Alpha-class subunit 2 (Y_{c1}) were significantly greater in jaundiced Gunn rats (Figure 4a), and injection with sulphadimethoxine augmented these effects (Figures 4b and 4c). In contrast, GST subunit 2 (Y_{c1}) was the only subunit significantly greater in the vermis of jaundiced Gunn rats (Figure 5a), and subunit concentrations in the vermis were not affected by sulphadimethoxine

injection (Figures 5b and 5c). In the lateral region of the cerebellar cortex, the fold increase for GST subunits in the sulphadimethoxine-injected animals (Figure 5b and 5c) was comparable with or exceeded the corresponding values in the chronically hyperbilirubinaemic animals at 60 days of age (Figures 3a and 3b).

DISCUSSION

Our major findings are: (1) age-dependent and region-specific increases in GST isoenzymes were seen in three regions of the cerebellar cortex of hyperbilirubinaemic animals; (2) GST concentrations, however, were not altered in the brainstem, thalamus/hypothalamus, cerebral cortex or liver of jaundiced Gunn rats; (3) in cerebellum from both non-jaundiced and jaundiced rats, the flocculus had the highest concentration of Mu-class GST subunit 4 (Y_{b2}) and vermis the lowest of all ages; (4) hyperbilirubinaemia increased the concentration of subunit 4 (Y_{b2}) in the flocculus and lateral hemispheres of the cerebellar cortex, but not the vermis; (5) sulphadimethoxine injection increased GST protein concentrations in the lateral regions of the cerebellum, but not the central cerebellar cortex.

In Gunn rats, Purkinje cell abnormalities are present by 7 days, reach a fully advanced stage of degeneration during the next 16 days and disappear between 12 and 30 days of age (Takagishi and Yamamura, 1989). Serum bilirubin concentrations in the Gunn rat parallel the observed Purkinje cell loss (Schutta and Johnson, 1969; Stobie et al., 1991). In the jaundiced Gunn rat, serum concentrations of bilirubin increase from birth to 20 days of age (230 μM) and then decline until about 45 days, where the concentration stabilizes (115 μM) (Schutta and Johnson, 1969; Stobie et al., 1991). The increase in GST activity and GST subunit protein concentrations also correlate with these changes in serum bilirubin levels, suggesting that GSTs may be involved in modulating bilirubin toxicity.

Schutta and Johnson (1969) showed that jaundiced Gunn rats treated with sulphadimethoxine had more abnormal Purkinje cells in the vermis of the cerebellar cortex than untreated jaundiced littermates. Abnormalities in neurons of other brain regions were rarely seen in untreated jaundiced Gunn rats. However, sulphadimethoxine injection caused abnormalities in neurons of the cerebral cortex, medial nucleus of the trapezoid body and anteroventral cochlear nucleus (Schutta and Johnson, 1969; Conlee and Shapiro, 1991). We have demonstrated that GST concentrations are significantly increased in the lateral regions, but not the vermis, in the cerebellar cortex of jaundiced rats by sulphadimethoxine injection (Figures 4 and 5). These data imply that displacement of bilirubin from serum albumin, which results in decreased serum and increased tissue concentrations of bilirubin, selectively alters GST expression in lateral regions of the cerebellar cortex. Thus there is a correlation between the presence of GSTs and reduced susceptibility to bilirubin-induced cytotoxicity. Further evaluation of the effects of sulphadimethoxine on GST expression in other brain regions, where neurons have been shown to be affected by increased tissue concentrations of bilirubin, may help in understanding the role that GSTs play in modifying bilirubin toxicity.

The increased concentration of specific GST subunits in the cerebellar cortex of jaundiced Gunn rats could be due to: (1) increased proliferation of a GST-containing cell population(s), (2) toxicity-induced selection for a GST-containing cell population(s) or (3) increased transcription of genes encoding GSTs. GSTs have been localized to glial cell populations (Abramovitz et al., 1988; Cammer et al., 1989; Tansey and Cammer, 1991a,b; Johnson et al., 1993), and it is known that bilirubin causes astrocytic proliferation in the cerebellar cortex (Sawasaki et al., 1976; Takagishi and Yamamura, 1987, 1989). This may contribute to the increased concentrations of GST in the cerebellar cortex. Another possible explanation could be toxicity-induced selection for GST-containing cell populations. Cells that have low levels or do not express GSTs may be more susceptible to bilirubin toxicity and the increased concentrations would result from the greater fraction of GST-containing cells present in the cerebellar cortex of jaundiced Gunn rats. If this were true, we would have expected a uniform increase in all GST subunit protein concentrations associated with hyperbilirubinaemia. Since our data indicate that there are region- and subunit-specific changes in the cerebellar cortex of the jaundiced Gunn rat, this may not be the case. The effects of sulphadimethoxine injection also demonstrated a region- and subunit-specific augmentation of GST levels in the cerebellar cortex of jaundiced rats (Figures 4 and 5), reinforcing the suggestion that these observed effects of hyperbilirubinaemia on GSTs are probably not due to selection for GST-containing cells. Finally, the changes in GST concentration may be due to an increase in the concentration of GST within a certain cell population(s).

Irrespective of the mechanism by which GSTs are increased, cellular proliferation or induction, there should be a corresponding increase in the binding capacity for bilirubin which may protect cells against bilirubin toxicity. In cerebellar cortex, the first line of defence would be Alpha-class GST 8–8 (YkYk) in endothelial cells, then Alpha- and Mu-class GSTs in astrocytes, then Mu-class GSTs in Bergmann glia and finally Alpha-class and microsomal GST in neurons (Johnson et al., 1993). For example, if GST concentrations are increased in the Bergmann glial cells, which are intimately associated with Purkinje cells, they may protect Purkinje cells from bilirubin toxicity. Furthermore, GSTs present in Purkinje cells could be upregulated as a defence against the cytotoxic effects of bilirubin. A similar protective mechanism could be envisaged for GST-positive cells at each level of defence. Thus GSTs may effectively reduce bilirubin neurotoxicity.

GSTs may also control the uptake and transport of numerous endogenous compounds including bilirubin, steroids, thyroid hormones and neurotransmitters, as well as the removal of toxins and harmful metabolic by-products in brain via GSH-dependent binding to GST (Listowsky et al., 1988; Abramovitz et al., 1988). Philbert et al. (1991) have recently described the regional and cellular distribution of GSH in the nervous system. In general, neuronal soma throughout the central nervous system were negative for GSH histochemical staining, but in the cerebellum, both granule cells and Purkinje cells were positively stained for GSH (Philbert et al., 1991). Furthermore, we have shown, by immunohistochemistry, that neurons throughout the brain contain Alpha-class and microsomal GST (Johnson et al., 1993). Microsomal GST was localized to the neuronal soma of Purkinje cells, brainstem neurons and neurons in the hippocampus, Alpha-class GST 1–1 (Y_aY_a) was localized to nuclei of Purkinje cells, as well as neurons in the brainstem and hippocampus, and Alpha-class GST 2–2 (Y_cY_c) antibody was consistently immunoreactive with the nucleolus, but neither the nucleus nor soma in these same neurons. Thus both GSTs and GSH have been shown to be present in cerebellar neurons. Philbert et al. (1991) also showed that GSH was present throughout the central nervous system neurophil white matter fibre tracts and noted that the choroid plexus was intensely stained for GSH. This pattern of GSH localization was consistent with localization of Alpha-, Mu- and Pi-class GSTs (Abramovitz et al., 1988; Cammer et al., 1989;

Tansey and Cammer, 1991a,b; Johnson et al., 1993). The co-localization of GST and GSH supports the proposed mechanism that GSTs may control the uptake and transport of various compounds via GSH-dependent binding to GST (Listowsky et al., 1988; Abramovitz et al., 1988).

The observations that, in the absence of glucuronidation, bilirubin is converted into polar derivatives that are excreted in the bile (Schmid and Hammaker, 1963) and that some of the derivatives are hydroxylated metabolites (Blanckaert et al., 1977), imply that a somewhat less efficient alternative metabolic pathway involving hepatic cytochrome *P*-450-dependent monooxygenase(s) may exist for bilirubin catabolism. Administration of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin to jaundiced Gunn rats results in decreased plasma bilirubin levels (Kapitulnik and Ostrow, 1977). Kapitulnik and colleagues (1987) subsequently demonstrated that jaundiced (jj) Gunn rats have higher concentrations of hepatic cytochrome *P*-450IA1 than non-jaundiced (Jj) Gunn rats, and that treatment of adult jaundiced Gunn rats with 3-methylcholanthrene decreased the plasma bilirubin levels from 12.6 to 7.3 mg/dl and caused a corresponding 200-fold increase in cytochrome *P*-4501IA1 (Kapitulnik et al., 1987). The presence of the cytochrome *P*-450IA family was detected in brain microsomes and shown to be induced with 3-methylcholanthrene by immunoblotting (Anandatheerthavarada et al., 1990). In addition, Odell and colleagues (1991) have reported the isolation of two distinct GSH conjugates of the dimethyl ester of bilirubin from bile of Gunn rats. Our data indicate that there are region- and GST-subunit-specific effects on the concentration of GST protein present in the cerebellar cortex of Gunn rats. These findings suggest that an alternative pathway of bilirubin catabolism involving the cytochrome *P*-450IA and GST families of enzymes may be present in brain.

Regulation of the GST expression in the cerebellar cortex of Gunn rats could also be secondary to the cytotoxicity associated with hyperbilirubinaemia. Pickett and colleagues (Rushmore et al., 1991) identified a 'responsive element' in the 5′-flanking regions of some Phase II enzyme genes which is involved in the control of gene expression by phenolic antioxidants. The 'antioxidant responsive element' (ARE) is present in the 5′-flanking regions of the *GST* Y_a and *NQO*$_1$ genes. The ARE responds to reactive oxygen products (including hydrogen peroxide) generated during redox cycling (Rushmore et al., 1991). The mechanism of this ARE-mediated response is not known. Bilirubin is a known antioxidant (Stocker et al., 1987a,b) and the increase in GST concentrations in the cerebellar cortex of the Gunn rats could be ARE-mediated. These data do not suggest that bilirubin directly interacts with the ARE, but bilirubin may, through an induced cytotoxicity, cause oxidative stress in the cerebellar cortex and subsequently increase the expression of GSTs.

The region-specific increase in cytosolic GST concentrations seen in untreated jaundiced animals, combined with the region-specific effects of the sulphadimethoxine injection, suggests that GSTs may play a role in neuroprotection. Specifically, the normal levels of Mu-class GST subunit 4 (Y_{b2}) and the increase in concentration of this GST in the cerebellum of jaundiced rats (Table 3) are correlated with regional differences in resistance to degeneration after exposure to other toxic or metabolic insults. The vermis is most susceptible to these insults, whereas the lateral lobes and flocculus are most resistant. 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. Region-specific cerebellar dysgenesis 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 humans. In non-human mammals, alcohol exposure *in utero* (Kornguth et al., 1979; Bonthius and West, 1991), methadone withdrawal (Zagon and McLaughlin, 1978), exposure to mutated diphtheria toxin (Riedel et al., 1990), hypothyroidism (Kornguth et al., 1967, 1979; Nicholson and Altman, 1972; 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. In each case, there is a region-specific loss of Purkinje cells due to a toxic or metabolic insult, and this pattern of Purkinje cell loss correlates inversely with the distribution of Mu-class GST subunit 4 (Y_{b2}). Thus the failure to increase specific GST isoenzymes in hyperbilirubinaemia or the absence of these enzymes from regions of the cerebellar cortex may confer susceptibility of that region to endogenous and/or exogenous neurotoxic agents.

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