HHS Public Access

Author manuscript

Alcohol Clin Exp Res. Author manuscript; available in PMC 2015 June 04.

Published in final edited form as:

Alcohol Clin Exp Res. 2004 November ; 28(11): 1622–1628.

Glutathione S-Transferase 8-8 Expression Is Lower in Alcohol-Preferring Than in Alcohol-Nonpreferring Rats

Tiebing Liang, **Kirk Habegger**, **John P. Spence**, **Tatiana Foroud**, **Julie A. Ellison**, **Lawrence Lumeng**, **Ting-Kai Li**, and **Lucinda G. Carr**

Departments of Medicine (TL, KH, JPS, LL, LGC) and Medical and Molecular Genetics (TF), Indiana University School of Medicine, Indianapolis, Indiana; Digital Gene Technologies, Inc. (JAE), La Jolla, California; and National Institute on Alcohol Abuse and Alcoholism (T-KL), National Institutes of Health, Bethesda, Maryland

Abstract

Objective—A primary focus of alcohol research is to provide novel targets for alcohol treatment by identifying genes that predispose individuals to drink alcohol. Animal models of alcoholism developed by selective breeding are invaluable tools to elucidate both the genetic nature and the underlying biological mechanisms that contribute to alcohol dependence. These selected lines (high alcohol preferring and low alcohol preferring) display phenotypic and genetic differences that can be studied to further our understanding of alcohol preference and related genetic traits. By combining molecular techniques, genetic and physiological factors that underlie the cause of alcoholism can be identified.

Methods—Total gene expression analysis was used to identify genes that are differentially expressed in specific brain regions between alcohol-naïve, inbred alcohol-preferring (iP) and -nonpreferring (iNP) rats. Quantitative reverse transcriptase–polymerase chain reaction, in situ hybridization, Western blot, and sequence analysis were used to further characterize rat glutathione S-transferase 8-8 (rGST 8-8).

Results—Lower expression of rGST 8-8 mRNA was observed in discrete brain regions of iP compared with iNP animals, and these expression differences were confirmed. To determine additional expression patterns of rGST 8-8, we used in situ hybridization. Rat GST 8-8 was highly expressed in hippocampus, the choroid plexus of the dorsal third ventricle and the lateral ventricle, and ependymal cells along the dorsal third ventricle and the third ventricle. Western blot analysis showed that rGST 8-8 protein levels were lower in the hippocampus and the amygdala of iP compared with iNP. A silent single-nucleotide polymorphism in the coding region and three single-nucleotide polymorphisms in the 3′-UTR were identified in the rGST 8-8 cDNA.

Conclusion—There is regional variation of rGST 8-8 expression in the brain, at both the mRNA and protein level, and the iP strain has lower innate rGST 8-8 levels than the iNP strain in discrete brain regions.

Reprint requests: Lucinda G. Carr, PhD, Departments of Medicine and Pharmacology, Indiana University School of Medicine, Medical Research and Library Building, Room 407, 975 W. Walnut Street, Indianapolis, IN 46202; Fax: 317-274-2695; lcarr@iupui.edu.

Keywords

Rat GST 8-8; Alcoholism; mRNA Expression; Polymorphism; Rat

Alcoholism is a complex disorder influenced by both environmental and genetic factors. The genetic contribution to alcoholism likely results from the action of multiple, possibly interacting genes. Identification of the genes that influence alcohol drinking is an important area of research in the alcohol field and has involved several strategies, including the use of human studies and the development of genetic animal models of alcoholism. Sequencing of the rat genome has revealed that most human genes have counterparts in the rat (Rat Genome Sequencing Project Consortium, 2004). Thus, identification of interesting genes in a rat model can provide candidate genes for specific diseases that can be evaluated further in humans.

Animal models of alcohol preference have been used to identify both chromosomal loci and candidate genes that may influence alcohol-drinking behavior (Carr et al., 1998; Foroud et al., 2000; Grisel et al., 2002; Liang et al., 2003). The alcohol-preferring (P) and -nonpreferring (NP) rat lines were developed through bidirectional selective breeding from a randomly bred closed colony of Wistar rats [Wrm: WRC(WI)BR] on the basis of alcohol consumption and preference (Li et al., 1991). In these lines, P rats display the phenotypic characteristics considered necessary for an animal model of alcoholism (Cicero, 1979). Subsequently, inbred P (iP) and NP (iNP) strains that have maintained highly discordant alcohol consumption scores were established.

Complementary methods, quantitative trait locus (QTL) analysis, and total gene expression analysis (TOGA; a registered trademark of Digital Gene Technologies) were conducted to identify genes that influence alcohol consumption in iP and iNP rats (Liang et al., 2003). A genome screen of the iP × iNP F2 animals identified QTLs on rat chromosomes 3, 4, and 8 (Bice et al., 1998; Carr et al., 1998). TOGA, which identifies differential expression of both known genes and 3′ expressed sequence tags (ESTs) (Lockhart and Barlow, 2001; Sutcliffe et al., 2000), identified rat glutathione S-transferase 8-8 (rGST 8-8) as one of the genes differentially expressed between iP and iNP. rGST 8-8 was selected for further investigation because (1) it was located near a QTL for alcohol preference identified in an iP × iNP F2 population that is syntenic with a QTL region for alcohol preference identified in the mouse (Belknap and Atkins, 2001; Phillips et al., 1998), (2) the expression difference between iP and iNP was notable, and (3) alcohol consumption induces the expression of GSTs (Schnellmann et al., 1984; Thibault et al., 2000).

Glutathione S-transferases (GSTs) are a large family of multifunctional proteins involved in transport, biosynthesis of endogenous compounds, and disposition of exogenous toxicant compounds. GST isoenzymes have been categorized into eight classes: GSTA, GSTM, GSTP, GSTS, GSTT, GSTZ, GSTK, and GSTO (Board et al., 1997, 2000; Meyer et al., 1991; Hayes and Pulford, 1995; Pemble et al., 1996). rGST 8-8, a class α isoenzyme that has recently been designated rGSTA4 (Hayes and Pulford, 1995; Meyer et al., 1989; Stenberg et al., 1992), has special catalytic specificity toward 4-hydroxyalkenal and acrolein

(Bjornestedt et al., 1995; Danielson et al., 1987). Because the GST 8-8 designation is widely used in the literature, this nomenclature is used throughout this article.

GSTs function as defense mechanisms against oxidative damage and lipid peroxidation, making GSTs relevant to the effects of alcohol (Hayes and Pulford, 1995). For example, alcohol treatment induces oxidative stress in astrocytes in primary culture and depletes the levels of glutathione (GSH) (Montoliu et al., 1995). Furthermore, oxidative stress is a probable mechanism involved in alcohol-induced neural damage. Similar to mGSTA4 and hGSTA4, rGST 8-8 seems to belong to a special subgroup of GSTs involved in the detoxification of highly cytotoxic compounds produced by lipid peroxidation, radical reactions, and other reactions elicited by oxidative stress (Berhane et al., 1994; Hubatsch et al., 1998; Singhal et al., 1994). Previous studies have also demonstrated that dopamine and other physiologically relevant catecholamines are inactivated by human GSTs (Baez et al., 1997; Hubatsch et al., 1998; Segura-Aguilar et al., 1997). Therefore, GSTs are of interest to the study of alcoholism because of their relevance to oxidative stress and catecholamine inactivation.

The objective of this study was to define better the expression of rGST 8-8 in iP and iNP rats, using multiple molecular approaches. TOGA and quantitative reverse transcription–polymerase chain reaction (qRT-PCR) were used to evaluate rGST 8-8 mRNA expression, and in situ hybridization was used to determine specific brain regions that express rGST 8-8 in the iP and iNP rats. Western blot analysis was used to analyze rGST 8-8 protein levels between these strains. Finally, sequence analysis was performed to screen rGST 8-8 cDNA for polymorphisms that might influence its expression.

MATERIALS AND METHODS

Dissection of Brain Regions

iP and iNP alcohol-naïve adult male rats were killed, and the entire brain was removed and dissected using the coordinates of Paxinos and Watson to produce four subregional tissue samples: (1) hypothalamus; (2) hippocampus; (3) caudate-putamen, nucleus accumbens, and olfactory tubercles; and (4) prefrontal, frontal, and parietal cortex. The prefrontal cortex is dissected by a coronal cut at Bregma 2.7 using a rat brain matrix; the cingulate and infralimbic cortices are separated from the rest of the tissue along the midline aspect of the corpus callosum and a diagonal cut outward from the apex of the callosum. The caudate putamen, nucleus accumbens, and olfactory tubercles are dissected from a 2-mm section generated by a coronal cut at 2 mm anterior to the optic chiasm (Bregma 1.70 mm) and a coronal cut at the optic chiasm (Bregma −0.26 mm). The nucleus accumbens is dissected bilaterally by cutting below the rhinal fissure, retaining the olfactory tubercles and trimming off the cortical tissue at the ventral and ventrolateral borders of the slice. From the remaining tissue, the caudate putamen (bilateral) is dissected below the corpus callosum, leaving the septum hanging in the center. The frontal and parietal cortices are dissected from this slice by trimming away the cingulate cortex (at the apex of the forceps minor corpus callosum) and a cut dorsal to the claustrum. The frontal and parietal cortices are separated by visual estimation. The hypothalamus is dissected from the tissue posterior to the optic chiasm by making an incision 2 mm to both the right and the left of the midline and a cut in

front of the mammillary bodies as the caudal limit. The entire hippocampus is dissected from the remaining brain by a midline incision between the hemispheres and rolling the hippocampus out of the cerebral cortex. The microdissected tissues are snap-frozen on dry ice and stored at −80°C until RNA isolation.

TOGA—RNA was isolated from the microdissected tissues of 10 iP and 10 iNP rats according to the RNeasy Midi manufacturer's protocol (Qiagen, Valencia, CA), and the isolated RNA was resuspended in DEPC-treated water and stored at −80°C. For the TOGA analysis, pooled iP and iNP RNA samples were assembled for each subregion. TOGA analysis was performed as previously described (Spence et al., 2004; Sutcliffe et al., 2000).

qRT-PCR—For qRT-PCR, the hippocampus, striatum, cortex, and hypothalamus were dissected from another group of five iP and five iNP alcohol-naïve adult male rats and RNA was isolated using the RNeasy Mini Kit (Qiagen). Pooled iP and iNP RNA samples were assembled for each subregion using 1.5 μg of RNA sample from each of the five iP and five iNP rats. The relative expression levels of rGST 8-8 in each brain region were determined by qRT-PCR using TaqMan chemistry and the ABI PRISM 7700 Sequence Detection System (PE Biosystems, Wellesley, MA). The fluorogenic TaqMan Minor Grove Binder probe and primers were designed and synthesized by the assay-by-design service of ABI as follows: FAM-labeled rGST 8-8 TaqMan Minor Grove Binder probe, ACCCTTGCAG-TAGCCA, primer 1F, 5′-GAAGTTCTAGTGACAGCGTGCTTTA-3′, and primer 1R, 5′-TGTAGCTGCTGCTGTGATTGG-3′. cDNA first was generated from 1 μg of each pooled RNA sample using TaqMan Reverse Transcription Reagents (Roche Molecular Systems, Branchbury, NJ). Each PCR contained cDNA template that was reverse-transcribed from 18 ng of total RNA, 0.25 μM of probe, 0.9 μM of forward and reverse primers, and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA).

Each sample was amplified for 40 cycles, and the cycle threshold was determined for each cDNA template. The cycle threshold refers to the cycle number at which the fluorescence of the amplified product reached an arbitrary threshold that was within the exponential phase of amplification. Relative values of expression were determined for each sample using the standard curve method. The mRNA expression levels of the iP versus iNP brain regions were analyzed by the method of two-way analysis of variance (ANOVA). Significant differences were found on the group effect (iP versus iNP), whereas the experiment effect and interaction term did not show any significance for all regions.

IN SITU HYBRIDIZATION—rGST 8-8 cDNA was cloned into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA). From this construct, antisense and sense cRNA probes, labeled with ^{35}S-UTP and ^{35}S-CTP, were prepared. The tissues were hybridized according to published procedures (Liang et al., 2003; Wirdefeldt et al., 2001). Briefly, 16-μm brain sections were dehydrated and covered with 100 μl of hybridization buffer [50% formamide, 50 mM of Tris (pH 8.0), 2.5 mM of EDTA (pH 8.0), 50 μg/ml of tRNA, 1× Denhardt's solution, 0.2 M of NaCl, and 10% Dextran sulfate] containing 1.0×10^6 cpm of labeled probe for 16 to 18 hr at 55°C. The sections were washed, dehydrated, and incubated in formamide buffer [0.3 M of NaCl, 50% formamide, 20 mM of Tris, 1 mM of EDTA (pH 7.5)] for 10 min at 60°C, RNase A for 30 min at 37°C, and graded SSC washes (2× SSC to

0.5× SSC), followed by dehydration. The dried sections were exposed to Kodak MR film (Kodak) for 3 to 4 days; selected slides were dipped in hypercoat LM-1 emulsion (Amersham, Piscataway, NJ) and exposed for 2 to 3 weeks at 4°C. The autoradiograph film was scanned with maximum resolution (2400 pixels) without adjustment for brightness and contrast. From these films, the hippocampus CA2 and CA3 region was selected to quantify. In total, four sections from each of five iP rats and four sections from each of five iNP rats were analyzed using the Image J software (http://rsb.info.nih.gov/ij/). For each section on the film, a threshold value on the gray scale was determined by measuring randomly selected areas of the hippocampus. These were then averaged and subtracted as background. A mean value of the CA2–CA3 region for each animal (four sections per animal) was determined, and the five animal means were averaged to create an iP and an iNP group mean, expressed in arbitrary density units. The two groups were compared by *t* test.

WESTERN BLOT ANALYSIS—The hippocampus, striatum, amygdala, nucleus accumbens, and substantia nigra were dissected from another group of five iP and five iNP rats, and the analysis was performed on individual animals. Protein extracts were prepared as described previously (Liang et al., 2003). Dr. J.A. Johnson (University of Wisconsin-Madison) provided rGST 8-8 antibody. Neuron-specific enolase (NSE; MW = 45 kD) was used as the internal control to normalize the amount of protein loaded in each lane. Approximately 30 μg of the protein supernatant was fractionated on a Novex 4 to 20% Tris-Glycine Gel and was transferred to a PVDF membrane (0.2 μm; Bio-Rad, Hercules, CA). The membrane was treated for 1 hr in TTBS blocking buffer (5% nonfat dry milk, 0.1% Tween-20, and 1× Tris-buffered saline), followed by incubation with rGST 8-8 primary antibody (1:1000 in TTBS) overnight at 4°C. The next day, NSE antisera (1:5000; Polysciences, Warrington, PA) was added and incubated for 1 hr at room temperature. After washing with TTBS for 1 hr, the membrane was incubated for 1 hr with horseradish peroxidase–conjugated secondary antibody (1:15,000), washed, and then developed using the Immun-Star HRP Chemiluminescent Kit (Bio-Rad). rGST 8-8 levels were quantified using the Image J software (http://rsb.info.nih.gov/ij/) with each lane normalized to NSE levels. Protein expression levels of iP and iNP rats were compared by ANOVA.

SEQUENCE ANALYSIS—Reverse transcription was used to generate cDNA (RETROscript kit; Ambion, Austin, TX) from pooled total RNA samples that were isolated from iP and iNP rats. On the basis of the rGST 8-8 cDNA sequence (accession no. X62660), three primer pairs were designed: 2F 5′-CCAATAAGGAAACTCTGAACCAG-3′ and 2R 5′-TTTCAAACA-CTGGGAAGTAACGG-3′, 3F 5′-CTAGCTTTAGCAGTGAAGAGGG-3′ and 3R 5′-TTTGTCATTGTTGGACAGAGTGG-3′, 4F 5′-CCAC-TATGTTGACGTGGTCAG-3′ and 4R 5′-TTAACAGTTTTT-CACTCTATTTAATTGG-3′. Using iP and iNP cDNA, these primers were used to amplify three overlapping DNA fragments, covering the entire rGST 8-8 cDNA. Resulting PCR products were purified (GenElute PCR Cleanup Kit; Sigma, St. Louis, MO) and sequenced (Thermo Sequenase Cycle Sequencing Kit; USB, Cleveland, OH).

GENOTYPE DETERMINATION AND GENE MAPPING—A dense map of markers in the chromosome 8 QTL region was previously used for genotyping the iP × iNP F2 (Bice et al., 1998). Thus, it was possible to map the rGST 8-8 single-nucleotide polymorphism (SNP) by selectively genotyping 88 animals at the extremes of the iP × iNP F2 alcohol consumption distribution (Carr et al., 1998). For genotyping, the fragment that contained the polymorphism at +727 was amplified from genomic DNA using the 5F 5′-TAAAGTGGCTACTGCAAGGGTC-3′ and 5R 5′-GGTTCTTTGAAGACCTTTACTCC-3′ primers. Amplified DNA fragments were slot-blotted onto Zeta-probe membranes (Bio-Rad) and baked at 80°C for 2 hr. Two allele-specific probes, NP 5′-GCAGCAGC-TA*C*AGAGCATTC-3′ and P 5′-GCAGCAGCTA*T*AGAGCATTC-3′, were end-labeled using [γ^{32}P]ATP. The rGST 8-8 alleles were distinguished using buffers that contained 3 M of tetramethylammonium chloride (DiLella and Woo, 1987). Membranes were hybridized for 1 hr at 55°C, and the critical washing temperature was 63°C for 20 min. Membranes were exposed to film for 3 to 18 hr at −70°C, and the resulting genotypes were recorded. The program MAPMAKER/EXP (Lander et al., 1987) was used to place the rGST 8-8 SNP within a recombination-based map of chromosome 8 markers genotyped in the 381 iP × iNP F2 animals.

RESULTS

Differential Expression

To investigate the mRNA expression difference between iP and iNP rats, we analyzed four brain regions using TOGA (Sutcliffe et al., 2000): the cortex, hippocampus, hypothalamus, and striatum. TOGA analysis initially detected 19,954 genes and ESTs. From this data set, 28 that exhibited 2-fold or greater change in expression were prioritized on the basis of their expression pattern and function reported in the literature (Liang et al., 2003; Spence et al., 2004). rGST 8-8 was selected to characterize further because of its chromosomal location in the mouse, the role that it plays in neuroprotection, and the effect that alcohol consumption has on inducing expression of GSTs (Schnellmann et al., 1984; Thibault et al., 2000). A decrease in rGST 8-8 expression was observed in the iP compared with the iNP rats in all four dissected regions, with the cortex exhibiting the lowest fold change (2.9) and the striatum having the highest fold change (4.8; Table 1). qRT-PCR analysis confirmed this finding, with the iP rats exhibiting significantly less GST 8-8 expression than the iNP rats in all four brain regions tested (hypothalamus, $p < 0.0001$; hippocampus, $p < 0.0001$; striatum, $p < 0.0001$; and cortex, $p < 0.0001$; Table 1). Although the fold differences were greater in the TOGA than in the qRT-PCR, this may be because different individuals dissected different groups of animals; one group of rats was dissected at Digital Gene Technologies for the TOGA, whereas the group of rats used for qRT-PCR was dissected at Indiana University School of Medicine.

rGST 8-8 mRNA Expression Pattern

To identify additional brain regions where GST 8-8 was expressed, we performed in situ hybridization in the iP and iNP rats (Fig. 1). GST 8-8 was expressed throughout the brain, with expression localized to specific brain regions and cells. An example of the distinctive pattern of expression of GST 8-8 is shown in Fig. 1. rGST 8-8 was highly expressed in the

choroid plexus of the dorsal third ventricle (D3V; Fig. 1A, 1 and 2), the lateral ventricle (LV; Fig. 1A, 3 and 4), and the CA2 and CA3 regions of the hippocampus (Fig. 1A, 3–6). Lower expression levels were detected in the cerebellum (data not shown), thalamus (Fig. 1A, 4), and cortex (Fig. 1A, 6). Comparison of rGST 8-8 mRNA expression between iP (Fig. 1A, 1, 3, and 5) and iNP (Fig. 1A, 2, 4, and 6) on the autoradiographs showed lower expression in iP than iNP rats. Quantification of rGST 8-8 expression in the CA2 and CA3 regions of the iP/iNP hippocampus (Fig. 1A, 3 and 4, and 1B, 1 and 2) revealed a 3-fold lower expression in iP (Fig. 1B, 1 and 3) than in iNP (Fig. 1B, 2 and 4; $p < 0.0001$) rats. At the cellular level, it is clear that rGST 8-8 is expressed in the pyramidal cells of CA2 and CA3 of iP/iNP rat brain (Fig. 1B, 1–4), the endothelial cells of the choroid plexus (Fig. 1B, 5), and the ependymal cells along the D3V (Fig. 1B, 5). Expression of rGST 8-8 in the ependymal cells is evident at the base of the third ventricle of iNP rats (Fig. 1B, 6).

rGST 8-8 Protein Levels Are Lower in the Hippocampus and Amygdala of iP Than of iNP Rats

To compare rGST 8-8 protein expression in iP and iNP rats, we analyzed five brain regions: hippocampus, striatum, amygdala, nucleus accumbens, and substantia nigra. Similar to rGST 8-8 mRNA levels, the level of protein expression in the amygdala was lower in iP rats than in iNP rats, with the iP rats having 1.6-fold lower levels than the iNP rats ($p < 0.020$; Fig. 2). The same trend was observed in the hippocampus, with a 1.2-fold difference between iP and iNP rats ($p < 0.048$; data not shown). No difference in expression was detected in the striatum ($p < 0.918$), nucleus accumbens ($p < 0.194$), or substantia nigra ($p < 0.607$) between the iP and iNP rats. It is clear from these studies that there are regional differences in the expression of rGST 8-8 in rat brain.

Polymorphism in the rGST 8-8 cDNA of iP and iNP Rats

For determining whether a sequence difference might underlie the expression difference observed between the iP and iNP rats, the rGST 8-8 cDNA was sequenced in each strain (Fig. 3). A silent SNP was identified in the coding region at +628, relative to the translation start site (+1), and three SNPs were discovered in the 3′-UTR. The iP sequence displayed differences in four positions (+628, +714, +727, and +756), whereas the iNP sequence was identical to the published rGST 8-8 cDNA sequence (Accession no. X62660; Table 2). An alignment of the iP and iNP sequences with another published GST sequence (XM_217195) indicated that the iNP GST 8-8 sequence was also more homologous to this sequence than was the iP sequence (Table 2).

rGST 8-8 Mapping and Association Studies

Using the SNP that was discovered at +727 in the iP and iNP rats, rGST 8-8 was mapped, using recombination-based methods, to chromosome 8, 2.9 cM distal to *D8Rat21* and 3.8 cM proximal to *D8Rat23*, which is adjacent to the chromosome 8 QTL previously identified (Bice et al., 1998; Carr et al., 1998). The polymorphism at +727 then was used to determine whether the high-drinking allele in the iP strain was associated with the high-drinking animals in the iP × iNP F2 population: A slight trend for the GST 8-8 iP allele to be associated with the high-drinking animals was observed ($p < 0.13$).

DISCUSSION

This is the first study to systematically compare expression of rGST 8-8 between two selectively bred rat strains. A difference in rGST 8-8 mRNA expression between the iP and iNP rats was discovered and confirmed. rGST 8-8 protein levels were lower in the amygdala and hippocampus of iP rats compared with iNP rats. Sequence analysis of the rGST 8-8 cDNA between the iP and iNP strains identified three polymorphisms in the 3′-UTR, which could underlie the difference in expression observed between the rat strains. Numerous reports have identified the eukaryotic mRNA 3′-UTR as a primary site for the regulation of mRNA stability (Loflin and Lever, 2001; Nair and Menon, 2000; Wang and Kiledjian, 2000). In addition, regulation by the 3′-UTR can occur at the level of mRNA subcellular localization and translation initiation (Grzybowska et al., 2001; Heese et al., 2002). A polymorphism was used as a marker to map rGST 8-8 to a locus on chromosome 8 near a previously identified provisional QTL for alcohol preference in the iP and iNP rats (Carr et al., 1998). The peak of the mouse chromosome 9 QTL is broad and encompasses the rat chromosome 8 QTL and the rGST 8-8 locus (Phillips et al., 1998).

Both regional and cellular differences in expression exist between the various classes of GSTs (Gandy et al., 1996; Hayes and Pulford, 1995; Jowsey et al., 2001; Otieno et al., 1997; Singhal et al., 1994). rGST 8-8 is expressed in many rat tissues, and its pattern of expression differs from that of other class *α* GSTs (Johnson et al., 1993; Meyer et al., 1989; Singhal et al., 1994). Although protein expression of GSTs has been studied in the rat brain, this is the first report of rGST 8-8 mRNA expression in rat brain. Rat GST 8-8 mRNA in the iP and iNP rats was highly expressed in choroid plexus of D3V and LV, similar to previously observed protein levels (Johnson et al., 1993). Similarly, expression of rGST 8-8 mRNA and protein was observed in the hippocampus of the iP and iNP rats (Fig. 1A, 3–6, and 1B, 1–4). In contrast, rGST 8-8 mRNA was observed in the ependymal cells along the third ventricle in the iP (data not shown) and iNP rats (Fig. 1B, 5 and 6), whereas the rGST 8-8 protein was not detected in these cells (Johnson et al., 1993). These data suggest that the regulation of rGST 8-8 expression is complex and that in some regions, high levels of mRNA translate into high levels of protein, whereas in other regions the mRNA and protein levels are not correlated.

GSTs represent a major group of detoxification enzymes, and their expression is a crucial factor in determining the sensitivity of cells to a broad spectrum of toxic compounds. The ability of rGST 8-8 to metabolize electrophiles generated during oxidative stress and its induction after exposure to allylamine suggests that this enzyme functions as a major defense mechanism (He et al., 1999; Misra et al., 1995). We found that rGST 8-8 was highly expressed in the choroid plexus and in the ependyma zone that lines the ventricles, regions that have the capability to modulate endo- and xenobiotics. Thus, it is very possible that rGST 8-8 has a neuroprotective role in the rat.

It is possible that the expression differences between the iP and iNP resulted from chance fixation of polymorphisms during selective breeding, which are not associated with the alcohol preference phenotype. The polymorphisms detected in the rGST 8-8 gene of the iP/iNP rats were not significantly associated with their drinking behavior. However, the

differential expression that we have detected between the iP and iNP rats may be a crucial factor in determining the sensitivity of cells in the brain to toxic chemicals, such as alcohol. The function of rGST 8-8 has not been elucidated in the brain regions where we have detected differential expression, and these brain regions are known to be important in the brain reward pathway. Because GSTs are multifunctional proteins that are involved in transport and biosynthesis of endogenous compounds and play a defensive role against oxidative damage as well as neuroprotection (Danielson et al., 1987; Hubatsch et al., 1998; Singhal et al., 1994), they could be playing a role in these rat brain regions that has yet to be discovered. It has been shown that alcohol exposure alters the glutathione/glutathione transferase (Schnellmann et al., 1984) and the glutathione/glutathione peroxidase-1 systems (Bailey et al., 2001) in response to ethanol-related oxidative stress.

The rGST 8-8 gene mapped adjacent to the provisional chromosome 8 QTL for alcohol preference identified in the iP/iNP rats. This region is syntenic with a QTL for alcohol preference identified on mouse chromosome 9 in the B × D RI mice (Belknap et al., 1997; Phillips et al., 1994) and confirmed in B6D2F2 mice (Belknap and Atkins, 2001; Phillips et al., 1998). It is also syntenic with a QTL for ethanol metabolism identified on mouse chromosome 9 in the B × D RI mice, and candidate genes in this region include a cluster of *Gsta* genes (Grisel et al., 2002). Thus, rGST 8-8 maps to a chromosomal region that has been implicated in alcohol preference in the rat and the mouse.

In conclusion, the relationship between rGST 8-8 and the detoxification of highly cytotoxic compounds produced by lipid peroxidation, radical reactions, and other reactions elicited by oxidative stress, such as alcohol consumption, provide a promising area for future research. Further studies are necessary to evaluate better the importance of rGST 8-8 to the modulation of alcohol preference. It would be interesting to determine whether ethanol administration differentially alters rGST 8-8 expression between the iP and iNP rats in future studies. Such findings may elucidate whether rGST 8-8 is involved in the drinking behavior of these rats.

Acknowledgments

Supported by U.S. Public Service Grants AA10707, AA07611, and AA00285 and Digital Gene Technologies.

References

Baez S, Segura-Aguilar J, Widersten M, Johansson AS, Mannervik B. Glutathione transferases catalyse the detoxication of oxidized metabolites (o-quinones) of catecholamines and may serve as an anti-oxidant system preventing degenerative cellular processes. Biochem J. 1997; 324:25–28. [PubMed: 9164836]

Bailey SM, Patel VB, Young TA, Asayama K, Cunningham CC. Chronic ethanol consumption alters the glutathione/glutathione peroxidase-1 system and protein oxidation status in rat liver. Alcohol Clin Exp Res. 2001; 25:726–733. [PubMed: 11371722]

Belknap JK, Atkins AL. The replicability of QTLs for murine alcohol preference drinking behavior across eight independent studies. Mamm Genome. 2001; 12:893–899. [PubMed: 11707775]

Belknap JK, Richards SP, O'Toole LA, Helms ML, Phillips TJ. Short-term selective breeding as a tool for QTL mapping: Ethanol preference drinking in mice. Behav Genet. 1997; 27:55–66. [PubMed: 9145544]

Berhane K, Widersten M, Engstrom A, Kozarich JW, Mannervik B. Detoxication of base propenals and other alpha, beta-unsaturated aldehyde products of radical reactions and lipid peroxidation by human glutathione transferases. Proc Natl Acad Sci U S A. 1994; 91:1480–1484. [PubMed: 8108434]

Bice P, Foroud T, Bo R, Castelluccio P, Lumeng L, Li TK, Carr LG. Genomic screen for QTLs underlying alcohol consumption in the P and NP rat lines. Mamm Genome. 1998; 9:949–955. [PubMed: 9880658]

Bjornestedt R, Tardioli S, Mannervik B. The high activity of rat glutathione transferase 8-8 with alkene substrates is dependent on a glycine residue in the active site. J Biol Chem. 1995; 270:29705–29709. [PubMed: 8530359]

Board PG, Baker RT, Chelvanayagam G, Jermiin LS. Zeta, a novel class of glutathione transferases in a range of species from plants to humans. Biochem J. 1997; 328:929–935. [PubMed: 9396740]

Board PG, Coggan M, Chelvanayagam G, Easteal S, Jermiin LS, Schulte GK, Danley DE, Hoth LR, Griffor MC, Kamath AV, Rosner MH, Chrunyk BA, Perregaux DE, Gabel CA, Geoghegan KF, Pandit J. Identification, characterization, and crystal structure of the Omega class glutathione transferases. J Biol Chem. 2000; 275:24798–24806. [PubMed: 10783391]

Carr LG, Foroud T, Bice P, Gobbett T, Ivashina J, Edenberg H, Lumeng L, Li TK. A quantitative trait locus for alcohol consumption in selectively bred rat lines. Alcohol Clin Exp Res. 1998; 22:884–887. [PubMed: 9660316]

Cicero, TJ. A critique of animal analogues of alcoholism. In: Majchrowicz, E.; Noble, EP., editors. Biochemistry and Pharmacology of Ethanol. Vol. 2. Plenum Press; New York: 1979. p. 533-560.

Danielson UH, Esterbauer H, Mannervik B. Structure-activity relationships of 4-hydroxyalkenals in the conjugation catalysed by mammalian glutathione transferases. Biochem J. 1987; 247:707–713. [PubMed: 3426557]

DiLella AG, Woo SL. Hybridization of genomic DNA to oligonucleotide probes in the presence of tetramethylammonium chloride. Methods Enzymol. 1987; 152:447–451. [PubMed: 3657583]

Foroud T, Bice P, Castelluccio P, Bo R, Miller L, Ritchotte A, Lumeng L, Li TK, Carr LG. Identification of quantitative trait loci influencing alcohol consumption in the high alcohol drinking and low alcohol drinking rat lines. Behav Genet. 2000; 30:131–140. [PubMed: 10979603]

Gandy J, Primiano T, Novak RF, Kelce WR, York JL. Differential expression of glutathione S-transferase isoforms in compartments of the testis and segments of the epididymis of the rat. Drug Metab Dispos. 1996; 24:725–733. [PubMed: 8818568]

Grisel JE, Metten P, Wenger CD, Merrill CM, Crabbe JC. Mapping of quantitative trait loci underlying ethanol metabolism in BXD recombinant inbred mouse strains. Alcohol Clin Exp Res. 2002; 26:610–616. [PubMed: 12045468]

Grzybowska EA, Wilczynska A, Siedlecki JA. Regulatory functions of 3′UTRs. Biochem Biophys Res Commun. 2001; 288:291–295. [PubMed: 11606041]

Hayes JD, Pulford DJ. The glutathione S-transferase supergene family: Regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Crit Rev Biochem Mol Biol. 1995; 30:445–600. [PubMed: 8770536]

He NSS, Awasthi S, Zhao T, Boor PJ. Role of glutathione S-transferase 8-8 in allylamine resistance of vascular smooth muscle cells in vitro. Toxicol Appl Pharmacol. 1999; 158:177–185. [PubMed: 10406932]

Heese K, Nagai Y, Sawada T. The 3′ untranslated region of the new rat synaptic vesicle protein 2B mRNA transcript inhibits translational efficiency. Brain Res Mol Brain Res. 2002; 104:127–131. [PubMed: 12225865]

Hubatsch I, Ridderstrom M, Mannervik B. Human glutathione transferase A4–4: An alpha class enzyme with high catalytic efficiency in the conjugation of 4-hydroxynonenal and other genotoxic products of lipid peroxidation. Biochem J. 1998; 330:175–179. [PubMed: 9461507]

Johnson JA, el Barbary A, Kornguth SE, Brugge JF, Siegel FL. Glutathione S-transferase isoenzymes in rat brain neurons and glia. J Neurosci. 1993; 73:2013–2023. [PubMed: 8478688]

Jowsey IR, Thomson AM, Flanagan JU, Murdock PR, Moore GB, Meyer DJ, Murphy GJ, Smith SA, Hayes JD. Mammalian class Sigma glutathione S-transferases: Catalytic properties and tissue-

specific expression of human and rat GSH-dependent prostaglandin D2 synthases. Biochem J. 2001; 359:507–516. [PubMed: 11672424]

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L. MAPMAKER: An interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics. 1987; 1:174–181. [PubMed: 3692487]

Li TK, Lumeng L, Doolittle DP, Carr LG. Molecular associations of alcohol-seeking behavior in rat lines selectively bred for high and low voluntary ethanol drinking. Alcohol Alcohol (Suppl). 1991; 1:121–124. [PubMed: 1845526]

Liang T, Spence J, Liu L, Strother WN, Chang HW, Ellison JA, Lumeng L, Li TK, Foroud T, Carr LG. Alpha-synuclein maps to a quantitative trait locus for alcohol preference and is differentially expressed in alcohol-preferring and -nonpreferring rats. Proc Natl Acad Sci U S A. 2003; 100:4690–4695. [PubMed: 12665621]

Lockhart DJ, Barlow C. Expressing what's on your mind: DNA arrays and the brain. Nat Rev Neurosci. 2001; 2:63–68. [PubMed: 11253360]

Loflin P, Lever JE. HuR binds a cyclic nucleotide-dependent, stabilizing domain in the 3′ untranslated region of Na(+)/glucose co-transporter (SGLT1) mRNA. FEBS Lett. 2001; 509:267–271. [PubMed: 11741601]

Meyer DJ, Coles B, Pemble SE, Gilmore KS, Fraser GM, Ketterer B. Theta, a new class of glutathione transferases purified from rat and man. Biochem J. 1991; 274:409–414. [PubMed: 1848757]

Meyer DJ, Lalor E, Coles B, Kispert A, Alin P, Mannervik B, Ketterer B. Single-step purification and h.p.l.c. analysis of glutathione transferase 8-8 in rat tissues. Biochem J. 1989; 260:785–788. [PubMed: 2764904]

Misra P, Srivastava SK, Singhal SS, Awasthi S, Awasthi YC, Boor PJ. Glutathione S-transferase 8-8 is localized in smooth muscle cells of rat aorta and is induced in an experimental model of atherosclerosis. Toxicol Appl Pharmacol. 1995; 133:27–33. [PubMed: 7597707]

Montoliu C, Sancho-Tello M, Azorin I, Burgal M, Valles S, Renau-Piqueras J, Guerri C. Ethanol increases cytochrome P4502E1 and induces oxidative stress in astrocytes. J Neurochem. 1995; 65:2561–2570. [PubMed: 7595552]

Nair AK, Menon KM. Regulatory role of the 3′ untranslated region of luteinizing hormone receptor: Effect on mRNA stability. FEBS Lett. 2000; 471:39–44. [PubMed: 10760509]

Otieno MA, Baggs RB, Hayes JD, Anders MW. Immunolocalization of microsomal glutathione S-transferase in rat tissues. Drug Metab Dispos. 1997; 25:12–20. [PubMed: 9010624]

Pemble SE, Wardle AF, Taylor JB. Glutathione S-transferase class Kappa: Characterization by the cloning of rat mitochondrial GST and identification of a human homologue. Biochem J. 1996; 319:749–754. [PubMed: 8920976]

Phillips TJ, Belknap JK, Buck KJ, Cunningham CL. Genes on mouse chromosomes 2 and 9 determine variation in ethanol consumption. Mamm Genome. 1998; 9:936–941. [PubMed: 9880656]

Phillips TJ, Crabbe JC, Metten P, Belknap JK. Localization of genes affecting alcohol drinking in mice. Alcohol Clin Exp Res. 1994; 18:931–941. [PubMed: 7978106]

Rat Genome Sequencing Project Consortium. Genome sequence of the Brown Norway rat yields insights into mammalian evolution. Nature. 2004; 428:493–521. [PubMed: 15057822]

Schnellmann RG, Wiersma DA, Randall DJ, Smith TL, Sipes IG. Hepatic mixed function oxygenase activity and glutathione S-transferase activity in mice following ethanol consumption and withdrawal. Toxicology. 1984; 32:105–116. [PubMed: 6540493]

Segura-Aguilar J, Baez S, Widersten M, Welch CJ, Mannervik B. Human class Mu glutathione transferases, in particular isoenzyme M2–2, catalyze detoxication of the dopamine metabolite aminochrome. J Biol Chem. 1997; 272:5727–5731. [PubMed: 9038184]

Singhal SS, Zimniak P, Sharma R, Srivastava SK, Awasthi S, Awasthi YC. A novel glutathione S-transferase isozyme similar to GST 8-8 of rat and mGSTA4–4 (GST 5.7) of mouse is selectively expressed in human tissues. Biochim Biophys Acta. 1994; 1204:279–286. [PubMed: 8142470]

Spence J, Liang T, Foroud T, David L, Carr LG. Total gene expression analysis (TOGA): A complementary approach to QTL analysis in selected rat strains. Addict Biol. 2004 in press.

Stenberg G, Ridderstrom M, Engstrom A, Pemble SE, Mannervik B. Cloning and heterologous expression of cDNA encoding class alpha rat glutathione transferase 8-8, an enzyme with high

catalytic activity towards genotoxic alpha, beta-unsaturated carbonyl compounds. Biochem J. 1992; 284:313–319. [PubMed: 1599415]

Sutcliffe JG, Foye PE, Erlander MG, Hilbush BS, Bodzin LJ, Durham JT, Hasel KW. TOGA: An automated parsing technology for analyzing expression of nearly all genes. Proc Natl Acad Sci U S A. 2000; 97:1976–1981. [PubMed: 10681428]

Thibault C, Lai C, Wilke N, Duong B, Olive MF, Rahman S, Dong H, Hodge CW, Lockhart DJ, Miles MF. Expression profiling of neural cells reveals specific patterns of ethanol-responsive gene expression. Mol Pharmacol. 2000; 58:1593–1600. [PubMed: 11093800]

Wang Z, Kiledjian M. The poly(A)-binding protein and an mRNA stability protein jointly regulate an endoribonuclease activity. Mol Cell Biol. 2000; 20:6334–6341. [PubMed: 10938110]

Wirdefeldt K, Bogdanovic N, Westerberg L, Payami H, Schalling M, Murdoch G. Expression of alpha-synuclein in the human brain: Relation to Lewy body disease. Brain Res Mol Brain Res. 2001; 92:58–65. [PubMed: 11483242]

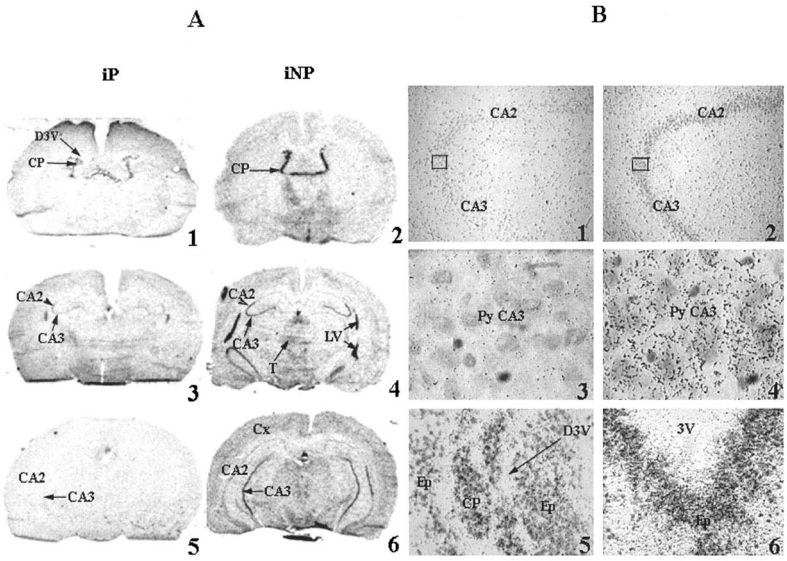

Fig. 1.

rGST 8-8 mRNA expression in rat brain. (A) ^{35}S-radiolabeled autoradiograph images of rGST 8-8 expression in iP (left) and iNP (right). Expression in choroid plexus (CP; 1 and 2) in D3V; CA2 and CA3 regions of hippocampus, LV, and thalamus (T; 3 and 4); and cortex (Cx) and CA2 and CA3 region of hippocampus (5 and 6). (B) Cellular localization of rGST 8-8 mRNA expression in rat brain. After dipping the ^{35}S-radiolabeled sections in emulsion and counterstaining with eosin Y and Mayer's hematoxylin, silver granula were observed. Expression of rGST 8-8 in iP (1) and iNP (2) CA2 and CA3 region of hippocampus. (3) Magnification of the square in 1. (4) Magnification of the square in 2 shows pyramidal cells of CA3 (Py CA3). (5) Magnification of 1A-2, CP in the D3V and ependymal cells along the D3V of iNP, noted with an arrow. (6) Expression is evident in the ependymal cells at the base of 3V of iNP rats. Magnification: ×2.5 in A; ×40 in B-1 and 2; ×800 in B-3 and 4; ×320 in B-5 and 6.

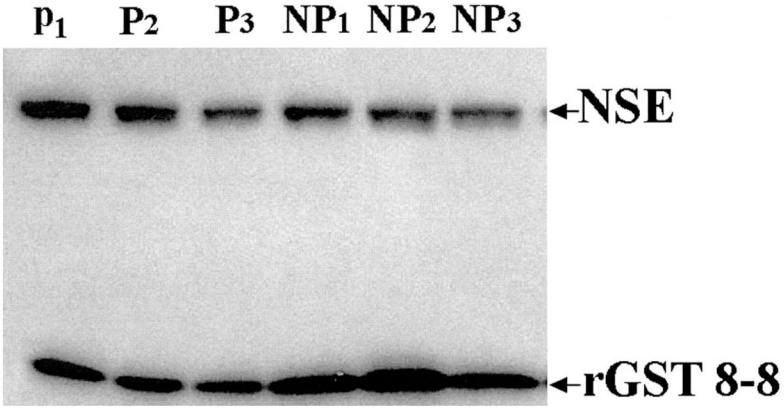

Fig. 2.

rGST 8-8 protein expression in the amygdala of iP and iNP rats using quantitative Western blot analysis. The blots were probed with both rGST 8-8 antibody and NSE antisera. The NSE signal was used to normalize the amount of neuronal protein loaded per lane.

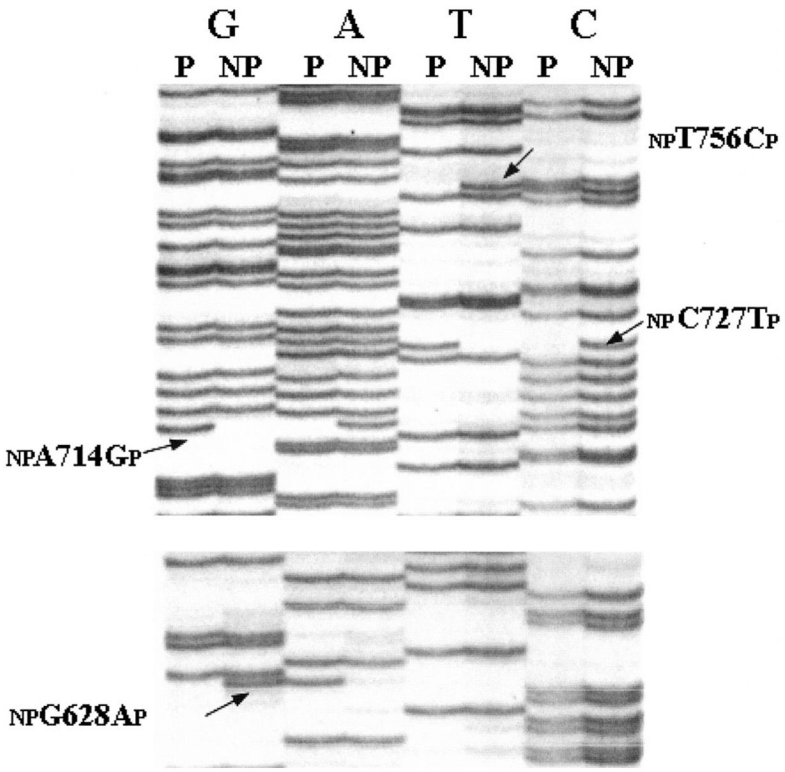

Fig. 3.

rGST 8-8 polymorphism. Sequence analysis of iP and iNP rats revealed three SNPs in the 3′-UTR of rGST 8-8 (+756, +727, and +714) and a silent SNP in the coding region at +628. Arrows denote the SNPs.

Table 1

Relative mRNA Expression Level in iNP vs. iP Brain Regions

	mRNA Fold Change of iNP/iP	
	TOGA[a]	**qRT-PCR[b]**
Hypothalamus	4.7	1.05
Hippocampus	3.1	1.31
Striatum	4.8	1.55
Cortex	2.9	1.59

[a] mRNA expression fold change of iNP/iP using the TOGA method.

[b] mRNA expression fold change of iNP/iP using the qRT-PCR method.

Table 2

Rat GST 8-8 Sequence Differences

	Position					
Name	**628**	**714**	**727**	**756**	**781**	**799**
rGST 8-8 iP	A	G	T	C	CTGA	A
rGST 8-8 iNP	G	A	C	T	CTGA	A
X62660	G	A	C	T	CTGA	A
XM_217195	G	A	C	T	G	Deletion