

Update 2011: This writing sample is from long ago. Now—after almost a year of continuous assignments in biomedical writing and editing for journals and NIH grant proposals—my approach has been greatly refined. I have adopted the standards of Mimi Zieger's *Essentials of Writing Biomedical Research Papers*, Angelica Hofmann's *Scientific Writing and Communication*, and William Gerin's *Writing the NIH Grant Proposal*. I have also been on the leading edge of writing NIH R01 proposals according to their new short form, introduced in 2010.

Characterization and distribution of glutathione receptors in primary visual cortex

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Abstract—Glutathione has been suggested to be one of the excitatory-amino-acid neurotransmitters in the brain (Ogita and Yoneda, 1987). We have attempted to characterize glutathione receptors in rat visual cortex and examine their distribution in rat, cat, and monkey primary visual cortex using in-vitro radioligand methods on 20- μ m-thick cortex sections. Saturation binding of radiolabeled glutathione in rat primary occipital cortex sections revealed a high-affinity site ($K_d = 5.4$ nM; $B_{max} = 235$ fmol/mg protein) and a denser low-affinity site ($K_d = 1.3$ μ M; $B_{max} = 1.3$ pmol/mg protein), the latter in the affinity range for excitatory amino acid receptors. Kinetic and competition experiments yielded similar K_d values. Competition studies of the low-affinity glutathione binding site showed a separate site as well as binding with affinity for the neurotransmitter candidates cysteine, aspartate, and glutamate. Excitatory-amino-acid receptor subtype affinity was shown for AMPA. Radiolabeled glutathione binding in adult rat visual cortex showed a relatively uniform distribution across all cortical layers. The distribution of radiolabeled glutathione binding in cat area 17 was densest in layer 4 from 13 days postnatal to adult. Distribution in adult monkey area 17 showed a marked preference for layer 4C.

Taken together with in-vivo evidence of uptake and possible retrograde transport of radiolabeled GSH and at least one of its constituent amino acids, cysteine, from terminals in area 17 into cell bodies in the lateral geniculate nucleus in rat (Arthur and Shaw, 1991), these data may suggest a role for glutathione as a geniculostriate neurotransmitter.

INTRODUCTION

The neurotransmitters that mediate sensory input to primary visual cortex are not known, and the evidence that does exist is contradictory. Glutamate can excite cells in all layers of visual cortex, including layer 4 (Krnjevic and Phillis, 1962), the predomi-

nant sensory input layer, and the excitatory amino acid antagonist kynurenic acid can block evoked responses in layer 4 of visual cortex (Hagihara *et al.*, 1988; Tsumoto *et al.*, 1986). However, receptor binding studies in adult visual cortex show relatively light binding densities for glutamate and for several of the excitatory amino acid receptor subtypes in layer 4 in rat (Monaghan and Cotman, 1982, 1985; Monaghan *et al.*, 1984), in cat (Shaw *et al.*, 1986, 1990), and in monkey (Shaw and Cynader, 1986; Shaw *et al.*, 1989, 1990, 1991) (for review see Tsumoto, 1990), while uptake experiments with [³H]glutamate in cat indicate that the geniculostriate projection is not glutamatergic (Baughman and Gilbert, 1981). The only receptors found to date in relatively high density in adult visual cortex layer 4 besides GABA_A and benzodiazepine receptors in cat (Shaw *et al.*, 1986) and presynaptic nicotinic acetylcholine receptors on geniculate terminals in cat (Prusky *et al.*, 1987) are for tachykinin peptides in rat and cat (Danks *et al.*, 1986; Mantyh *et al.*, 1984, 1989; March and Shaw, 1990), which are postsynaptic in cat (March and Shaw, 1990). Tachykinins, however, are so far known only as neuromodulators or as producing slow excitatory synaptic potentials (reviewed in Jessel and Womack, 1985), which is not suggestive of a role as a geniculostriate neurotransmitter. Preliminary data suggest that the NMDA subtype of EAA receptor is concentrated in sub-layer 4C β of monkey striate cortex (Shaw *et al.*, 1991), and could therefore conceivably play a role in geniculostriate synaptic transmission, although these receptors do not appear to play such a role in the rat or cat (Tsumoto, 1990).

Reduced glutathione (GSH) is a tripeptide

(gamma-glutamyl-cysteinyl-glycine) that may play a novel role in neurotransmission comparable to that found for the dipeptide N-acetyl-aspartyl-glutamate (NAAG). In the case of the lateral olfactory tract (LOT), evidence such as release and excitation in pyriform cortex had suggested that aspartate or glutamate were the neurotransmitters of the LOT, but instead they are more likely to be the breakdown products of the true neurotransmitter in this pathway, NAAG (Blakely *et al.*, 1987; French-Mullen *et al.*, 1985; Zollinger *et al.*, 1988), which is now also implicated as a major neurotransmitter in all retinal target zones including the lateral geniculate nucleus (LGN) and superior colliculus (Moffett *et al.*, 1991).

Various data suggest a role for GSH as a neurotransmitter in addition to its other established roles, such as detoxification. Glutathione can act as a coenzyme, where it may cycle between the predominant reduced form and an oxidized, disulfide form (GSSG), or act as an S-conjugate (for review see Meister, 1989). Though GSH is found in particularly large amount in brain (Reichelt and Fonnum, 1969; Orłowski and Karkowsky, 1976) and is necessary for maintenance of mitochondrial function in brain (Jain *et al.*, 1991), there is little detoxification by GSH conjugation found there (Orłowski and Karkowsky, 1976). In rat brain, GSH peroxidase is confined largely to the nuclei of only some neurons (Ushijima *et al.*, 1986) and the GSH S-transferases are found only in astrocytes (Sejno *et al.*, 1986). GSH is concentrated in glia, in axons and terminals, or in both in rodent and primate brain (Slivka *et al.*, 1987). A strong field-potential source may be induced in rat visual-cortex layer 6 in response to in-vitro application of GSH to layer 4 in cortical slices (Teyler, personal communication), presumably via apical dendrites of layer 6 pyramidal cells. GSH binding sites are seen in crude synaptic membrane preparations from rat brain, showing regional variation (Ogita *et al.* 1986b; Ogita and Yoneda 1987, 1988). These sites include a possible GSH receptor with a Kd value typical of EAA receptors, as well as a putative Na-independent, temperature dependent uptake site. GSH also shows affinity for glutamate binding sites, with selectivity for the AMPA and AP4 receptors (Ogita and Yoneda, 1987; Oja, 1988) and for the NMDA receptor antagonist-preferring site (Ogita and Yoneda, 1990). Cysteine is released in a calcium-dependent manner from depolarized rat

cortical slices, and may thus be a degradation product of released GSH (Keller *et al.*, 1989).

Most recently in our laboratory in-vivo micro-injection of [³H]GSH (labeled on the glycine residue) into cat and rat primary visual cortex produced uptake of radiolabel to visual system thalamic nuclei in rats and to the dorsal LGN in one cat. Possible retrograde uptake to cell bodies, indicative of a neurotransmitter function (Streit, 1980), was demonstrated in the dorsal LGN and lateral posterior nucleus in the rats (Arthur and Shaw, 1991). At least one of the constituent amino acids of GSH, cysteine, also showed possible retrograde uptake.

In this paper we report in-vitro binding studies of radiolabeled GSH in thin sections of rat, cat, and monkey primary visual cortex. We characterize two specific receptor sites in rat and show a predominance of binding in cortical layer 4 in cat and monkey, supporting the view that GSH may function as a neurotransmitter of the visual system, in particular as a geniculostriate neurotransmitter.

MATERIALS AND METHODS

[³⁵S]GSH (31.4–112.5 Ci/mmol), labeled on the cysteine residue; [³H]GSH (1–0.853 Ci/mmol), labeled on the glycine residue; and [³H]glutamate (36–49 Ci/mmol) were obtained from New England Nuclear (NEN, Boston, MA). All other chemicals were obtained from Sigma (St. Louis, MO)

All experiments were performed on rat primary occipital cortex (Oc1) (Paxinos and Watson, 1986) and cat area 17 (Tusa *et al.*, 1981; Snider, 1961). Adult Sprague-Dawley rats weighing 200 to 600 g were anesthetized with halothane and killed by decapitation. Colony cats from 6 to 230 days postnatal age were sacrificed with an overdose of sodium pentobarbital and rapidly perfused through the heart with cold phosphate buffer solution (PBS) followed in most cases by a PBS/0.2% formaldehyde solution. The brains were dissected out and frozen in liquid Freon or isopentane for storage at -20 to -60 °C. Specific binding of [³H]GSH in rat cortex showed no decrease from storage up to one year. Coronal sections (20 µm) were cut on a cryostat and thaw-mounted onto subbed glass slides.

Sections were pre-incubated in coplin jars in cold (4 °C) 100 mM Tris-acetate buffer at pH 8.0 with 0.1% formaldehyde, which had no effect on

specific binding, followed by two five-minute rinses in the same buffer, with the intent to remove as much endogenous ligand as possible. The slides were then placed face up on a plastic tray, dried in a stream of cold air, and then 500 μ l of incubation solution containing radiolabeled ligand was dripped onto each section. Incubations were carried out at room temperature in 100 mM Tris-acetate buffer at pH 8.0 for 120 minutes, except where noted below, to maximize specific binding as determined in preliminary tests. At high concentrations of radiolabeled GSH, 100 mM diethyl disulfide was added to the incubation buffer in order to neutralize the reducing agent dithiothreitol which was included in the stock solution by the manufacturer (10 mM), and which had the effect of reducing the binding affinity of GSH. The inclusion of acivicin to block the possible degrading action of endogenous plasma-membrane-bound gamma-glutamyl transpeptidase (GGTP) (Hill *et al.*, 1985) was omitted because it was determined that up to 10 mM acivicin showed no effect on specific binding under these in-vitro conditions. Incubations for autoradiography included 0.2% bovine serum albumen in order to reduce binding to nonspecific sites. Some time course and autoradiography incubations were performed at 4 °C to eliminate binding to possible Na-independent uptake sites (Ogita and Yoneda, 1989). Incubation was terminated by a single 6-second wash in cold (4 °C) buffer and rapid drying in a stream of cool air, except for autoradiography, in which three 5-second washes were used.

Total binding assays were run in at least triplicate; non-specific binding was determined in triplicate by addition of 50 μ l of 10^{-1} M GSH and 10^{-1} M GSSG to give a final concentration of 2×10^{-2} M total displacer. This high amount of displacer was determined to be necessary due to the presence of high capacity, low affinity, non-receptor "specific" binding (see Results). Twenty μ l samples of incubation medium were removed to determine free ligand concentration. The washed and dried sections were scraped onto a small circle of glass microfibre filter paper (Whatman FG/B), and placed directly in counting vials in 4 ml of Formula 963 (NEN). Vials were capped, shaken, left overnight, and the amount of bound receptor was determined in an LS 6000 IC Beckman Scintillation Counter (efficiency 55% for tritium, 95% for 35 S). In the saturation binding experiments alternate sections were used for determina-

tion of protein content by the methods of Lowry *et al.* (1951).

Sections were processed for autoradiography by apposition to Amersham Hyperfilm- 3 H followed by conventional development and fixing. For cat and monkey, original sections were then stained for cytochrome oxidase levels by the method of Wong-Riley (1979), to delineate layer 4 of area 17 (Carroll and Wong-Riley, 1984; Wong-Riley, 1979). Quantitative analysis of laminar binding density of [35 S] on autoradiographic films was done using an Imaging Research Inc. (St. Catherine's, ONT Canada) image analysis system, "MCID", calibrated with [14 C] standards (ARC, St. Louis, MD; .002 – 35.0 μ Ci/g). A density profile was constructed across all cortical layers in one representative section of striate cortex from each animal.

Saturation binding analysis was performed using the single-site Eadie-Hofstee method described by Zivin and Waud (1982) with the following modification. For dual binding sites, corrected Bmax and Kd values calculated from the two isolated components of the curve were used to determine intercept values for recalculation of Bmax and Kd values by the dual-site method of Hunston (1975). Dual-site displacement data was analysed by the method of Bylund (1986) to obtain separate Kd values. Hill coefficients and kinetic constants were calculated as described by Bylund (1980). For mean values for parameters from transformed data, standard errors were calculated. For untransformed data, standard errors were calculated for each point and used to aid drawing of curves and to calculate significance by Student's t-test. Linear plots for transformed data were determined by a linear regression program (SIGMAPLOT). Each line drawn for a single component of a dual component plot was determined by linear regression on the subset of points attributable to that component only, with the following exceptions. In the case of the high-concentration dual-component kinetic association curve, new points were first derived for the early component by mathematical stripping of the slower exponential component by a modification of the method of Lawson (1986). The fitting of the lines for the dual-site competition data was performed by the graphic method of Rosenthal (1967). The fitting of the line for the dual-site Eadie-Hofstee plot was determined from the values derived from the Hunston (1975)

method as described above.

RESULTS

Characterization experiments

In rat visual cortex, high- and low-concentration time course and saturation experiments were conducted in order to reveal high- and low-affinity receptor subtypes, respectively. Displacement experiments were conducted in both rat and cat visual cortex, and a low-concentration time course was conducted in cat. Under these conditions (pH 8, 120 minutes) there was no displacement of [³H]GSH binding by glutamate, and no displacement of Na-independent [³H]glutamate binding by GSH, allowing characterization of binding sites separate from other excitatory-amino-acid (EAA) receptors. Two receptor subtypes were revealed, as well as a low-affinity, high-capacity (LAHC) site. Such "specific" but non-receptor LAHC binding often appears as a second binding-site component in data from binding experiments conducted at high concentrations (Cuatrecasas and Hollenberg, 1976). A competition assay for crossover with other EAA receptors or neurotransmitters was conducted at 20-minutes incubation at pH 7.4, which minimized the LAHC binding.

Specific binding was significantly reduced by inclusion of NaCl, CaCl₂, MgCl₂, MgSO₄, KCl, Tris-hydrochloride, or Tris-citrate in the incubation medium. Non-specific binding defined by displacement ranged from 10 to 25% of total.

Figure 1 shows data from time course experiments in rat visual cortex that revealed a low-affinity GSH receptor (Figure 1A) and a high affinity GSH receptor (Figure 1B). In the high-concentration time course experiments (5 μM) specific binding reached equilibrium by 90 minutes; dissociation half-time was 1.5 minutes. Figure 1A shows association and dissociation experiments at high concentration. There were two components of each of the two curves shown, representing receptor and non-receptor binding (see saturation experiments, below). The first component of the observed rate constant (K_{ob}) was used with the second component of the dissociation rate constant (K_{-1}) to calculate the association rate constant (k_{+1}), while the remaining components, not representing binding to a receptor, could not be used to calculate kinetic constants. Values for K_{ob} , K_{-1} ,

and K_{+1} were 0.067 ± 0.012 min⁻¹, 0.010 ± 0.003 min⁻¹, and 0.011 μM⁻¹min⁻¹, respectively, which resulted in a K_d of 0.88 μM. In the low-concentration time course experiments at 5 nM at 4°C, specific binding reached equilibrium by 120 minutes; dissociation half-time was 1 minute (Figure 1B). Values for K_{ob} , K_{-1} , and K_{+1} were calculated as described above: 0.014 ± 0.002 min⁻¹, 0.009 ± 0.001 min⁻¹, and 0.001 nM⁻¹min⁻¹, respectively, yielding a K_d of 9 nM. The remaining components of each of the two curves could not be used to calculate kinetic constants. The second component of the association curve violated pseudo-first order assumptions of a constant free ligand concentration (Bylund, 1980), which requires that the free ligand concentration, [F], be much larger than the receptor concentration in that incubation volume [R]. Since this component represented the more slowly-associating low-affinity receptor subtype and LAHC site (see saturation experiments), the corresponding ratios of [F]/[R], as calculated from B_{max} values obtained in the saturation experiments (described below), were 2.2 and 0.1, respectively. These ratios would have produced large deviations in [F]. In addition, the first component of the dissociation curve also includes the confounding non-receptor LAHC binding, which rapidly dissociates.

Competition with 18 nM [³⁵S]GSH by non-radiolabeled GSH in rat (Figure 2A) yielded, upon two-site analysis (Figure 2B), a K_d for the low-affinity receptor of 1.3 ± 0.5 μM, in accordance with the K_d values from the time course and saturation experiments, as well as showing the LAHC site (with apparent K_d of 92 ± 2 μM). Competition with GSSG was the same as for GSH in rat, with a slightly higher affinity for the LAHC binding (Figure 3A). Three other substances showed affinity for [³H]GSH binding in rat at 1 mM (Figure 3A), in the following order: cysteine, S-methyl-GSH, and cysteine sulfinate (CSA). Under these conditions (pH 8, 120 minutes) the following substances failed to show displacement of [³H]GSH binding from 1 μM to 1 mM. These were L-glutamate, L-aspartate, D/L-homocysteic acid, quinolinic acid, D/L-2-amino-3-phosphonopropionic acid (AP3), D/L-2-amino-4-phosphonobutyric acid (AP4), D/L-2-amino-5-phosphonopentanoic acid (AP5), gamma-D-glutamylglycine (gamma-DGG), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and

quisqualic acid (QUIS). N-methyl-D-aspartate (NMDA) showed no significant displacement at 1 mM. A pH of 8 is optimal for binding of AP3, AP4, and AP5 to EAA receptors (Fagg *et al.*, 1988).

Figure 4 shows data from saturation binding experiments that reveal the low-affinity GSH receptor (Figure 4A) and the high-affinity GSH receptor (Figure 4B). Eadie-Hofstee analysis of the high-concentration saturation experiment (Figure 4A; see Methods) yielded a K_d of $1.3 \pm 0.3 \mu\text{M}$ and a B_{max} of $1.3 \pm 0.2 \text{ pmoles/mg protein}$. The second component shown in the Eadie-Hofstee plot in Figure 4A is the non-receptor LAHC binding site with a very high K_d of $17.2 \pm 7.8 \mu\text{M}$ and a large B_{max} of $31.4 \pm 11.4 \text{ pmoles/mg protein}$. The low-concentration saturation experiment (Figure 4B) was performed at 45 minutes incubation to minimize binding to the low-affinity receptor and the LAHC site, for the reason described above. Eadie-Hofstee analysis (Figure 4B) yielded a single component with a low K_d of $5.4 \pm 0.8 \text{ nM}$ and a smaller B_{max} of $0.24 \pm 0.02 \text{ pmoles/mg protein}$.

The quality of saturation-binding data can be assessed by inspection of the $SD_{(\text{Erad})}$ values and the Hill coefficient. $SD_{(\text{Erad})}$ values give an estimate of the bias in B_{max} and K_d values inherent in single-component Eadie-Hofstee plots. If the $SD_{(\text{Erad})}$ is more than 0.2, the data will be of little value. The Hill coefficient is an indication of cooperativity: a value of 1.0 indicates that ligand-receptor interaction occurs via a bimolecular reaction obeying a simple mass action law. Higher or lower values suggest positive or negative cooperativity, respectively. Hill coefficient and $SD_{(\text{Erad})}$ values for each separate component are as follows: 0.99 and 0.07, respectively, for the high-affinity receptor; 0.98 and 0.09 for the low-affinity receptor; 0.98 and 0.11 for the LAHC site; and a Hill coefficient of 0.92 for the high-concentration saturation experiment as a whole.

In a single experiment with adult cat area 17, competition with $2 \mu\text{M}$ [^3H]GSH (pH 8, 120 min) (Figure 3B) showed similar specificity and LAHC binding to that in rat brain. A possible difference from rat was a small but significant displacement by glutamate at 1 mM at pH 8 ($p < 0.02$).

To further characterize possible interactions between GSH and EAA neurotransmission under physiological conditions, we conducted competition experiments at 20 minutes incubation at pH 7.4, in

which there was a reduction of the LAHC binding as shown by a competition curve (data not shown), and at pH 6.9, which is optimal for glutamate receptor binding (Fagg *et al.*, 1988). At pH 7.4 there were significant displacements at 1 mM as shown in Figure 5. There was no significant displacement by D/L-AP3, D/L-AP4, NMDA, kainic acid (KA), or CSA. At pH 6.9 there was significant displacement as shown in Figure 5, with no displacement by L-glutamate, L-aspartate, D/L-AP3, D/L-AP4, or KA. Control specific binding of [^3H]GSH with 20-minute incubations, reducing the LAHC binding, showed ratios relative to binding at pH 7.4 of 3.6 at pH 6.9 and 2.5 at pH 8.0. At pH 6.9 there was also displacement ($p < 0.05$) of [^3H]glutamate binding by 1 mM GSH.

GSH binding site distribution

Quantitation of specific binding of [^3H]GSH in rat brain showed regional variation with significantly less binding in more lateral, non-visual cortex, brainstem, and white matter ($p < .01$). In cat brain, non-specific binding of [^{35}S]GSH, determined by the addition of 10^{-2} M non-radiolabeled GSH, showed densest binding in white matter and brainstem, whereas total binding was consistently higher in grey matter than in white matter.

The laminar distribution of GSH total-binding sites in visual cortex was determined by autoradiography for adult rats, for cats at postnatal ages of 6, 13, 30, 46, 61, 90, and 230 days, and for one adult monkey. These incubations were performed with 10 nM [^{35}S]GSH at pH 7.4 for 30 minutes to reduce LAHC binding. Rat cortex showed relatively uniform binding across all layers (Figure 6A). Cat visual cortex showed the highest density of binding in layer 4, confined to area 17, from after 6 days postnatal age. Figure 6B shows a representative autoradiograph and Figure 7 shows quantitative densitometry of layers at each age studied. In 6-day old cat area 17 no distinct laminar pattern of [^{35}S]GSH binding was seen (Figure 7A). At all later ages, densest binding was shown in middle layers of area 17 (Figure 7). Cytochrome oxidase staining for layer 4 was obtained in the 46, 61, and 90 day old cats, which shows that the band of densest [^{35}S]GSH binding corresponded consistently with layer 4 in area 17 (see for example Figure 6B,C). Quantitative analysis of binding density, shown in Figure 7,

showed a peak in middle layers after 6 days of age, with binding showing an increase from 6 to 61 days of age and then a reduction at 90 and 230 days of age, with layer 4 binding density becoming more discrete and slightly less prominent at the latter two ages. The region of maximum binding density for the 46, 61, and 90 day old sections corresponded with the cytochrome oxidase band. Adult monkey area 17 showed a distinct [³⁵S]GSH laminar binding preference for layer 4C, as shown by the corresponding cytochrome oxidase stained section (Figure 6D,E).

DISCUSSION

We believe the present results give the first demonstration of glutathione receptor binding sites in whole brain. For the low affinity GSH receptor binding site the K_d values (0.9 to 1.3 μM) and B_{max} (1.3 pmol/mg protein) are typical of EAA receptors (Foster and Fagg, 1984). For the high-affinity GSH binding site the K_d values (5.4 to 9 nM) and B_{max} (0.24 pmol/mg protein) are typical of peptide and neuromodulator receptors (Shaw *et al.*, 1986).

An explanation for the marked decrease in [³H]GSH specific binding seen at physiological concentrations of Na⁺ and Mg²⁺ is suggested as follows. For opioid receptor binding in brain, Na⁺ decreases agonist binding to 37% at 50 mM and abolishes binding by 200 mM, due to a decrease in affinity (with a corresponding increase in antagonist affinity), and there is also a general reduction in binding caused by cations (Simon *et al.*, 1975; Young and Kuhar, 1979). For tachykinin peptides in rat cortex, receptor binding is reduced to 60% at 150 mM NaCl, KCl, and NH₄Cl (Cascieri *et al.*, 1985). Even binding of glutamate to the Na-dependent high affinity glutamate uptake site in rat cortex synaptosomes is steadily decreased to 50% over the Na⁺ concentration range of 20 to 200 mM (Bennett *et al.*, 1973). Thus Na⁺ and other cations can reduce receptor and uptake site binding at physiological concentrations and pH. It is conceivable that effects such as these may be dramatically enhanced at non-physiological pH. Such a situation is suggested for GSH binding at pH 8.0 in the present study. The experiments of Ogita and Yoneda (1987) support the idea that GSH receptors are not Na⁺ sensitive at pH 7.4, since [³H]GSH binding was not decreased by 100 mM CH₃COONa in synaptic membranes, a

preparation which may exclude metabolic uptake sites and LAHC binding. The alternative possibility that reduction by NaCl is due to Cl⁻ is also suggested by the reduction of [³H]GSH binding consistently found with Cl⁻ salts (NaCl, CaCl₂, MgCl₂, and KCl) and with Tris-HCl.

It is unlikely that the possible GSH receptor binding sites described here reflect binding of GSH to its metabolizing enzymes or uptake sites, for the following reasons. (1) The K_d values for GSH binding were in the low nM and low μM range, while K_m values for the various GSH S-transferases are 0.2 to 2 mM in rat liver (Habig *et al.*, 1974) and their activity is low in brain (Orlowski and Karkowsky, 1976); the K_m for GSH peroxidase is 18 μM in rat liver (Flohe *et al.*, 1972) and is largely confined to neuronal nuclei (Ushijima, 1986); the K_m for the mitochondrial high-affinity GSH transporter is 60 μM in liver (Martensson *et al.*, 1990); the putative blood-brain and blood-CSF GSH transport site, which may instead be the extracellular GSH-degrading enzyme gamma-glutamyl transpeptidase, has a K_m of about 6 mM in rat brain (Kannan *et al.*, 1990; see discussion by Jain *et al.*, 1991); and acivicin had no effect on binding. (2) GSSG had a similar affinity for the low-affinity GSH binding site, as it does for the high-affinity site in astrocytes (Guo *et al.*, personal communication, see below). (3) There was no necessity for the -SH moiety in the binding, nor is there necessity for the gamma-glutamyl structure in synaptic membrane binding (Ogita and Yoneda, 1987).

Likewise the possible receptor binding sites are unlikely to represent degradation of [³⁵S]GSH into [³⁵S]cysteine or of [³H]GSH into [³H]glycine and subsequent binding to cysteine or glycine uptake sites because (1) the brain cysteine uptake site is Na-dependent (Hwang and Segal, 1979), and (2) the K_m for the low-affinity Na-independent cortical glycine uptake site was originally found to be 300 μM (Johnston and Iversen, 1971), and is found to be 8 μM in isolated cortical astrocytes and 10 μM in isolated cortical neurons (Hannuniemi and Oja, 1981). The binding is also unlikely to represent [³H]glycine binding to the strychnine-insensitive NMDA receptor glycine binding site because the K_d for this site is 80 nM in rat cortex sections (Miyoshi *et al.*, 1990). In addition, GSH is not degraded in synaptic membrane preparations in Tris-acetate buffer (Ogita *et al.*, 1986a), and opioid peptides are

not degraded in lightly fixed, whole brain section incubations in Tris-HCl (Young and Kuhar, 1979).

The low-affinity receptor may correspond to the higher-affinity component of the two GSH binding sites characterized by Ogita *et al.*, who published values of approximately 1.9, 0.6, and 0.8 μM for the K_d and 9.6, 2.5, and 4.0 pmoles/mg protein for the B_{max} (Ogita *et al.*, 1986b; Ogita and Yoneda, 1987, 1988, respectively), and which is similarly displaced by cysteine. Our LAHC site (K_d 17 to 92 μM ; B_{max} 31 pmol/mg protein) may or may not correspond to their lower-affinity, temperature-dependent, Na-independent putative uptake site because, although the K_d and B_{max} values are similar to ours (K_d : 5.9, 12.6, 11.0 μM ; B_{max} : 21.4, 28.5, 27.6 pmoles/mg protein), our LAHC binding was not potentiated by cysteine as is theirs (Ogita and Yoneda, 1989). From our present experiments we cannot discern whether the LAHC binding or the low-affinity receptor binding is temperature dependent because the non-mass-action component of the 4 °C time courses could be due to either or both of these sites. We suggest that synaptic membrane preparations may eliminate the LAHC binding. Such a preparation would also eliminate the high-affinity GSH receptor if it is a hormone receptor or is confined to astrocytes (see below), but in any case the high concentration range covered by the saturation binding analysis done by Ogita *et al.* would miss this receptor, as we have demonstrated in the present study.

Guo *et al.* (1991) have demonstrated GSH binding sites on cultured astrocytes and in white matter in rat brain. Using low-concentration [^{35}S]GSH saturation binding they find a high-affinity receptor on both fibrous and protoplasmic cortical astrocytes in culture ($K_d = 9 \text{ nM}$). The additional presence of our low affinity receptor and LAHC binding site on astrocytes was supported by these authors' displacement data (Guo, personal communication), which yielded separate K_d values of 2.3 μM and 46 μM (Bylund, 1986), and by cellular resolution obtained with colloidal gold decoration of biotinyl-GSH binding (Guo and Shaw, 1991), which showed very high density binding on astrocytes. The presence of a low-affinity receptor in synaptic membranes (Ogita *et al.*, 1986) makes it unlikely that the low-affinity receptor we have found is confined to astrocytes, but the possibility that the high-affinity receptor is confined to astrocytes remains, allowing

one to speculate that GSH is involved in a novel form of neuron-glial signalling (Marrero *et al.*, 1989; Usowicz *et al.*, 1989; Lieberman *et al.*, 1989; for reviews see Barres, 1989, and Kimelberg, 1988) in addition to neuronal signalling. Guo *et al.* (1991) also obtain whole rat brain section distributions of specific binding of biotinylated GSH confined largely to white matter, and a lesser amount in brainstem. This contrasts with our autoradiograms which show densest specific [^{35}S]GSH binding in grey matter and densest non-specific binding in white matter and brainstem. Why biotinylated GSH has a preference for white matter is not understood.

Most studies of the distributions of receptor types, including EAA subtypes, in more highly-developed visual cortex such as in cat (Shaw *et al.*, 1986) and particularly monkey (Shaw and Cynader, 1986; Shaw *et al.*, 1991) show greater densities in specific layers, many of which will change to a new laminar pattern during the critical period for plasticity. Rat visual cortex shows less distinct (Monaghan and Cotman, 1982, 1985; Monaghan *et al.*, 1984) or uniform (Miyoshi *et al.*, 1990) lamination for EAA receptor binding, as well as for many other radioligands (Shaw, unpublished). Our [^{35}S]GSH autoradiographic distributions in visual cortex are consistent with these trends.

In adult monkey area 17, cytochrome oxidase staining is highest in the thin sublayer 4A, in sublayer 4C α , and in the lower part of sublayer 4C β (Carroll and Wong-Riley, 1984), as indicated in Figure 6E. The [^{35}S]GSH binding in comparison, then, showed a preference for sublayer 4C (Figure 6D), which is exclusively stellate cells receiving geniculate terminations, and this binding is not found in sublayer 4B, a layer that receives no geniculate terminations and contains the line of Gennari (Lund, 1973; Gilbert, 1983). This pattern is consistent with a role as a geniculostriate neurotransmitter.

Cat area 17 layer 4 [^{35}S]GSH binding was somewhat more diffuse than in the monkey, which is consistent with the presence in the cat of geniculate afferents to layers immediately above and below layer 4, unlike in the monkey, and the lack of a line of Gennari (Gilbert, 1983). The general increase in binding from 6 to 61 days of age is coincident with the major synaptogenesis that occurs during this period (Winfield, 1981), while the reduction in binding at 90 and 230 days of age is coincident with the

succeeding synapse elimination (for review see Payne *et al.*, 1988). The lack of a binding peak in 6-day old cat area 17 occurs at a time when geniculate innervation is much more uniform, and synapses are fewer (Kato *et al.*, 1983; Payne *et al.*, 1988). At 32 days of age the most distinctive [³⁵S]GSH binding density in layer 4 was seen, at which time in the cat the adult pattern of geniculate innervation has been reached (Kato *et al.*, 1983; Payne *et al.*, 1988), and the critical period for plasticity is peaking (Hubel and Weisel, 1970; for review see Cynader *et al.*, 1990).

If GSH does act as a neurotransmitter at the sensory-input synapses to layer 4 in primary visual cortex, then the uniform distribution in the rat must be explained. Layer 4 is the predominant input zone for specific afferents from the LGN, but in the rat there is also geniculate input to at least layers 1, 3, and 6 (for review see Sefton and Dreher, 1985), and such terminals may also have GSH receptors. There are also lateral posterior nucleus projections to at least layers 5 and 6 in the rat (Sefton and Dreyer, 1985). It has been suggested that extrageniculate visual input may represent a phylogenetically older and less specifically organized pathway (Diamond and Hall, 1969). In the rat, then, extrageniculate visual input may rival the geniculate input in terms of number of receptors. Since *in vivo* uptake experiments in rats (Arthur and Shaw, 1991) show possible retrograde uptake of radiolabeled GSH from Oc1 to the lateral posterior nucleus, there is support for the notion that such extrageniculate cortical synapses also have GSH receptors. It therefore seems possible to speculate that glutathione may be a geniculostriate neurotransmitter in the adult monkey, cat, and rat.

None of the neurotransmitters for any of the EAA receptor subtypes have been identified with certainty, and displacement of [³H]GSH binding by subtype-specific ligands or by putative transmitters may indicate a physiological role for GSH through interaction with EAA receptors (Coyle *et al.*, 1986). For EAA reviews see Foster and Fagg (1984), Griffiths (1990), Mayer and Westbrook (1987), and Monaghan *et al.* (1989). At pH 7.4, the neurotransmitter candidates glutamate, aspartate, and cysteine (Keller *et al.*, 1989; Li and Jope, 1989) partially displaced [³H]GSH binding. Of the six different EAA subtype-specific ligands tested, only AMPA partially displaced [³H]GSH at pH 7.4, indicating a possible AMPA receptor affinity. This suggestion is

supported by the observation that AMPA was a more effective competitor than glutamate, because the affinity of glutamate for AMPA receptors is four times lower than the affinity of AMPA itself (IC_{50} s = 0.3 μ M and 1.3 μ M, respectively) (Foster and Fagg, 1984). Cysteine is a candidate neurotransmitter for the AP4 receptor (Pullan *et al.*, 1987), and although L-AP4 itself did not show displacement of [³H]GSH, freezing of the tissue and chlorine-free incubation would have abolished the predominant AP4 putative uptake site and levels of postsynaptic AP4 receptor would be too low to detect (Bridges *et al.*, 1986; Fagg *et al.*, 1983), thus GSH affinity for AP4 receptors is not ruled out. Failure of L-AP3 to displace [³H]GSH indicates a lack of affinity for the ACPD (QUIS metabotropic) receptor (Schoepp and Johnson, 1989). Failure of CSA indicates lack of affinity for the CSA receptor (Pin *et al.*, 1987; Iwata *et al.*, 1982a,b). To compare our results to the literature, Oja *et al.* (1988) and Varga *et al.* (1989) found displacement of [³H]AMPA by GSH, and Ogita and Yoneda found displacement of [³H]GSH by AMPA and by AP4 (1987) and affinity of GSH for the NMDA receptor antagonist-preferring site (Fagg *et al.*, 1988; Monaghan *et al.*, 1988) by its displacement of [³H]3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP) binding (1990). At pH 6.9 the increased binding of [³H]GSH may have been due to an increase of GSH receptor binding, since glutamate and aspartate did not displace this binding. At this pH [³H]GSH binding is displaced by NMDA as well as by two other ligands with affinity for the NMDA receptor agonist-preferring site: QUIS (Foster and Fagg, 1984) and CSA (Pullan *et al.*, 1987). These displacements may indicate an affinity for the NMDA agonist receptor. More likely, these displacements may indicate a structural similarity of the recognition site of the GSH molecule to cysteine (which displaced [³H]GSH at pH 6.9, 7.4, and 8), and therefore AP4 receptor affinity, as well as AMPA receptor and NMDA antagonist receptor affinity. This latter possibility is suggested because (1) NMDA showed no displacement of [³H]GSH at pH 7.4; (2) NMDA has a lower affinity for NMDA receptors than does glutamate (Foster and Fagg, 1987), which did not displace the GSH binding at pH 6.9; (3) Ogita and Yoneda (1990) show GSH affinity for only the antagonist-preferring site of the NMDA receptor; (4) QUIS has greater affinity for the NMDA

receptor antagonist-preferring site (Ferkany and Coyle, 1983), for the AMPA receptor (for which GSH affinity at pH 7.4 was shown), and for AP4 receptors (Ogita and Yoneda, 1986); and (5) CSA also has affinity for the AP4 receptor (Pullan *et al.*, 1987).

Binding, uptake (Arthur and Shaw, 1991), and electrophysiology (Teyler, personal communication) of GSH in visual cortex suggest that glutathione may be a neurotransmitter in this system, particularly for the geniculostriate projection. In reference to the reported abolishment of visually- and electrically-evoked responses of neurons of layer 4 in cat striate cortex by the excitatory amino acid receptor antagonist kynurenic acid (Hagihara *et al.*, 1988; Tsumoto, 1986), it is possible that this broad-spectrum antagonist (Monaghan *et al.*, 1989) is actually acting in this pathway as an antagonist of glutathione receptors, blocking the effects of endogenous release of GSH from geniculo-striate terminals. It is also possible that glutathione may be rapidly metabolized by known brain enzymes into a series of seven substances, all of them neurotransmitter/modulator candidates — gamma-glutamyl-amino acids and glutamate; cysteine and glycine; cysteine sulfinate; cysteic acid; and taurine, in that order (Hill *et al.*, 1985; Meister, 1983; Rassin and Gall, 1987) — all possibly within the period of a single postsynaptic potential (Jain *et al.*, 1991; Kozak and Tate, 1982; Legay *et al.*, 1987; Misra, 1983). It is therefore interesting to speculate that glutathione neurotransmission could be of a novel and highly complex form. Glutathione receptor activation itself may, in some systems, be metabotropic, since no sodium flux was found upon application to rat striatum (Luini, *et al.*, 1984). Glutathione may also activate AMPA and AP4 receptors, while antagonizing NMDA receptors (Ogita and Yoneda, 1987, 1990; Varga *et al.*, 1989). NMDA receptor antagonism would produce a decrease in tonic background voltage dependence and reduction of facilitation of action potentials (Sah *et al.*, 1989), and a decrease in glycine-site affinity (Hood *et al.*, 1990). NMDA receptor antagonism has also been suggested to reduce induction of long-term potentiation and developmental plasticity (reviewed in Monaghan *et al.*, 1989; Bear, 1988). Next, gamma-glutamyl amino acids and some free glutamate could activate the full range of glutamate receptors (Varga *et al.*, 1989), and reverse all antagonist

effects on NMDA receptors (Hood *et al.*, 1990). Cysteine may then activate AP4 receptors, which may include presynaptic autoreceptors that reduce release of glutamate (Monaghan *et al.*, 1989). Glycine may then increase NMDA-receptor mediated responses (Bonhaus *et al.*, 1989; Thompson *et al.*, 1989), including switching the receptor toward the agonist-preferring state (Monaghan *et al.*, 1988), facilitating action potentials (Sah *et al.*, 1989), and lowering the threshold for induction of long-term potentiation (Oliver *et al.*, 1990) and of developmental synaptic plasticity (reviewed by Monaghan, 1989). Then CSA may activate the CSA receptor (Iwata *et al.*, 1982a,b; Pin *et al.*, 1987; for review see Griffiths, 1990), which may stimulate cyclic AMP production (Baba *et al.*, 1988). Excitation may be produced by CA, possibly via KA and AP4 receptors (Pullan *et al.*, 1987). Finally taurine, produced last, may have an inhibitory effect (reviewed in McGeer *et al.*, 1987). Glutathione neurotransmission may even be modulated in a novel manner: the initial enzyme in the metabolic cascade, gamma-glutamyl-transpeptidase, is modulated by somatostatin and LHRH in rat brain (Vali and Vijayan, 1990). Such a hypothesized neurotransmission cascade, reminiscent of military MIRVs (Multiple Independently-targeted Re-entry Vehicles), we refer to as Multiple Independently-targeted Sequential Synaptic Transmission (MISST). Such MISST neurotransmission could allow a wide range of modulation and of regional variation by the modulation of the enzymes involved and by the differential expression of sets of enzymes and receptors by a given neuron or synapse. If true, some neurotransmitter candidates, in some pathways, may turn out to be secondary to glutathione release.

Acknowledgements—This study was supported in part by a Canadian MRC grant and a B.C. Health Care Research Foundation Operating Grant to C.S. We thank Mala Glenwright and Derrick March for technical assistance, and Drs. S. Kehl, D. Mathers, K. Curry, M. Wilkinson, and T. Tyler for helpful comments.

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Figure Legends

Fig. 1. Association and dissociation rates of radio-labeled GSH binding in cryostat sections of rat visual

cortex. Data is transformed to calculate Kobs from the association slope and K-1 from the dissociation slope. On the ordinate, Be is binding at equilibrium, while B is binding at the time indicated. Sections were incubated in radiolabeled GSH for various times up to 120 minutes. Remaining sections were then introduced into an "infinite dilution" of buffer and incubated for further times up to 40 minutes. Non-specific binding was defined by the addition of 10^{-2} M GSH and 10^{-2} M GSSG. (A) Data from specific binding at a free ligand concentration of $5 \mu\text{M}$ [^3H]GSH (room temperature) to reveal low-affinity receptor kinetics. The curves represent normalized data from two separate experiments conducted in quadruplicate. Regression lines are drawn only for the possible receptor components. The late component of the association curve and the early component of the dissociation curve show binding to the LAHC non-receptor site (see Results). For the first component of the association plot, linear regression was performed on points derived from mathematically stripping off the slower component by a modification of the method of Lawson (1986). Linear regression was performed on the last 8 points of the dissociation. (B) Data from specific binding at free ligand concentration of 5 nM [^{35}S]GSH (4°C) to reveal low-affinity receptor kinetics. Regression lines are drawn only for the possible receptor components. The late component of the association curve shows a violation of pseudo-first order assumptions (see Results), while the early component of the dissociation curve reflects non-receptor (LAHC) kinetics (see Results). The curves represent normalized data from three separate experiments conducted in triplicate. Linear regression was performed on the first 5 points of the association and last 4 points of the dissociation.

Fig. 2. Competition of GSH with 18 nM [^{35}S]GSH and its derived "B versus B.I" plot to reveal the separate Kd values for the possible receptor binding site and nonreceptor LAHC site. Cryostat sections of adult rat visual cortex were incubated for 120 minutes at room temperature (pH 8) in 18 nM [^{35}S]GSH and various concentrations of nonlabeled GSH as indicated. The graphs represent normalized data from three separate experiments conducted in triplicate. (A) Competition curve. (B) "B versus B.I" plot, where the negative inverse of the slopes of the

first and second components yield the separate Kd values. Extraction of the lines for the two separate components was performed by the method of Rosenthal (1967).

Fig. 3. Competition curves for [^3H]GSH binding in adult rat (A) and cat (B) cryostat sections of visual cortex. Sections were incubated for 120 minutes at room temperature (pH 8) in $2 \mu\text{M}$ [^3H]GSH and various concentrations of competitors as indicated. The curves in (A) represent normalized data from two separate experiments conducted in at least triplicate. The curves in (B) are from one experiment conducted in at least triplicate. Note that the extended section of the curves at high competitor concentrations represents the LAHC non-receptor binding site.

Fig. 4. Saturation binding curves and Eadie-Hofstee plots of [^3H]GSH and [^{35}S]GSH binding sites on cryostat sections of adult rat visual cortex. Nonspecific binding was defined by the addition of 10^{-2} M GSH and 10^{-2} M GSSG. (A) High free-ligand concentration range ([^3H]GSH) to reveal parameters for low-affinity receptors. The graphs represent combined data from two separate experiments conducted in quadruplicate at room temperature, 120 minutes incubation, pH 8. In the Eadie-Hofstee plot the component with steeper slope (no line drawn) represents the LAHC non-receptor site. The line for the possible receptor component was determined by the method of Hunston (1975). (B) Low free-ligand concentration range ([^{35}S]GSH), to reveal parameters for high-affinity receptors, incubated for 45 minutes (pH 8, room temp.) to minimize binding to the much denser LAHC sites to retain mass action assumptions (see Results). The graphs represent combined data from three separate experiments conducted in triplicate. The Eadie-Hofstee line is drawn by linear regression.

Fig. 5. Percent control binding of [^3H]GSH in rat visual cortex with significant displacement ($p < .05$) by various 1 mM competitors at pH 7.4 and at pH 6.9. Control binding is relative to binding at pH 7.4 without competitors. Cryostat sections of adult rat Ocl1 were incubated in Tris-acetate with $2 \mu\text{M}$ [^3H]GSH and competitors for 20 minutes (room temp.) to minimize LAHC binding. The graph repre-

sents normalized data from three separate experiments conducted in triplicate. Only significant displacement is shown.

Fig. 6. Autoradiographic distribution of [^{35}S]GSH binding in rat, cat, and monkey visual cortex. Slide-mounted coronal sections (20 μm) from frozen tissue were incubated for 30 minutes at 8-10 nM [^{35}S]GSH at room temperature, pH 7.4. Bar = 1 mm. In cat and monkey sections successfully stained for cytochrome oxidase levels, as shown, the position of the band of densest staining (layer 4, circle) corresponds to the position of densest binding of [^{35}S]GSH as shown in the autoradiographs. (A) Adult rat brain autoradiograph from a coronal section at the level of the SC and medial geniculate, lines indicating approximate borders of Oc1. (B,C) 61-day old cat visual cortex autoradiograph (B) and corresponding cytochrome oxidase stained section (C). (D,E) Adult monkey area 17 autoradiograph (D) and corresponding cytochrome oxidase stained section (C) with arrows indicating, from top to bottom, layers 4A, 4C α , and 4C β .

Fig. 7. Quantitative analysis of laminar binding density on autoradiographic film across cat area 17 cortex radially from white matter to pia at various postnatal ages. Optical density was calibrated using ARC [^{14}C] standards (0.002 - 35.0 $\mu\text{Ci/g}$). The graph is a histogram with each point showing the mean value for a bin dimension of 0.145 mm radially and approximately 0.460 mm tangentially. The histograms for all ages are aligned with the center of layer 4, as determined by cytochrome oxidase and Nissl staining. The approximate width of layer 4 is indicated by the horizontal bar. The pia (right side) and the boundary of the white matter (left side) are indicated by the vertical bars crossing the histogram for each age. Width in mm on the ordinate is provided for scaling, with its origin at an arbitrary point in the white matter. Variations in the thickness of cortex and positioning of anatomical layers depends on the particular sample taken at any given age. All samples are taken from the medial bank, with ages 6, 30, 46, and 61 days taken from the ventral gyrus, age 13 days from the dorsal gyrus, and ages 90 and 230 days from the sulcus. A peak in binding density is seen in layer 4 at all ages except day 6. (A) Density profile at postnatal ages of 6, 13, and 30 days. (B) Density

profile at postnatal ages 46, 61, 90, and 230 days.