Oxidative stress and diminished glutathione pools play critical roles in the pathogenesis of neurodegenerative diseases, including Alzheimer and Parkinson disease. Synthesis of glutathione, the most abundant mammalian antioxidant, is regulated at the substrate level by cysteine, which is synthesized from homocysteine via the transsulfuration pathway. Elevated homocysteine and diminished glutathione levels, seen in Alzheimer and Parkinson disease patients suggest impairments in the transsulfuration pathway that connects these metabolites. However, the very existence of this metabolic pathway in the brain is a subject of controversy. The product of the first of two enzymes in this pathway, cystathionine, is present at higher levels in brain and Parkinson disease patients suggest impairments in the transsulfuration pathway that connects these metabolites. However, the very existence of this metabolic pathway in the brain is a subject of controversy. The product of the first of two enzymes in this pathway, cystathionine, is present at higher levels in brain as compared with other organs. This, together with the reported absence of the second enzyme, γ-cystathionase, has led to the suggestion that the transsulfuration pathway is incomplete in the brain. In this study, we incubated mouse and human neurons and astrocytes and murine brain slices in medium with [35S]methionine and detected radiolabel incorporation into glutathione. This label transfer was sensitive to inhibition of γ-cystathionase. In adult brain slices, ~40% of the glutathione was depleted within 10 h following γ-cystathionase inhibition. In cultured human astrocytes, flux through the transsulfuration pathway increased under oxidative stress conditions, and blockade of this pathway led to reduced cell viability under oxidizing conditions. This study establishes the presence of an intact transsulfuration pathway and demonstrates its contribution to glutathione-dependent redox-buffering capacity under ex vivo conditions in brain cells and slices.

An inescapable consequence of aerobic energy metabolism is the generation of incompletely oxidized products, the reactive oxygen species, which, due to their inherent reactivity, can be damaging in a cellular milieu. Reactive oxygen species are also generated in response to specific triggers (viz. growth factors in intracellular signaling pathways and in intercellular communication as in the chemical warfare between cells of the immune system and pathogen) (1). Cells have an arsenal of antioxidant molecules and enzymes to neutralize reactive oxygen species and to shield themselves from oxidative damage. The brain, representing ~2% of body mass, consumes a disproportionately large fraction of the total oxygen (~20%) and may be more susceptible to oxidative damage than other organs. Aberrations in redox homeostasis are a hallmark of several neurodegenerative disease and are also associated with neuropsychiatric disorders (viz. schizophrenia and autism) (2–5).

Glutathione is a major antioxidant present at 1–10 mM concentrations in tissue (6). The limiting reagent in the synthesis of glutathione is cysteine, which is synthesized from homocysteine via the transsulfuration pathway (Fig. 1). In liver, ~50% of the cysteine in glutathione is derived from homocysteine via the transsulfuration pathway (7, 8). The importance of this pathway in glutathione-based redox homeostasis is further supported by a decrease in the steady-state glutathione concentration in liver (to 63%) and brain (to 71%) in transgenic mice with homozygous disruption of the cystathionine β-synthase gene (9). Homocysteine is a sulfur-containing nonprotein amino acid that is maintained at relatively low concentrations. Elevated levels of homocysteine are correlated with cardiovascular diseases (10), neural tube defects (11), and neurodegenerative disorders, including Parkinson disease (12) and Alzheimer disease (13, 14).

The reciprocal changes in homocysteine and glutathione levels seen in some neurodegenerative disorders suggest a possible dysfunction in the transsulfuration pathway that connects these metabolites. However, the very existence of this pathway in the brain is controversial. In a comparative study on methionine metabolism, the activity of brain cystathionine γ-lyase was reported to be >100-fold lower than in liver (15). This has been widely cited as evidence for the negligible importance or even absence of the transsulfuration pathway in brain. This conclusion appeared to be supported by a recent report on the absence of detectable cystathionine γ-lyase protein or activity in the mouse brain, although low RNA expression was observed (16), and by the inability of methionine to serve as a cysteine donor for glutathione synthesis in cultured rat neurons (17). In contrast, other studies provided evidence for the presence of cystathionine γ-lyase activity in brain (18–20). To our knowledge, the presence of an intact transsulfuration pathway in the human brain has not been addressed experimentally.

Given the prominent role of glutathione in defense against oxidative stress (3), the postulated roles of the transsulfuration enzymes in biogenesis of H₂S (21), a neuromodulator (22), and the association of elevated homocysteine with Alzheimer disease and Parkinson disease (12, 14), the controversial existence

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Transsulfuration in the Brain

of the transsulfuration pathway in the brain is a clinically important issue that merits resolution. Herein, we have used sensitive metabolic labeling and inhibitor studies to examine the conversion of methionine to glutathione in cultured human and murine neurons and astrocytes and in neonatal and adult murine brain slices. The results establish the existence of an intact transsulfuration pathway in the brain and furnish interesting insights into developmental and cell-specific differences in transsulfuration capacity.

EXPERIMENTAL PROCEDURES

Brain Slice Preparation—Slices were prepared from adult (6–8-week-old males) C57BL/6 mice and 2-day-old mouse pups. Adult mice were anesthetized with isoflurane, and pups were anesthetized by immersion in an ice water bath. Following decapitation, the brain was rapidly removed and placed in ice-cold modified Ringer’s solution for 3–4 min before slicing. Ringer’s solution consisted of 120 mM NaCl, 25 mM NaHCO₃, 3.3 mM KCl, 1.2 mM NaH₂PO₄, 1.8 mM CaCl₂, 2.4 mM MgSO₄, 10 mM dextrose and was bubbled with 95% O₂, 5% CO₂ before use. The cerebellum was then removed with a cut perpendicular to the anteroposterior axis, and the brain was glued anterior end up to the tissue slicer stage (VibraSlice, Camden Instruments). The tissue was submerged in ice-cold oxygenated Ringer’s solution containing neocortical and striatal regions were cut. The slices were transferred with a spatula to dishes containing DMEM/F-12 (Invitrogen) with 100 units/ml penicillin and 100 μg/ml streptomycin bubbled with 95% O₂, 5% CO₂ and maintained at 32 °C in a water bath.

For experiments in cystine-free medium, DMEM lacking pyruvate, glutamate, glutamine, methionine, and cystine (Invitrogen) was supplemented with 0.5 mM sodium pyruvate (HyClone), 2 mM glutamine (Invitrogen), 48 μM glutamate (Sigma), 115 μM methionine (cell culture grade, Sigma), and 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen). For control experiments, the same medium additionally supplemented with 150 μM cystine (cell culture grade; Sigma) was employed.

Measurement of Radiolabel Incorporation from Methionine to Glutathione in Brain Slices—Brain slices were preincubated for 1 h in the DMEM/F-12 medium described above with or without 2.5 mM propargylglycine (PPG). Then [³⁵S]methionine (1000 Ci/mmol; Amersham Biosciences) was added to the incubation medium (1 μl/ml; diluted 1:4 with sterile phosphate-buffered saline (PBS) from 10 μCi/μl solution). The medium was bubbled with a 95% O₂, 5% CO₂ mixture, and the temperature was maintained at 32 °C. Slices were collected at 5 and 10 h following the addition of [³⁵S]methionine and then rinsed with PBS and transferred to preweighed tubes. For the 0 h time point, slices were collected immediately after they were prepared. Slices were homogenized in 200 μl of metaphosphoric acid solution (16.8 mg/ml HPO₄, 2 mg/ml EDTA, and 9 mg/ml NaCl) and stored at −80 °C until further use.

For radiolabeling experiments in cystine-free medium, [³⁵S]methionine was added at 0 h, and the slices were collected at the indicated times.

Murine Neocortical Neuronal Cultures—Neuronal cultures were prepared from embryonic day 17–19 C57BL/6 mice. The dam was anesthetized with isoflurane, and the fetuses were removed and placed in ice-cold Hanks’ balanced salt solution (HBSS). Brains were rapidly removed, placed in ice-cold Neurobasal medium (NBM; Invitrogen), and the neocortex was dissected bilaterally. Tissue was dissociated with a 10-ml plastic pipette and incubated in NBM containing 0.25% trypsin for 15 min at 37 °C in a 5% CO₂ incubator. Following digestion, the tissue was diluted 1:1 with trypsin inhibitor solution (Sigma) containing 10% (final) fetal bovine serum (FBS) and 100 μg/ml (final) DNase I (Sigma) and placed on ice for 20 min. The tissue was then washed with 3 × 5 ml of ice-cold NBM, triturated using a sterile Pasteur pipette, and filtered through a 70-μm filter. Cells were cultured in flasks (at 10 × 10⁶ cells/75 cm²) or in 6-well culture plates (2 × 10⁶ cells/well) at 37 °C in a 5% CO₂ incubator. Culture flasks and plates were coated with 50 μg/ml poly-α-lysine before use. The culture medium consisted of NBM containing 2% B-27 supplement (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin. Every 4 days, 50% of the medium in each well was replaced with fresh medium.

Murine Astrocytes—Astrocytes were prepared from 1–2-day-old C57BL/6 mouse pups. The pups were anesthetized by immersion in an ice water bath, brains were rapidly removed and placed in ice-cold HBSS, and the neocortex was dissected bilaterally. The tissue was dissociated gently using a 10-ml plastic pipette and rinsed twice with ice-cold HBSS. The tissue was then incubated in HBSS containing trypsin (0.25% final) and DNase I (200 units final) for 30 min at 37 °C in a 5% CO₂ incubator. Ice-cold FBS (10%) was then added, and the tissue was rinsed twice with ice-cold HBSS. The tissue was triturated with a 5-ml pipette and filtered through a 40-μm filter. The cells were resuspended in culture medium and plated on flasks at a cell density of 10 × 10⁶ cells/75 cm². The culture medium consisted of DMEM/F-12 (Invitrogen), 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cultures were incubated at 37 °C in a 5% CO₂ incubator and were replated when near confluence at least three times before using in experiments. For final replating, cells were incubated in flasks (10 × 10⁶ cells/75 cm²) or 6-well culture plates (2 × 10⁶ cells/well).

Isolation and Cultivation of Human Fetal Astrocytes and Neurons—Human fetal astrocytes and neurons were isolated from first and early second trimester human fetal brain tissue from elective abortus specimens. These were obtained in full compliance with the ethical guidelines of both the National Institutes of Health and the University of Nebraska Medical Center.

Astrocytes were isolated from fetal brain tissue cortices as previously described (23). Briefly, brain tissue was dissected and mechanically dissociated by teasing through a Nitex bag and 40-μm sieves. The cell suspension was centrifuged, resuspended in DMEM/F-12 supplemented with 10% FBS and an
antibiotic mixture containing penicillin and streptomycin, and cultured at a density of $2 \times 10^6$ cells/150 cm$^2$. The adherent astrocytes were treated with trypsin and cultured under similar conditions to enhance the purity of replicating astroglial cells.

Human fetal cortical neurons were prepared as previously described (24, 25) with minor modifications. Briefly, dissociated brain tissue was incubated with 0.25% trypsin for 30 min, neutralized with 10% FBS, and further dissociated by trituration. The resulting single-cell suspension was cultured on poly-d-lysine-coated plates in NBM supplemented with antibiotics and B-27 supplement.

All preparations of astrocytes and neurons were characterized for purity by immunostaining for glial fibrillary acidic protein (an astrocyte marker), microtubule-associated protein-2, neurofilament, and NeuN for neurons and 4’,6-diamidino-2-phenylindole to stain nuclei (for cell counting). The purity of neurons ranged from 80 to 90%, whereas astrocytes were routinely >98% pure.

$[^{35}]$Methionine Labeling in Cell Culture—Before starting experiments, the medium was changed (100% for astrocytes and 50% for neurons). $L-[^{35}]$Methionine (1000 Ci/mmol) was diluted 1:4 with sterile PBS from a 10 mCi/ml stock solution, and 1 mCi/ml was added to the culture medium to a final activity of 2 mCi/ml. When used, PPG or aminoethoxyvinylglycine (AVG; Sigma) was added to a final concentration of 2.5 mM, and tertiary butylhydroperoxide ($t$-BuOOH; Sigma) in Me$_3$SO was added at a concentration of 200 mM. Cells were incubated at 37 °C in an atmosphere of 5% CO$_2$. At the desired time points, medium was aspirated, and cells were washed with cold PBS and harvested by scraping and suspended by pipetting. For analysis of thios, an aliquot of the cell suspension was mixed with an equal volume of metaphosphoric acid solution. To measure protein concentration, an aliquot of the cell suspension was mixed with an equal volume of lysis buffer (0.1M sodium phosphate, pH 7.4, containing 0.1% Triton X-100, 10 mM chloromethylketone, and 5 mM phenylmethylsulfonyl fluoride). Samples were stored at −80 °C until further treatment.

Effect of PPG and AVG on Cell Viability—To measure the influence of PPG and AVG (2.5 mM each) on cell viability, human astrocytes and neurons were incubated with or without effectors for the desired times, and their viability was assessed. The influence of the transsulfuration pathway on cell viability under oxidative stress conditions was assessed in human astrocytes due to the reader availability of these cells. The cells were grown in 6-well plates, and the medium was changed (2 ml/well) at the beginning of the experiment. Cells were incubated for 12 h with or without 2.5 mM PPG. Then $t$-BuOOH or H$_2$O$_2$ was added to a final concentration ranging from 0.1 to 3 mM, and cells were incubated for 12 h. Control samples received an equal volume of the vehicle (i.e., Me$_3$SO for $t$-BuOOH or PBS for H$_2$O$_2$). Cell viability was determined using the methylthiazolyldiphenyl-tetrazolium bromide (Sigma) assay (26).

HPLC Analysis of Thiol Compounds—For analysis of thiol compounds, the metaphosphoric acid fixed samples of cells and brain slices were thawed and vortexed, and proteins were sedimented by centrifugation. Thiol metabolites in protein-free extracts were derivatized with monoiododiacetic acid followed by 2,4-dinitrofluorobenzene and analyzed by HPLC using a $\mu$-Bondapak-NH$_2$ 300 × 3.9-mm column (Waters) with a methanol/acetic acid gradient as described previously (8). Incorporation of $[^{35}]$S-methionine into thiol compounds was evaluated by measuring radioactivity in the corresponding chromatographic fractions. Results were normalized to protein concentration or to the weight of the brain slices. The protein concentration in samples was measured using the Bradford reagent (Bio-Rad) with bovine serum albumin as a standard.

The identity of the peak attributed to GSH (by comigration with an authentic sample by HPLC) was further confirmed by mass spectrometric analysis (negative ion mode). The presence of a parent ion with $m/z = 530.2$ confirmed the presence of the dinitrobenzene derivative of carboxymethylated GSH.

RESULTS

Glutathione Content in Astrocytes and Neurons—Oxidized and reduced glutathione levels were measured in cultured murine and human cells (Table 1). The average glutathione content in human astrocytes was 177 ± 10 mmol/g of protein and was ∼2-fold higher than in neurons with an average glutathione concentration of 93 ± 13 mmol/g of protein. In mice, the astrocyte glutathione content (86 ± 7 mmol/g of protein) was ∼1.4-fold higher than in neurons (62 ± 8 mmol/g of protein). The GSH/GSSG ratio, an indicator of redox poise, was similar in the two murine cell types and higher in human astrocytes than in neurons.

Inhibition of Cystathionine γ-Lyase Depletes the Glutathione Pool in Cultured Astrocytes and Neurons—PPG is a mechanism-based inhibitor of cystathionine $\gamma$-lyase and inhibits the endogenous route for provision of cysteine via the transsulfuration pathway (Fig. 1). The addition of PPG to neuronal and astrogial cell cultures led to a reduction in intracellular glutathione concentration and in radiolabel transfer from $[^{35}]$S-methionine (Fig. 2A). The average glutathione levels in both human and mouse astrocytes were 87% of untreated controls and 69% in murine neurons. PPG treatment did not alter the GSH/GSSG ratio in any of these cell types. The observed diminution in glutathione pools in these cells is consistent with the presence of an intact transsulfuration pathway. However, the magnitude of change indicates that the contribution of the transsulfuration pathway to cysteine synthesis is lower in cultured neurons and astrocytes than in hepatoma cells or that PPG is a less effective inhibitor in these cells versus hepatoma cells (8).

Incorporation of Radiolabel from Methionine into Glutathione in Cultured Astrocytes and Neurons—A more sensitive test for the existence of an intact transsulfuration pathway is detection of radioactivity in glutathione in cells cultured in the pres-
ence of $^{35}\text{S}$methionine. The only known route for the transfer of radiolabel from methionine to glutathione under these conditions is via synthesis of cysteine in the transsulfuration pathway (Fig. 1). Cultured human neurons and astrocytes showed a time-dependent increase in incorporation of $^{35}\text{S}$ radiolabel in the glutathione pool (Fig. 2B). The addition of PPG inhibited incorporation of radioactivity to $47 \pm 7\%$ in human astrocytes compared with untreated controls ($p < 0.01$) (Fig. 2A). The addition of AVG inhibited incorporation of radioactivity to a similar extent ($51 \pm 3\%$) (not shown). The effect of PPG on the kinetics of radiolabel incorporation into glutathione in human neurons was not determined due to the paucity of cells. Murine neurons and astrocytes also exhibited a time-dependent increase in radiolabel incorporation, which was sensitive to inhibition. PPG decreased incorporation of radioactivity to $48 \pm 10\%$ ($p < 0.04$) in murine neurons and to $61 \pm 9\%$ ($p < 0.06$) in murine astrocytes relative to untreated controls. Together, these results demonstrate that cultured astroglia and neurons have a functional transsulfuration pathway. Neither AVG nor PPG affected cell viability over a 48-h period (not shown).

Incorporation of Radiolabel from Methionine into Glutathione in Murine Brain Slices—To eliminate the possibility that expression of the transsulfuration pathway is induced during culture of astrocytes and neurons and is not relevant to metabolism in brain, we performed similar experiments with neonatal and adult murine coronal brain slices containing, primarily, neocortical and striatal regions (Table 2). A time-dependent decrease in GSH levels was observed that could be due to tissue injury during slice preparation. After 5 and 10 h of incubation, glutathione levels in brain slices were $\sim$2-fold lower in adults than in neonates (Table 2). Between the 5 and 10 h incubation time points, an $\sim 20\%$ decrease in the net glutathione concentration was observed, which could be explained by a moderate swelling of brain slices as indicated by a $20 \sim 30\%$ increase in their wet weight compared with the 0 h time point. A decrease in the GSH/GSSG ratio from $48 \pm 5$ to $32 \pm 3$ was observed in the adult brain, whereas the ratio in neonatal samples was similar at 5 and 10 h ($11 \pm 0.8$ and $10 \pm 1.4$, respectively).
Treatment of brain slices with PPG resulted in changes in the glutathione pool size and in the extent of radioactivity incorporated into glutathione (Fig. 3). In neonatal slices, glutathione decreased to 77 ± 4% (n = 6, p < 0.03) of untreated controls in 10 h, whereas glutathione-specific radioactivity decreased to 44 ± 7% (n = 6, p < 10⁻⁵). In adult brain slices, glutathione decreased to 62 ± 3% (n = 6, p < 10⁻⁴) in 10 h and was accompanied by a proportional decrease in radioactivity incorporation. Consequently, the specific GSH radioactivity in adult slices treated with PPG was virtually unchanged. Exposure of brain slices to [³⁵S]methionine resulted in a 1.8-fold increase in radiolabel incorporation into glutathione between the 5- and 10-h incubation period (Table 3).

An age dependence was observed in cysteine concentration with adult brain slices having an ~7-fold higher concentration than neonatal slices (Table 2). The cysteine concentrations measured in adult brain slices incubated in culture medium are ~20-fold higher than those measured previously in freshly prepared brain homogenates (9). The magnitude of the change in cysteine content was strikingly different in neonatal and adult brain slices treated with PPG (Table 2). In neonatal brain slices, the cysteine concentration was not significantly affected by PPG treatment. In contrast, the cysteine concentration decreased to 55 ± 3% (n = 6, p < 10⁻⁷) and 19 ± 4% (n = 6, p < 10⁻⁶) of untreated controls after 5 and 10 h of PPG treatment of adult slices.

To test whether the observed cysteine accumulation in adult brain slices (Table 2) resulted from increased uptake of this amino acid from the culture medium (which contained 150 μM cysteine),³ the incubation was repeated in cysteine-free medium. An increase in intracellular cysteine was not observed, although GSH levels declined as in control medium (Table 4). These results indicate that cysteine accumulation in brain slices is a consequence of enhanced cystine transport, which probably results from exposure of cells in brain slices to high cystine concentrations, which is not the case in intact brain. Incubation of brain slices in cysteine-free medium containing [³⁵S]methionine increased the specific radioactivity in intracellular cysteine and GSH 11- and 4-fold, respectively (Table 4). These results demonstrate that transport and the transsulfuration pathway contribute to the intracellular cysteine pool in brain slices. Thus, in the absence of extracellular cysteine, the proportion of methionine-derived cysteine that is used for GSH synthesis increases.

It should be noted that brain slices contain a mixture of neuronal and glial cell types that have different capacities for cysteine transport and, presumably, transsulfuration flux. This in turn would influence the sensitivity of different cell types to PPG and to cystine restriction. Our analyses therefore represent the average metabolic changes for a heterogeneous mixture of brain cells but are nevertheless consistent with the presence of an intact transsulfuration pathway in the brain.

### TABLE 2

| Sample          | GSH-specific radioactivity | n  | p value |
|-----------------|----------------------------|----|---------|
| Neonatal brain slices | 100 ± 7                    | 7  | <0.002  |
| Adult brain slices     | 100 ± 8                    | 5  | <0.02   |

³ According to the vendor, the medium contains 100 μM each of cysteine and cystine. However, our analysis revealed that cysteine was completely oxidized at the initiation of the experiment.
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Influence of extracellular cystine on intracellular cysteine and GSH concentration and radiolabeling from [35S]methionine in adult mouse brain slices

Data are presented as mean ± S.E. n, number of independent measurements. NA and ND, not applicable and not detected, respectively.

| Incubation time | Cystine in medium (150 µL) | Cysteine Concentration | Radioactivity | GSH Concentration | Radioactivity | n |
|----------------|---------------------------|------------------------|---------------|-------------------|---------------|---|
| 6 h            |                           | NA                     | 26 ± 4        | 13.8 ± 3          | 631 ± 24      | 6 |
| 1 h            |                           | +                      | 1032 ± 29     | 15.8 ± 0.3        | 631 ± 24      | 2 |
| 1 h            |                           | –                      | 139 ± 14      | 68.7 ± 0.3        | 556 ± 32      | 2 |
| 5 h            |                           | +                      | 1062 ± 126    | 21.0 ± 1.6        | 250 ± 11      | 4 |
| 5 h            |                           | –                      | 56 ± 7        | 233 ± 2           | 267 ± 16      | 4 |

The absolute concentration of intracellular cysteine observed in this experiment is lower than reported in Table 2 and is probably due to differences in the media that were employed (see “Experimental Procedures”).

A common thread connecting the progression of neurodegenerative diseases is redox imbalance and oxidative stress, which through free radical generation and induction of lipid and protein oxidative damage, leads to significant neuronal destruction as seen in Alzheimer disease and Parkinson disease (27). Glutathione is a major antioxidant that appears to be synthesized by all cells. In contrast, the transsulfuration pathway that provides an endogenous route for synthesis of cysteine, the limiting reagent in the biosynthesis of glutathione, has a more restricted distribution. It is believed that only cells that have a high turnover of glutathione and therefore a high demand for cysteine harbor this pathway. Redox buffering is an important homeostatic function in all cells, and the importance of glutathione in protecting the brain from oxidative damage is underscored by the decreased activities of other antioxidant systems (viz. catalase and superoxide dismutase) in this organ (28). A marked diminution in nigral glutathione levels is observed in Parkinson disease-afflicted brains and appears to be an early marker of brain pathology (2). Based on these observations, it is surprising that fundamental issues regarding metabolic pathways that govern glutathione homeostasis in the brain remain clouded in controversy.

Ascorbate, another small molecule antioxidant, is present at relatively high concentrations in brain, and its metabolism is linked to that of glutathione. Thus, compensatory changes are observed in the ascorbate pool size in transgenic mice, which contain diminished glutathione pools due to the heterozygous disruption of the γ-glutamylcysteine ligase gene (29) or homozygous disruption of the cystathionine β-synthase gene (9). This interrelationship is also observed in normal adult mice, in which a decrease in glutathione levels stimulates ascorbate synthesis (30). Unlike mice, which have the ability to synthesize ascorbate from glucose 6-phosphate due to the pres-
ence of L-gulonolactose oxidase, humans are unable to synthesize this vitamin and would appear to be unable to compensate for reduced glutathione levels by a concomitant increase in the ascorbate pool. Hence, in humans, depletion of glutathione pools may have more serious consequences for redox homeostasis.

The first enzyme in the transsulfuration pathway, cystathionine β-synthase, is present in the brain, albeit its activity is ~30-fold lower than in liver (9). In contrast, the published reports make contradictory claims about the existence of the second enzyme, cystathionine γ-lyase, in the brain. Homozygous disruption of cystathionine β-synthase results in a decrease in brain glutathione and cysteine levels (9) and a significant increase in plasma homocysteine levels (31). Similarly, diminution of the brain glutathione and cystathionine pools were observed in rats maintained on a pyridoxine-deficient diet (32). Although these observations could be taken as evidence for the presence of an intact transsulfuration pathway, it could also be argued that disruption of this pathway in liver and kidney by dietary or genetic manipulations led to decreased cysteine supply to the brain, accounting for the diminished glutathione pool size.

In this study, we have employed a sensitive metabolic radio-labeling method to demonstrate the presence of an intact transsulfuration pathway in cultured human and murine astrocytes and neurons and in murine brain slices. In culture, glutathione levels are higher in astrocytes than in neurons, and this difference is more marked in human cells (Table 1). Similar differences in glutathione pool size have been reported in cells cultured from neonatal rat brain (33, 34) and chick brain (35, 36). Astrocytes are believed to be the major source of extracellular glutathione in brain (3) and are also important for augmenting neuronal reserves of glutathione by provision of cysteine generated by the action of the ectoenzyme, γ-glutamyltranspeptidase (34). The observed decrease in glutathione concentration in cells treated with the cystathionine γ-lyase inhibitors, PPG and AVG, is consistent with the presence of an intact transsulfuration pathway, which is confirmed by the appearance of radioactivity in glutathione in cells exposed to [35S]methionine (Fig. 2). An issue with the use of any pharmacological inhibitor is that specificity for the targeted enzyme is unlikely to be absolute. We note, however, that the primary evidence for the presence of an intact transsulfuration pathway is the transfer of radiolabel from methionine to glutathione, which, based on our understanding of sulfur metabolism, is only explained by the presence of both enzymes in this pathway. The response of cells to inhibition by AVG or PPG is consistent with the results from metabolic labeling studies.

The responsiveness of the transsulfuration pathway to oxidative stress conditions was further examined in astrocytes (due to their random availability as compared to neurons) and found to be similar to the behavior observed in human hepatoma cells (8). Thus, an increased flux through the transsulfuration pathway paralleled by an increase in glutathione synthesis was observed (Fig. 4), which constitutes an autocorrective response to the glutathione pool initially diminished by oxidative stress.

Our results are consistent with several earlier studies. Injection of rats with PPG resulted in a 3–14-fold increase in cystathionine concentration in different regions of the brain (20), and direct intracerebral injection of [35S]cystathionine yielded radiolabeled cysteine (19). Furthermore, partial restoration of the glutathione pool size depleted by glucose starvation was achieved by feeding cystathionine to astroglial cells (37). On the other hand, a number of studies have reported the absence of cystathionine γ-lyase in rodent brain (16, 38). A plausible explanation for the negative results is the >100-fold lower concentration of cystathionine γ-lyase in brain versus liver and kidney (39), rendering activity assays (38) or comparative Western blot analysis of cell extracts from these organs (16) insensitive measures for detection of this enzyme. It should be noted that in one of these studies that reported the absence of detectable cystathionine γ-lyase in the brain, the mRNA for this gene was observed by Northern analysis (16). Furthermore, the expressed sequence tag data base reveals multiple entries for the γ-cystathionase mRNA in brain.

A caveat with our detection of transsulfuration activity in neuronal and astroglial cells is that culture conditions can induce cultured cells to express genes that are otherwise repressed in their normal environment. To address this issue, we have extended our studies to brain slices dissected from neonatal and adult mice.

The addition of PPG had a less significant effect on the glutathione and cysteine contents in neonatal brain slices, whereas a marked decrease in both sulfur metabolites was observed in adult brain slices (Table 2). The greater sensitivity of the glutathione pool size in adult brain to inhibition by PPG is consistent with the more robust expression of cystathionine β-synthase in postnatal versus neonatal brain (40). In contrast, γ-cystathionase activity remains low and virtually unchanged following birth (39). Further confirmation of the presence of the transsulfuration activity is provided by detection of [35S]-labeled glutathione in brain slices exposed to [35S]methionine (Table 3). These studies reveal that despite the relatively low concentrations of the transsulfuration pathway enzymes in brain versus liver, they nevertheless play a quantitatively significant role in glutathione homeostasis in adult brain. Hence, inhibition of cysteine synthesis leads to a decrease in the glutathione pool size. Our knowledge of methionine transport in brain cells is limited. The solute carrier 6 (SLC6) gene family is known to encode sodium-dependent transporters for broad specificity neutral amino acids (e.g., B0AT2 in mice (41)) and for branched chain amino acids and methionine (SBAT1 in humans (42)).

Increased expression of rat cystathionine β-synthase but not γ-cystathionase after birth is paralleled by the postnatal increase in the cystathionine content in this organ (43) and indicates that flux through the transsulfuration pathway is limited by γ-cystathionase. In fact, cystathionine concentrations are higher in the brain than in other organs (44), and significant regional differences are observed within this organ (43, 45, 46). Primate brains have ~10-fold higher cystathionine than rodent brains (40, 46), and the concentration is ~2-fold higher in human versus monkey brain (44). Higher cystathionine concentration has been measured in white versus gray matter in human brain (47). These observations suggest that regulation of flux through the transsulfuration pathway is different in the brain versus other tissues, which allows accumulation of the interme-
diate, cystathionine. The significance of high cystathionine concentrations in brain is not known, and pharmacological studies have suggested a potential role for this amino acid as a neuromodulator (48, 49). Interestingly, cystathionine is virtually absent in homocystinuric patients deficient in cystathionine β-synthase who exhibit disorders of the central nervous system, including seizures (50).

The significance of the transsulfuration pathway under oxidative stress conditions is exemplified by the diminished viability of astrocytes when the pathway is inhibited (Fig. 4). Under steady state conditions, blockade of the transsulfuration pathway by PPG or AVG is without discernable effect on viability (not shown), which is expected based on the high intracellular concentration of glutathione that is maintained under these conditions (Table 2). However, under oxidizing conditions, which necessitate a change in the dynamics of glutathione synthesis, provision of cysteine, the limiting reagent for glutathione, is without effect on viability (51). As with the cystathionine synthase who exhibit disorders of the central nervous system, including seizures (50).

In summary, our demonstration of a functional transsulfuration pathway in the brain's redox buffering capacity warrants investigations to up-regulation of the transsulfuration pathway. To our knowledge, this has not been evaluated in patients (with respect to increased susceptibility to diseases linked to higher reactive oxygen species) or studied in patient-derived cells.

The significant link between the transsulfuration pathway and the brain's redox buffering capacity warrants investigations into potential neuroprotective benefits of nutritional modulation of redox homeostasis.

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