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Supplementary Information for

The glutathione cycle shapes synaptic glutamate activity

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Supplementary Information Text

Materials and Methods

Cell culture and reagents. Cells were maintained at 37 °C in 5% CO2/95% atmosphere and medium replaced every 3 days. For glutathione and glutamate quantification, cell viability analysis and oxidative stress assay, cells were sub-cultured in 12-well plates at a density of 4.2 x 10^5 cells/well. For electrophysiological recording, cells were seeded onto 12 mm diameter poly-D-lysine coated glass coverslips in 24-well plates and grown for 48 hours before experiments. The media was then replaced with fresh media and treated with 25 μM acivicin, 200 μM buthionine sulfoximine (BSO) or 5 μM pyroglutamate (PGA) for glutathione and glutamate measurement. Alpha-methylaminoisobutyric acid (MeAIB) was treated 1 to 4 hours before the electrophysiological recording. Acivicin was obtained from BIOMOL. BSO and PGA were obtained from Sigma. MeAIB was purchased from Chem-Impex International. All animal procedures related to were approved by the Johns Hopkins University Animal Care and Use Committee.

Measurement of glutathione. Total and oxidized glutathione were determined by the Tietze method with minor modifications (1). Cells were washed in ice cold Mg/Ca-free PBS, scraped and suspended in phosphate buffer with fresh 5% metaphosphoric acid, then sonicated. A portion of lysate was suspended in 0.1% N-lauroylsarcosine and used for analysis of protein content by Bradford assay (2). The remaining solution was centrifuged 15 minutes at 20,000g and the soluble fraction was used for detection of glutathione content. Proteins in the remaining fraction were precipitated with 50 mg/mL metaphosphoric acid, removed by centrifugation, and supernatants neutralized with 200 mM triethanolamine. For oxidized glutathione measurement, an aliquot of the supernatant was incubated 60 minutes at room temperature with 10 mM 2-vinylpyridine (2-VP) to

scavenge reduced glutathione. The rate of increase in absorbance at 415 nm, which measures the reduction of 5-5'-dithiobis (2-nitrobenzoic acid) by glutathione, reflects the total glutathione content (or oxidized glutathione when 2-VP is added). The concentration of total and oxidized glutathione content in cells was calculated by a calibration curve with standards.

Measurement of glutamate. Cells were collected in phosphate buffer (100 mM sodium phosphate, 1 mM ethylene diamine tetraacetate, pH 7.5) and sonicated and lysates centrifuged to remove insoluble debris. Determination of glutamate was performed by Kusakabe's method (3). 2-10 nmol of L-glutamate standards and the cell lysate were mixed with reaction buffer (final concentrations of 36.8 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.1), 2.19x10-5 U L-glutamate oxidase, 1 U peroxidase, 0.8 mM 3.5-dimethoxy-N-ethyl-N-(2-hydroxy-3-sulphopropyl) aniline (sodium salt) (DAOS), 0.8 mM aminoantipyrine). After incubating at 22 °C for 30 minutes, the mixture was measured at a wavelength of 570 nm with a microplate spectrophotometer. The concentration of L-glutamate was then calculated using a standard calibration curve, normalized to protein sample concentrations, and levels expressed as percentage relative to controls.

mEPSC recordings. Whole-cell patch clamp recordings were performed at 22 °C from primary cortical neurons at days *in vitro* of 12-16. Coverslips containing the neurons were loaded into an upright microscope (Olympus BX-61-WI) fitted with a Warner RC-26 submerged recording chamber. Patch pipettes (4-6 Mohm) were filled with cesium solution containing 115 mM cesium methanesulfonate, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10 mM disodium phosphocreatine, 5 mM tetraethylammonium chloride, 3 mM adenosine 5'-triphosphate magnesium, 2.8 mM sodium chloride, 0.5 mM guanosine 5'-triphosphate sodium, and 0.4 mM ethylene glycol tetraacetic acid (pH 7.2, 285-290 mOsm/kg). Recordings were made at room temperature in artificial cerebrospinal fluid (aCSF) (pH 7.3, osmolarity 305 +/- 5 mOsm/kg)

perfused at 2 mL/min containing 150 mM NaCl, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10 mM glucose, 3 mM potassium chloride, 2 mM calcium chloride, and 1.3 mM magnesium chloride. Putative neurons were identified under visual guidance with infrared differential interference contrast optics (Olympus BX-61-WI) with a 40x water-immersion objective. The image was captured with an infrared-sensitive EMCCD camera (IXON DU885K) and displayed on a monitor. In addition, the image was sent to a computer with a serial connector and captured (Metamorph Advanced). Whole-cell patch clamp recordings were made with a headstage (CV-7B) connected to a computer-controlled amplifier (MultiClamp 700B, Axon Instruments), digitized through a Digidata 1440A Analog/digital converter and acquired at a sampling rate of 10 kHz. All current steps to set membrane potential and elicit action potentials were delivered through the recording pipette and controlled by Clampex 10 (Axon). Electrode potentials were adjusted to zero before recording. mEPSCs were recorded in the presence of tetrodotoxin (TTX, 1 μM, Tocris, Ellisville, MO, USA or Alomone, Jerusalem, Israel) and picrotoxin (0.1mM, Sigma-Aldrich, St. Louis, MO, USA). TTX was incubated in the culture for 10 min, followed by at least a 10 min lead in to establish baseline before recordings were made. All voltage clamp recordings were performed at a holding potential of -70mV. mEPSC events were detected using the software MiniAnalysis (Synaptosoft, Decatur, GA, USA) with a 5 pA amplitude threshold and all mEPSCs were verified visually. Data for analysis was excluded if access resistance is more than 50 Mohm or membrane resistance is less than 100 Mohm. Cumulative probability histograms were tested by Kolmogorov-Smirnov Test for each recording from at least 50 events. Mean values for mEPSC frequency and amplitude between groups were analyzed by Tukey-Kramer HSD test. All results are shown as mean +/- S.E.M.

Statistical analysis. All data were expressed as the mean+/-S.E. Statistical differences in glutathione, glutamate measurement and miniature EPSC analysis were done by Tukey-Kramer HSD test, except where noted. Statistical differences between two groups were determined with

Student's t test, and statistical differences among three groups or more were determined using a one-way analysis of variance (ANOVA), two-way ANOVA, and an ANOVA with repeated measures, followed by Bonferroni's multiple comparison test; $p<0.05$ was regarded as statistically significant.

Isolation of synaptosomes

Synaptosomes were isolated as described previously (4). Briefly, mice were euthanized by decapitation and whole brains homogenized in 20 vol. of 0.32 M sucrose. After centrifugation at 1000 x g for 10 min, the supernatant (S1) was centrifuged for 20 min at 17.500 x g to yield the supernatant S2 and the pellet P2 (crude synaptosomal fraction). P2 was suspended in 10 vol of 0.32 M sucrose and 10 ml of the solution layered on a 0.8 and 1.2 M two-step discontinuous gradient, which was centrifuged at 61,000 x g for 2 h to separate the synaptosomes from the mitochondria (which forms the pellet). The synaptosomes form the interface between the 1.2 M and 0.8 M sucrose layers.

Supplementary Figures

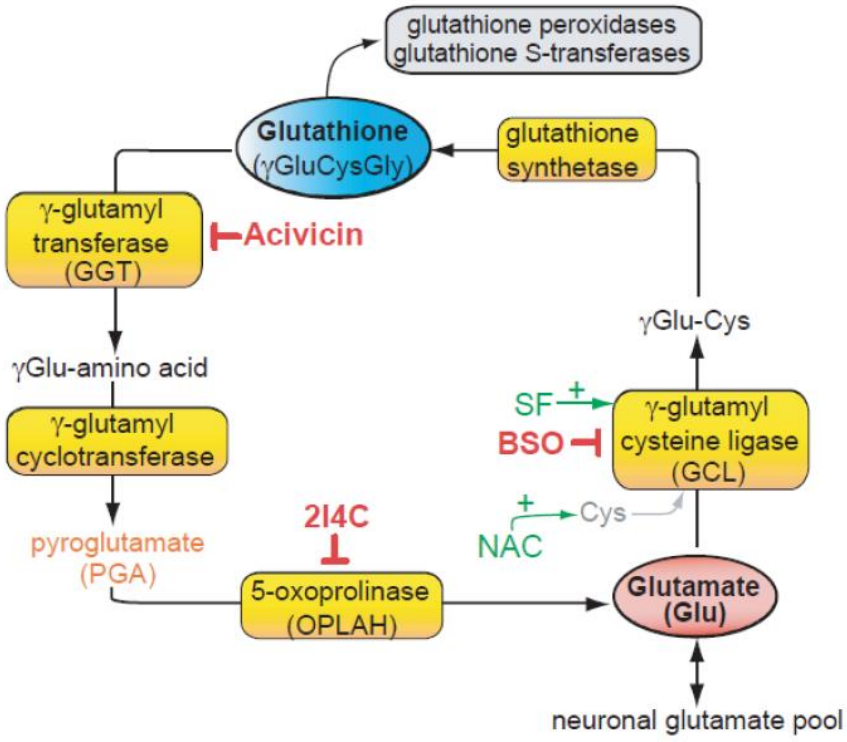

Fig. S1. The gamma-glutamyl cycle (glutathione cycle). Glutamate is used as a substrate for glutathione synthesis by GCL and glutathione synthetase. Glutamate is liberated from glutathione through the action of GGT, glutamyl cyclotransferase and OPLAH. Inhibitors (acivicin, 2I4C, and BSO) are shown with their respective targets. PGA is a metabolite of GGT and precursor of glutamate in this cycle. OPLAH, 5-oxoprolinase; GCL, gamma-glutamyl cysteine ligase; 2I4C, 2-imidazolidone-4-carboxylate; BSO, buthionine sulfoximine; NAC, N-Acetylcysteine; SF, sulforaphane; PGA, pyroglutamate.

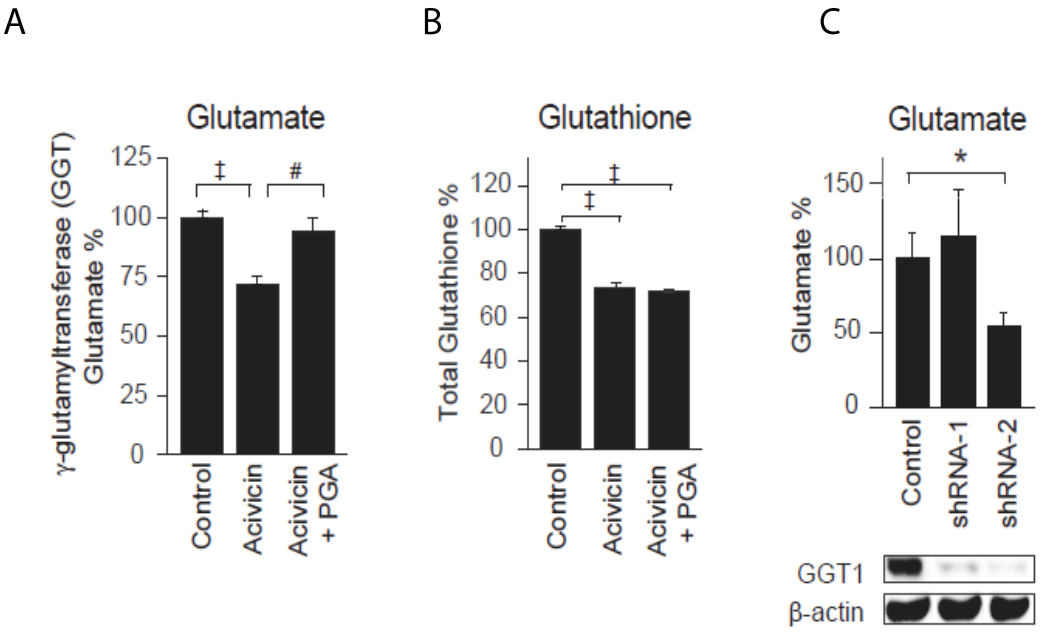

Fig. S2. Inhibition of γ-glutamyltransferase diminishes glutamate levels. (*A,B*) Acivicin treatment of cortical neurons (25 μM, 24 h) decreases glutamate and glutathione levels. Co-administration of 5 μM pyroglutamate (PGA) selectively rescues the glutamate decrease. (*C*) shRNA targeting of γ-glutamyltransferase in N2A neurons decreases glutamate. shRNA-2 had greater suppression of protein expression than shRNA-1. *, $p<0.05$, #, $p<0.01$, † $p<0.001$, ‡ $p<0.0001$ by 1-way ANOVA with Tukey-Kramer posthoc.

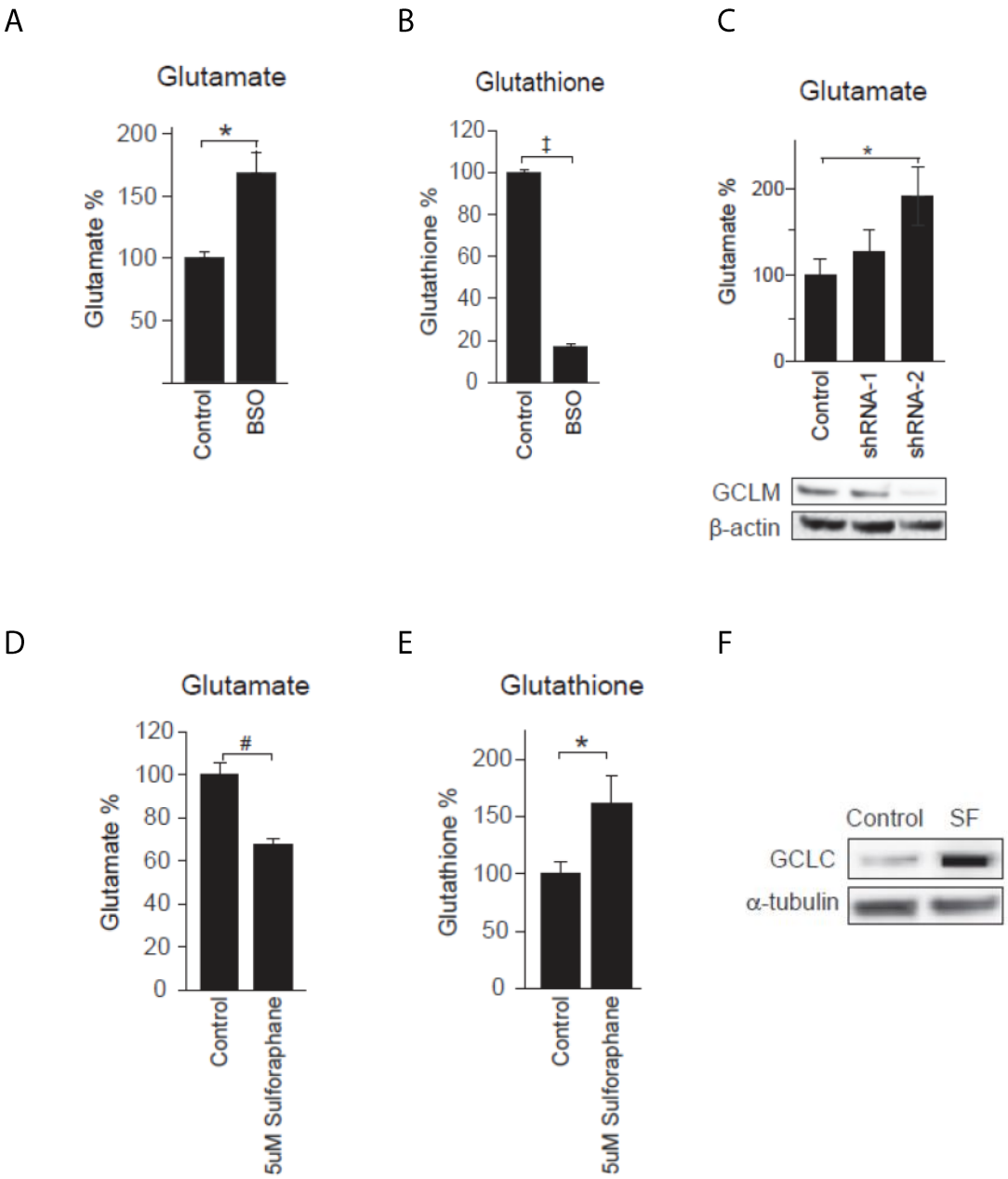

Fig. S3. Targeting GCL to modulate glutathione and glutamate levels. (*A*,*B*) BSO, which blocks GCL, decreases glutathione and increases precursor glutamate. Cortical neurons were treated 24 h with 200 μM BSO (*A*,*B*), or N2A neurons were treated with shRNA to GCL (*C*). shRNA-2 gave greater suppression of GCL protein than shRNA-1. (*D*-*F*) Sulforaphane increases GCL and acutely increases glutathione and decreases glutamate. Cortical neurons were treated 24 h with 5 μM sulforaphane. Lysates were immunoblotted with anti-GCLC antibody. BSO, buthionine sulfoximine; GCL, glutamate-cysteine ligase. *, $p<0.05$, #, $p<0.01$, † $p<0.001$, ‡ $p<0.0001$ by 1-way ANOVA with Tukey-Kramer posthoc.

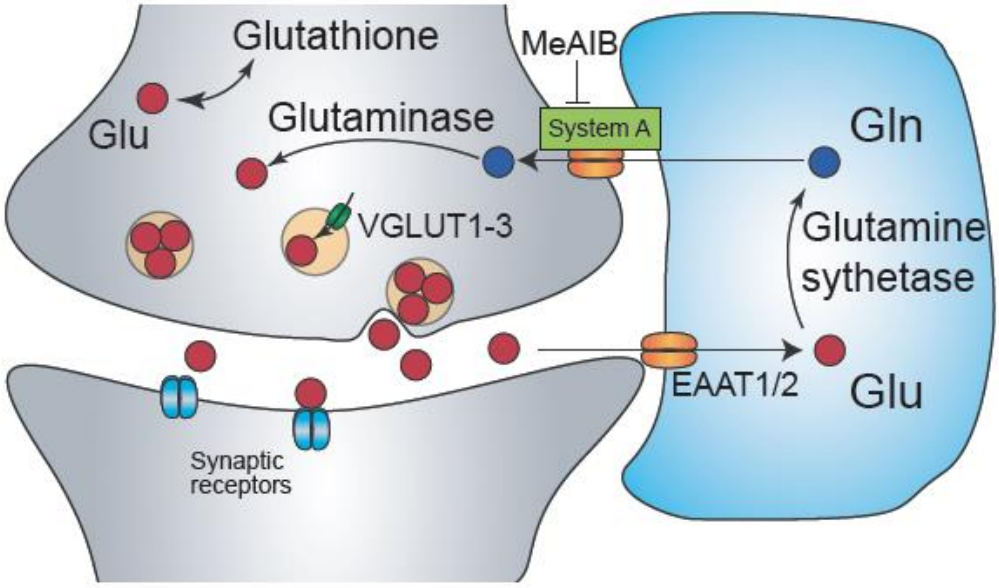

Fig. S4. Model for the glutathione cycle complementing the glutamine-glutamate shuttle. Schema of cycling of glutamate-glutamine and glutamate-glutathione. Solid red circles indicate glutamate and solid blue circles indicate glutamine. MeAIB blocks system A transporters that import glutamine.

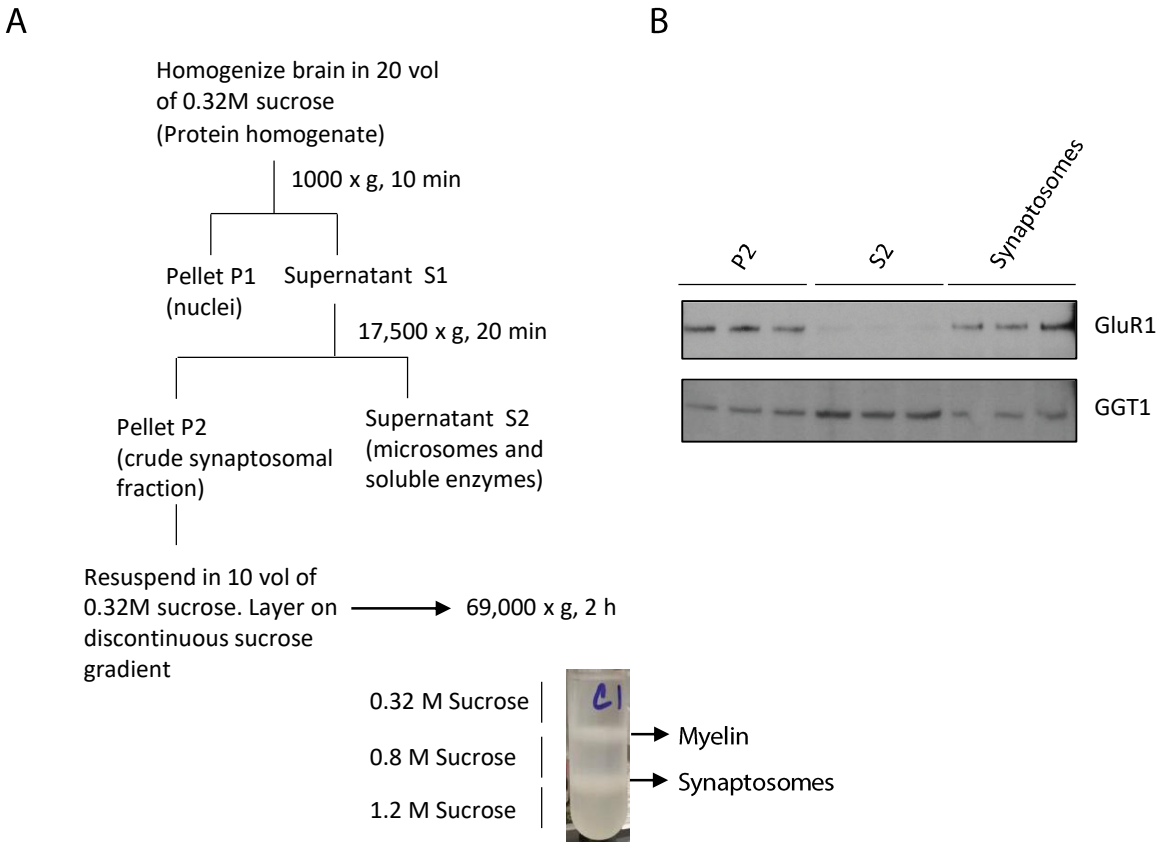

Fig. S5. Gamma-glutamyl transferase (GGT1) is associated with the synaptosomal fraction. (*A*) Subfractionation protocol to isolate synaptosomes. After discontinuous sucrose density gradient centrifugation, the synaptosomes band at the interface of the 0.8 M and 1.2 M sucrose layers. (*B*) Western blot showing localization of GGT1 to the synaptosomal fraction (n=3). GGT1 is also present in the soluble fraction, S2. GluR1 (the AMPA receptor) was used as a positive control, present only in the synaptosomal fractions and absent from the soluble fraction, S2.

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