Glutathione shapes glutamateric transmission

Minori Koga[1], Greg M. Parker[1,2], Lynda D. Hester[2], Yu Taniguchi[1], Atsushi Kamiya[1], Solomon H. Snyder[2,1,3], Akira Sawa[1], Thomas W. Sedlak[1]

[1] Dept. of Psychiatry and Behavioral Sci., [2] The Solomon H. Snyder Dept. of Neurosci., [3] Dept. of Pharmacol., Johns Hopkins Univ. Sch. of Med., Baltimore, MD 21205

ABSTRACT

Glutamate is the most abundant excitatory neurotransmitter, present at up to 80-90% of cortical synapses, and participating in many physiologic and pathologic processes ranging from learning and memory to stroke. The tripeptide, glutathione, is one third glutamate and present at up to 10 millimolar intracellular concentrations in brain, contributing to anti-oxidant and anti-inflammatory defense. Because of the substantial amounts of brain glutathione and its rapid turnover, we hypothesized that glutathione is a relevant reservoir of glutamate, and could influence synaptic excitability. We find that diminishing the liberation of glutamate by the glutathione cycle produces decreases in cytosolic glutamate, decreased frequency of miniature excitatory post synaptic potentials (mEPSC), and diminished depolarization-associated calcium flux. In contrast, pharmacologically decreasing the biosynthesis of glutathione leads to increases in cytosolic glutamate, increased frequency of mEPSC, and increased depolarization-associated calcium release. The glutathione cycle can compensate for decreased excitatory neurotransmission when the glutamate-glutamine shuttle is inhibited. Glutathione may be a physiologic reservoir of glutamate neurotransmitter that bridges anti-inflammatory pathways and glutamatergic functioning.

INTRODUCTION

Glutamate is the most abundant excitatory transmitter in the central nervous system, utilized at 50-70% of cortical synapses.[1, 2] Glutamate participates in diverse physiological processes, such as developmental plasticity and long-term potentiation as well as brain diseases: epilepsy, stroke, amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease and schizophrenia.[3] Glutathione is a tripeptide of glutamate, cysteine and glycine, occurring in neurons at concentrations of 2-10 mM; it is the most abundant low molecular weight thiol of bacteria, plant and animal cells.[4-6] As such, it regulates critical cellular processes such as metabolism of estrogens, prostaglandins, leukotrienes and xenobiotic drugs. Glutathione is well known as an anti-oxidant agent, providing a major line of defense against oxidative and other forms of stress, largely as a cofactor for the glutathione peroxidase and S-transferase enzyme families.[7-10]

Glutathione metabolism is governed by the glutathione cycle (Supplementary Figure 1), in which glutamate is added and liberated at discrete steps.[4, 11] Glia serve as a major supplier of cysteine for neuronal glutathione synthesis, and 50-60% of glutamate neurotransmitter is derived from the glutamine-glutamate shuttle between neurons and glia, with substantially smaller amounts of glutamate transmitter derived from glycolysis.[12-14] However, this shuttle is

not the only means to replenish supply of neuronal glutamate; when it is inhibited, neurons quickly restore glutamate neurotransmission by an ill-defined endogenous mechanism.[15] The authors speculated that neurons might be making use of a storage buffer of glutamate.[15] We hypothesize that the glutathione cycle may be one such glutamate reserve, especially considering its high concentration and short half-life.

We previously reported that in addition to its support of antioxidant function, the glutathione cycle also serves as a reservoir of neural glutamate.[16] Decreasing the liberation of glutamate from the glutathione cycle lead to decreased cortical neuron glutamate, while decreasing the utilization of glutamate leads to increased total glutamate by about 25%. These shifts in glutamate pools could be achieved without increasing oxidative stress or cell death. In the present study we sought to expand this concept by determining if shunting glutamate from the glutathione cycle can shape excitatory neurotransmission. We employed selective inhibitors of different steps of the glutathione cycle and the glutamine-glutamate shuttle and show that glutathione serves as a source for a material portion of glutamatergic neurotransmission.

MATERIALS AND METHODS

Cell culture and reagents

Dissociated cortical neuron cultures from Sprague-Dawley rats were prepared as described previously.[17] Primary cortical neurons were maintained in Neurobasal medium® (Life Technologies Corporation) supplemented with 1x B-27 (life technology). 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 105 cells/well. For electrophysiological recording, cells were seeded onto 12 mm diameter poly-D-lysine coated glass coverslips at a density of in 24-well plates and grown for 48 hours before experiments. The media was then replaced with fresh media and treated with 25 uM acivicin, 100 mM 2-imidazolidone-4-carboxylate (2I4C), 200 uM buthionine sulfoximine (BSO) or 5uM 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. 2I4C, BSO and PGA were obtained from Sigma. MeAIB was purchased from Chem-Impex International. shRNA to GCLM (bp 1256-74 & 1223-41 for shRNA 1&2, respectively), and GGT1 (bp 925-43 & 967-85) were cloned into pSuper vector and N2A neurons were transfected with Lipofectamine 2000 according to the manufacturer's instructions. All animal procedures related to were approved by the Johns Hopkins University Animal Care and Use Committee.

Measurement of glutathione

Total and oxidized glutathione was determined by the Tieze method with minor modifications.[18] Cells were washed in PBS, scraped and suspended in phosphate buffer then sonicated. A portion of lysate was suspended in 0.1% N-lauroylsarcosine and used for analysis of protein content by Bradford assay.[19] 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 ethylenediaminetetraacetate, pH 7.5) and sonicated and lysates centrifuged to remove insoluble debris. Determination of glutamate was performed by Kusakabe's method.[20] 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.

Calcium imaging

2 days after transfection of pCMV-GCaMP3, cortical neurons were treated with artificial cerebrospinal fluid (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5mM glucose) containing 0.5 μM TTX (Tocris) for 30 min at 37 °C. 100 mM potassium chloride in aCSF was then applied to the calcium concentration was measured by previous methods.[21-24] Time-lapse fluorescent images were recorded through 20x objective lens every 90 milliseconds by using a fluorescence microscope (Olympus IX81). The series of fluorescence images were collected by line-scan across the apical dendrite at 10-μm distance from cell bodies and analyzed by Slidebook software (Intelligent Imaging Innovations). Basal pixel fluorescence intensity was defined as the average of the last five

frames before treatment of 100 mM potassium chloride (F0) and raw pixel fluorescence values in each frame (F) were measured. ΔF/F represents the value of (F – F0)/F.

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 current 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 (1 μM, Tocris, Ellisville, MO, USA or Alomone, Jerusalem, Israel) and picrotoxin (0.1mM, Sigma-Aldrich, St. Louis, MO, USA). 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 for behavioral tests were determined with Student's t-test. 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.

RESULTS

To test the hypothesis that glutathione is a significant reservoir for glutamate, we treated neuronal cells with molecular inhibitors targeting enzymes of the glutathione metabolic cycle: L-2-imidazolidone-4-carboxylate (2I4C), acivicin, L-buthionine sulfoximine (BSO), and sulforaphane. Glutathione and glutamate were quantified by Ellman's procedure and glutamate oxidase methods.[25, 26] If glutathione constitutes a glutamate reservoir, then inhibiting GGT with acivicin should lead to decreased cellular glutamate, as this enzyme is upstream of the ultimate liberation of glutamate from the cycle (Figure 1a & Supplementary Figure 1).

As we had previously demonstrated in cell lines,[16] total glutamate levels declined in primary cortical neurons treated with acivicin (Supplementary Figure 2a-b). To confirm the specificity of this effect, shRNA targeting of GGT also decreased glutamate levels (Supplementary Figure 2c). Additionally, we find that the decrease in glutamate brought by acivicin could be rescued by administration of pyroglutamate [5-oxoproline], a downstream metabolite in the glutathione pathway that is a precursor of glutamate (Supplementary Figure 1). Pyroglutamate selectively repleted glutamate, but not glutathione (Supplementary Figure 2a-b).

To determine if glutamate availability from the glutathione cycle could shape excitatory transmission, we measured the frequency of miniature excitatory postsynaptic currents (mEPSCs), a reflection of presynaptic drive. Acivicin treatment (24 h) significantly decreased mEPSC frequency (Figure 1a-d). To demonstrate specificity of the effect, we sought to rescue the decreased mEPSC frequency by pretreating with pyroglutamate (PGA), the precursor of glutamate that is synthesized downstream of GGT, which is blocked by acivicin (Figure 1a). Pyroglutamate rescued the effect on mEPSC frequency (Figure 1b-d). As we previously demonstrated, acivicin treatment at these concentrations did not elicit oxidative stress or affect cell viability.[16]

Glutamate cysteine ligase (GCL) is the rate limiting step for glutathione synthesis, and utilizes glutamate as a substrate. Inhibition of GCL with buthionine sulfoximine (BSO) increased neuronal glutamate (Supplementary Figure 4a), consistent with our prior findings in cell lines.[16] BSO treatment depleted glutathione rapidly, reflecting its short half-life (1-4 h) as a substrate of multiple enzymes (Supplementary Figure 4b). This was confirmed by shRNA to GCLC, the target of BSO, which increased glutamate levels (Supplementary Figure 4c). To further test the role of GCL in modulating glutathione and glutamate, we utilized sulforaphane, which increases GCL expression through activation of the nrf2 pathway.[27] While BSO inhibition of GCL decreased glutathione and increased glutamate, sulforaphane induced GCL, and decreased glutamate while increasing glutathione (Supplementary Figure 4d-f). We previously demonstrated that these doses and durations of treatment do not alter cortical neuron viability or increase oxidative stress.[16] BSO was utilized to test whether increasing the liberation of glutamate from the glutathione cycle could shape excitatory transmission. The BSO induced increased in glutamate was accompanied by an increase in mEPSC frequency (Figure 2b-d).

The glutamate-glutamine shuttle (Supplementary Figure 4) between neurons and glia contributes 50-60% of glutamate neurotransmitter,[12, 13, 28] with intracellular glycolytic products contributing to the remainder. In the shuttle, glutamate is converted to glutamine in

astrocytes, and then exported to neuronal system A transporters, where it is converted to glutamate intracellularly by phosphate activated glutaminase.[29] However, glutaminase knockout mice[30] or blockade of system A transporters with methylaminoisobutyric acid (MeAIB)[15] fail to block excitatory neurotransmission. We explored whether the glutathione cycle can complement the actions of the glutamate-glutamine shuttle by blocking import of glutamine by the system A transporter,[15] which imports glutamine into neurons, after which it is converted to glutamate (Figure 3a).

As expected, MeAiB decreased mEPSC frequency (Figure 3b-d). Acivicin, which diminishes the availability of glutathione-derived glutamate, decreased average mEPSC frequency significantly, though not to the same extent as MeAIB (Figure 3d). mEPSC frequency declined even further when acivicin and MeAIB were co-administered, consistent with glutamate derived from the glutathione cycle having capacity to shape excitatory neurotransmission when glutamine supply is restricted (Figure 3c-d).

We next examined whether glutamate from the glutathione cycle could rescue mEPSC when glutamine supply was restricted (Figure 4a). BSO, which augments glutamate levels, also increased mEPSC frequency (Figure 4b-d), while glutamine restriction by MeAIB decreased mEPSC frequency. However, administration of BSO could rescue the decreased mEPSC frequency brought by MeAIB (Fig 4d, green line), consistent with the glutathione cycle being

able to compensate for decreased availability of glutamine, an established source of glutamate neurotransmitter.

DISCUSSION

We previously reported that the glutathione cycle may serve as a reservoir of total neuronal glutamate.[16] Treatments that decrease the liberation of glutamate from the glutathione cycle lead to decreased intracellular glutamate, whereas decreasing utilization of glutamate for glutathione synthesis increases it.[16] We now report that shunting of glutamate derived from glutathione can shape excitatory neurotransmission. Inhibiting GCL, which utilizes glutamate to synthesize glutathione, leads to increased glutamate and mEPSC frequency. Decreasing the release of glutamate from the glutathione cycle by blocking GGT leads to diminished mEPSC. We have previously demonstrated that these fluxes of cytosolic glutamate can occur without detectable increases in oxidative stress or altered cell viability.[16] Thus the glutathione pathway is poised to contribute glutamate without impacting neuronal viability unless there are massive, sustained deficits.[16, 31]

The glutathione pathway may also supply glutamate when glia derived glutamine is blocked by MeAIB, which inhibits system A transporters. While glia-derived glutamine provides 50-60% of glutamate neurotransmitter, when the pathway is blocked with MeAIB excitatory

transmission abruptly decreases but rapidly recovers, consistent with endogenous neuronal sources of glutamate neurotransmitter.[15] We suggest glutathione is one such endogenous source, as further impairing glutamate liberation from the cycle diminishes synaptic activity induced by MeAIB, while increasing glutamate availability can rescues the impairment by MeAIB. A reservoir capacity of glutathione may be utile during periods of sustained synaptic activity.

Alterations in neuronal glutamate levels, such as by fluxes to and from a glutathione reservoir, might have an impact upon glutamate neurotransmitter. Vesicular glutamate transporters have a much lower affinity for glutamate 0.5-3.5 mM than plasma membrane transporters GLT1/EAAT2, whose Km is 4-40 uM [32, 33]. Furthermore, as glutamate neurotransmitter typically does not saturate postsynaptic receptors, modest impacts upon release frequency may influence synaptic strength [34-37]. Our findings also affirm prior reports that increasing intracellular glutamate concentration in presynaptic terminals leads to greater excitatory post synaptic currents (EPSC) [36, 37]. In our specific approach, we suggest that the glutathione cycle may be one such source of this glutamate. This may provide some mechanistic implications to interpret magnetic resonance spectroscopy techniques in human subjects, in which total regional brain glutamate may be determined, though not provide details on excitatory neurotransmission [38, 39]. "Phasic" axons that fatigue in their glutamate

neurotransmitter release have lower glutamate levels than “tonic” glutamate axons with greater glutamate levels [40]. Glutamine levels are similar in both, suggesting that significant reservoirs of glutamate exist in neurons independent of glutamine. Localized glutathione synthesis would be expected to have an even more pronounced effect, and it has been suggested that non-soma areas contain more glutathione [41].

These findings may be relevant to human disease. Glutamatergic dysfunction has been implicated in schizophrenia by multiple lines of evidence.[42-46] Several investigators have reported aberrant glutathione levels in schizophrenia patients, including medication naïve subjects.[47-50] Mice lacking the modifier subunit of GCL, have a 60% reduction in glutathione, accompanied by abnormal cortical gamma synchrony, decreased parvalbumin interneurons (PV-IN) and behavioral phenotypes relevant to schizophrenia.[51-53] Despite substantial glutathione deficits, the mice are outwardly healthy. Additionally, rare deficiencies of glutathione cycle enzymes (gamma-glutamylcysteine ligase, glutathione synthetase, 5-oxoprolinase, and gamma-glutamyl transferase) have all been associated with neuropsychiatric and cognitive impairments, although detailed phenomenological characterization has not been reported.[9, 54, 55] A role for glutathione as a glutamate reservoir may be a bridge between distinct lines of research that implicate glutamatergic

dysfunction and aberrant glutathione levels in neuropsychiatric conditions such as schizophrenia.

We suggest that two drugs available for human use, sulforaphane, which increases glutathione, and pyroglutamate, which is converted to glutamate in the glutathione cycle, may be therapeutically beneficial. Sulforaphane[56] is a potent inducer of the Nrf2 transcription factor, has excellent blood brain barrier penetration,[57] and might expand the size of the glutathione reservoir by increasing expression of GCL, the rate liming step in glutathione biogenesis. Sulforaphane has also been reported to improve symptoms of autistic spectrum disorder.[58] Pyroglutamate[59] is a glutamate precursor whose CSF concentration is 120 uM,[60] rivaling the extracellular glutamine concentration of 400 uM (basal glutamate is 2-3 uM). Oral administration of pyroglutamate has been found to benefit age-associated memory impairment,[61] alcoholic encephalopathy,[62] and delirium induced by anticholinergic medication.[63] Pyroglutamate may be a promising therapeutic candidate for cognitive dysfunction in schizophrenia and other conditions with glutathione disturbances.

FIGURE LEGENDS

Figure 1 | Blocking efflux of glutamate from the glutathione cycle decreases mEPSC. (a) Diagram of the glutathione cycle and inhibition of gamma-glutamyl transferase (GGT) by acivicin, which is upstream of the liberation of glutamate. Details appear in Supplementary Figures 1 and 2. (b) Representative mEPSC traces in primary cortical neurons treated 24 h with vehicle, 25 uM acivicin and/or 5 uM pyroglutamate (PGA). Acivicin decreased mEPSC frequency which can be recovered by pyroglutamate. (c) Distribution of mEPSC frequency in cortical neurons treated with acivicin. (d) Cumulative probability plots of mEPSC frequency. Acivicin, which decreases efflux of glutamate from the glutathione cycle, decreased mEPSC frequency, a reflection of presynaptic drive. Pyroglutamate restores glutamate, and presynaptic drive (see also Supplementary Figure 2). #, $p<0.01$, ‡, $p<0.0001$ by Steel-Dwass All Pairs test.

Figure 2 | Efflux of glutamate from the glutathione cycle can increase mEPSC. (a) Diagram of the glutathione cycle and inhibition of glutamate cysteine ligase (GCL) by buthionine sulfoximine (BSO), which shuttles free glutamate into glutathione. Details appear in Supplementary Figures 1 and 4. (b) Representative mEPSC traces in primary cortical neurons treated 24 h with vehicle or 200 uM BSO. (c) Distribution of mEPSC frequency in cortical neurons treated with BSO. (d) Cumulative probability plots of mEPSC frequency.

BSO which increases efflux of glutamate from the glutathione cycle, increased mEPSC frequency, a reflection of presynaptic drive (see also Supplementary Figure 4). ‡, $p<0.0001$ by Steel-Dwass All Pairs test.

Figure 3 | The glutathione cycle supports excitatory transmission, but to a smaller degree than glutamine derived glutamate. (a) Scheme of glutamate-glutamine cycling and blockade of system A glutamine transporters by MeAiB. Details appear in Supplementary Fig 6. (b) Representative traces of mEPSC recordings in primary cortical neurons treated with 25 uM acivicin (24 h) and/or 25 mM MeAiB (2 h). (c) Distribution of mEPSC frequency in cortical neurons treated with acivicin and/or MeAiB. (d) Cumulative probability plots of mEPSC frequency. MeAiB decreased mEPSC frequency to a greater degree than acivicin, with both treatments having additive effects for presynaptic drive frequency. ‡, $p<0.0001$ by Steel-Dwass All Pairs test.

Figure 4 | The glutathione cycle can rescue restrictions of glutamine derived glutamate.

(a) Scheme of glutamate-glutamine cycling and blockade of system A glutamine transporters by MeAiB. Details appear in Supplementary Fig 6. (b) Representative traces of mEPSC recordings in primary cortical neurons treated with 25 uM BSO (24 h) and/or 25 mM MeAiB

(2 h). (c) Distribution of mEPSC frequency in cortical neurons treated with BSO and/or MeAiB. (d) Cumulative probability plots of mEPSC frequency. MeAiB, which restricts glutamine derived glutamate, inhibits mEPSC frequency (blue). Decreases in mEPSC frequency induced by were rescued by pre-treatment with BSO (green), which increased mEPSC frequency (red). ‡, $p<0.0001$ by Steel-Dwass All Pairs test.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Reis HJ, Guatimosim C, Paquet M, Santos M, Ribeiro FM, Kummer A *et al.* Neuro-transmitters in the central nervous system & their implication in learning and memory processes. *Curr Med Chem* 2009; **16**(7)**:** 796-840.

2. Kalivas PW. The glutamate homeostasis hypothesis of addiction. *Nat Rev Neurosci* 2009; **10**(8)**:** 561-572.

3. Mattson MP. Glutamate and neurotrophic factors in neuronal plasticity and disease. *Ann N Y Acad Sci* 2008; **1144:** 97-112.

4. Forman HJ, Zhang H, Rinna A. Glutathione: Overview of its protective roles, measurement, and biosynthesis. *Molecular Aspects of Medicine* 2009; **30**(1-2)**:** 1-12.

5. Aoyama K, Watabe M, Nakaki T. Regulation of Neuronal Glutathione Synthesis. *Journal of Pharmacological Sciences* 2008; **108**(3)**:** 227-238.

6. Janáky R, Cruz-Aguado R, Oja SS, Shaw CA. Glutathione in the Nervous System: Roles in Neural Function and Health and Implications for Neurological Disease. In: Lajtha A, Oja SS, Schousboe A, Saransaari P (eds). *Handbook of Neurochemistry and Molecular Neurobiology*. Springer US2007, pp 347-399.

7. Dickinson DA, Forman HJ. Glutathione in defense and signaling: lessons from a small thiol. *Ann N Y Acad Sci* 2002; **973:** 488-504.

8. Hayes JD, Flanagan JU, Jowsey IR. Glutathione transferases. *Annu Rev Pharmacol Toxicol* 2005; **45:** 51-88.

9. Ballatori N, Krance SM, Notenboom S, Shi S, Tieu K, Hammond CL. Glutathione dysregulation and the etiology and progression of human diseases. *Biological Chemistry* 2009; **390**(3)**:** 191-214.

10. Chinta SJ, Kumar MJ, Hsu M, Rajagopalan S, Kaur D, Rane A *et al.* Inducible Alterations of Glutathione Levels in Adult Dopaminergic Midbrain Neurons Result in Nigrostriatal Degeneration. *Journal of Neuroscience* 2007; **27**(51)**:** 13997-14006.

11. Meister A. Selective modification of glutathione metabolism. *Science* 1983; **220**(4596)**:** 472-477.

12. Hamberger AC, Chiang GH, Nylen ES, Scheff SW, Cotman CW. Glutamate as a CNS transmitter. I. Evaluation of glucose and glutamine as precursors for the synthesis of preferentially released glutamate. *Brain Res* 1979; **168**(3)**:** 513-530.

13. Thanki CM, Sugden D, Thomas AJ, Bradford HF. In Vivo Release from Cerebral Cortex of [14C]Glutamate Synthesized from [U-14C]Glutamine. *Journal of Neurochemistry* 1983; **41**(3)**:** 611-617.

14. Kanai Y, Hediger M. The glutamate/neutral amino acid transporter family SLC1: molecular, physiological and pharmacological aspects. *Pflugers Archiv European Journal of Physiology* 2004; **447**(5)**:** 469-479.

15. Kam K, Nicoll R. Excitatory Synaptic Transmission Persists Independently of the Glutamate Glutamine Cycle. *Journal of Neuroscience* 2007; **27**(34)**:** 9192-9200.

16. Koga M, Serritella AV, Messmer MM, Hayashi-Takagi A, Hester LD, Snyder SH *et al.* Glutathione is a physiologic reservoir of neuronal glutamate. *Biochemical and Biophysical Research Communications* 2011; **409**(4)**:** 596-602.

17. Hayashi-Takagi A, Takaki M, Graziane N, Seshadri S, Murdoch H, Dunlop AJ *et al.* Disrupted-in-Schizophrenia 1 (DISC1) regulates spines of the glutamate synapse via Rac1. *Nat Neurosci* 2010; **13**(3)**:** 327-332.

18. Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: Applications to mammalian blood and other tissues. *Analytical Biochemistry* 1969; **27**(3)**:** 502-522.

19. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72:** 248-254.

20. Kusakabe H, Midorikawa Y, Fujishima T, Kuninaka A, Yoshino H. Purification and properties of a new enzyme, L-glutamate oxidase, from Streptomyces sp X-119-6 grown on wheat bran. *Agricultural and Biological Chemistry* 1983; **47**(6)**:** 1323-1328.

21. Mao T, O'Connor DH, Scheuss V, Nakai J, Svoboda K. Characterization and subcellular targeting of GCaMP-type genetically-encoded calcium indicators. *PLoS One* 2008; **3**(3)**:** e1796.

22. Porcher C, Hatchett C, Longbottom RE, McAinch K, Sihra TS, Moss SJ *et al.* Positive feedback regulation between gamma-aminobutyric acid type A (GABA(A)) receptor signaling and brain-derived neurotrophic factor (BDNF) release in developing neurons. *The Journal of biological chemistry* 2011; **286**(24)**:** 21667-21677.

23. Tian L, Hires SA, Mao T, Huber D, Chiappe ME, Chalasani SH *et al.* Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. *Nature methods* 2009; **6**(12)**:** 875-881.

24. Akerboom J, Chen TW, Wardill TJ, Tian L, Marvin JS, Mutlu S *et al.* Optimization of a GCaMP calcium indicator for neural activity imaging. *J Neurosci* 2012; **32**(40)**:** 13819-13840.

25. Rahman I, Kode A, Biswas SK. Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. *Nat Protocols* 2007; **1**(6)**:** 3159-3165.

26. Chapman J, Zhou M. Microplate-based fluorometric methods for the enzymatic determination of L-glutamate: application in measuring L-glutamate in food samples. *Analytica Chimica Acta* 1999; **402**(1-2)**:** 47-52.

27. Dinkova-Kostova AT, Abramov AY. The emerging role of Nrf2 in mitochondrial function. *Free Radic Biol Med* 2015; **88**(Pt B)**:** 179-188.

28. Reimer RJ, Chaudhry FA, Gray AT, Edwards RH. Amino acid transport system A resembles system N in sequence but differs in mechanism. *Proceedings of the National Academy of Sciences of the United States of America* 2000; **97**(14)**:** 7715-7720.

29. Rector FC, Jr., Seldin DW, Copenhaver JH. The mechanism of ammonia excretion during ammonium chloride acidosis. *The Journal of clinical investigation* 1955; **34**(1)**:** 20-26.

30. Masson J, Darmon M, Conjard A, Chuhma N, Ropert N, Thoby-Brisson M *et al.* Mice Lacking Brain/Kidney Phosphate-Activated Glutaminase Have Impaired Glutamatergic Synaptic Transmission, Altered Breathing, Disorganized Goal-Directed Behavior and Die Shortly after Birth. *The Journal of Neuroscience* 2006; **26**(17)**:** 4660-4671.

31. Baranano DE, Rao M, Ferris CD, Snyder SH. Biliverdin reductase: a major physiologic cytoprotectant. *Proceedings of the National Academy of Sciences of the United States of America* 2002; **99**(25)**:** 16093-16098.

32. Takamori S. VGLUTs: 'Exciting' times for glutamatergic research? *Neuroscience Research* 2006; **55**(4)**:** 343-351.

33. Liguz-Lecznar M, Skangiel-Kramska J. Vesicular glutamate transporters (VGLUTs): the three musketeers of glutamatergic system. *Acta Neurobiol Exp (Wars)* 2007; **67**(3)**:** 207-218.

34. Otis TS. Vesicular Glutamate Transporters In Cognito. *Neuron* 2001; **29**(1)**:** 11-14.

35. Pozo K, Goda Y. Unraveling Mechanisms of Homeostatic Synaptic Plasticity. *Neuron* 2010; **66**(3)**:** 337-351.

36. Ishikawa T, Sahara Y, Takahashi T. A Single Packet of Transmitter Does Not Saturate Postsynaptic Glutamate Receptors. *Neuron* 2002; **34**(4)**:** 613-621.

37. Yamashita T, Ishikawa T, Takahashi T. Developmental Increase in Vesicular Glutamate Content Does Not Cause Saturation of AMPA Receptors at the Calyx of Held Synapse. *Journal of Neuroscience* 2003; **23**(9)**:** 3633-3638.

38. Cai K, Haris M, Singh A, Kogan F, Greenberg JH, Hariharan H *et al.* Magnetic resonance imaging of glutamate. *Nature Medicine* 2012; **18**(2)**:** 302-306.

39. Rae CD. A guide to the metabolic pathways and function of metabolites observed in human brain 1H magnetic resonance spectra. *Neurochem Res* 2014; **39**(1)**:** 1-36.

40. Shupliakov O, Atwood HL, Ottersen OP, Storm-Mathisen J, Brodin L. Presynaptic glutamate levels in tonic and phasic motor axons correlate with properties of synaptic release. *Journal of Neuroscience* 1995; **15**(11)**:** 7168-7180.

41. Slivka A, Mytilineou C, Cohen G. Histochemical evaluation of glutathione in brain. *Brain Research* 1987; **409**(2)**:** 275-284.

42. Akbarian S, Sucher NJ, Bradley D, Tafazzoli A, Trinh D, Hetrick WP *et al.* Selective alterations in gene expression for NMDA receptor subunits in prefrontal cortex of schizophrenics. *J Neurosci* 1996; **16**(1)**:** 19-30.

43. Gao XM, Sakai K, Roberts RC, Conley RR, Dean B, Tamminga CA. Ionotropic glutamate receptors and expression of N-methyl-D-aspartate receptor subunits in

subregions of human hippocampus: effects of schizophrenia. *Am J Psychiatry* 2000; **157**(7)**:** 1141-1149.

44. Moghaddam B, Adams B, Verma A, Daly D. Activation of glutamatergic neurotransmission by ketamine: a novel step in the pathway from NMDA receptor blockade to dopaminergic and cognitive disruptions associated with the prefrontal cortex. *J Neurosci* 1997; **17**(8)**:** 2921-2927.

45. Tsai G, Passani LA, Slusher BS, Carter R, Baer L, Kleinman JE *et al.* Abnormal excitatory neurotransmitter metabolism in schizophrenic brains. *Arch Gen Psychiatry* 1995; **52**(10)**:** 829-836.

46. Kantrowitz JT, Javitt DC. N-methyl-d-aspartate (NMDA) receptor dysfunction or dysregulation: The final common pathway on the road to schizophrenia? *Brain Research Bulletin* 2010; **83**(3-4)**:** 108-121.

47. Do KQ, Cabungcal JH, Frank A, Steullet P, Cuenod M. Redox dysregulation, neurodevelopment, and schizophrenia. *Current Opinion in Neurobiology* 2009; **19**(2)**:** 220-230.

48. Do KQ, Trabesinger AH, Kirsten-Kruger M, Lauer CJ, Dydak U, Hell D *et al.* Schizophrenia: glutathione deficit in cerebrospinal fluid and prefrontal cortex in vivo. *Eur J Neurosci* 2000; **12**(10)**:** 3721-3728.

49. Matsuzawa D, Obata T, Shirayama Y, Nonaka H, Kanazawa Y, Yoshitome E *et al.* Negative correlation between brain glutathione level and negative symptoms in schizophrenia: a 3T 1H-MRS study. *PLoS One* 2008; **3**(4)**:** e1944.

50. Yao JK, Leonard S, Reddy R. Altered glutathione redox state in schizophrenia. *Dis Markers* 2006; **22**(1-2)**:** 83-93.

51. Steullet P, Cabungcal J-H, Kulak A, Kraftsik R, Chen Y, Dalton TP *et al.* Redox Dysregulation Affects the Ventral But Not Dorsal Hippocampus: Impairment of

Parvalbumin Neurons, Gamma Oscillations, and Related Behaviors. *Journal of Neuroscience* 2010; **30**(7)**:** 2547-2558.

52. Kulak A, Cuenod M, Do KQ. Behavioral phenotyping of glutathione-deficient mice: relevance to schizophrenia and bipolar disorder. *Behav Brain Res* 2012; **226**(2)**:** 563-570.

53. Duarte JM, Kulak A, Gholam-Razaee MM, Cuenod M, Gruetter R, Do KQ. N-Acetylcysteine Normalizes Neurochemical Changes in the Glutathione-Deficient Schizophrenia Mouse Model During Development. *Biol Psychiatry* 2011**:** (in press).

54. Ristoff E, Larsson A. Patients with genetic defects in the gamma-glutamyl cycle. *Chemico-Biological Interactions* 1998; **111-112:** 113-121.

55. Njalsson R, Ristoff E, Carlsson K, Winkler A, Larsson A, Norgren S. Genotype, enzyme activity, glutathione level, and clinical phenotype in patients with glutathione synthetase deficiency. *Human Genetics* 2005; **116**(5)**:** 384-389-389.

56. Keum YS. Regulation of the Keap1/Nrf2 system by chemopreventive sulforaphane: implications of posttranslational modifications. *Ann N Y Acad Sci* 2011; **1229:** 184-189.

57. Zhao J, Kobori N, Aronowski J, Dash PK. Sulforaphane reduces infarct volume following focal cerebral ischemia in rodents. *Neuroscience Letters* 2006; **393**(2â€“3)**:** 108-112.

58. Singh K, Connors SL, Macklin EA, Smith KD, Fahey JW, Talalay P *et al.* Sulforaphane treatment of autism spectrum disorder (ASD). *Proceedings of the National Academy of Sciences* 2014; **111**(43)**:** 15550-15555.

59. Kumar A, Bachhawat AK. Pyroglutamic acid: throwing light on a lightly studied metabolite. *Curr Science* 2012; **102**(2)**:** 288-297.

60. Eckstein JA, Ammerman GM, Reveles JM, Ackermann BL. Analysis of glutamine, glutamate, pyroglutamate, and GABA in cerebrospinal fluid using ion pairing HPLC with positive electrospray LC/MS/MS. *J Neurosci Methods* 2008; **171**(2)**:** 190-196.

61. Grioli S, Lomeo C, Quattropani MC, Spignoli G, Villardita C. Pyroglutamic acid improves the age associated memory impairment. *Fundamental and Clinical Pharmacology* 1990; **4**(2)**:** 169-173.

62. Sinforiani E, Trucco M, Cavallini A, Gualtieri S, Verri AP, Spignoli G. Reversibility of cognitive disorders among chronic alcoholics in phases of withdrawal. Effect of arginine pyroglutamate. *Minerva Psichiatr* 1985; **26**(4)**:** 339-346.

63. Blin O, Audebert C, Pitel Sv, Kaladjian A, Casse-Perrot C, Zaim M *et al.* Effects of dimethylaminoethanol pyroglutamate (DMAE p-Glu) against memory deficits induced by scopolamine: evidence from preclinical and clinical studies. *Psychopharmacology* 2009; **207**(2)**:** 201-212.

Figure 1

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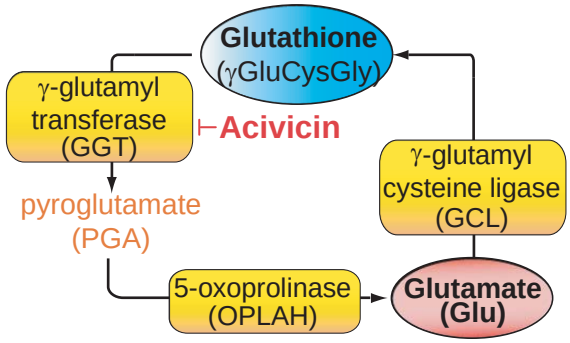

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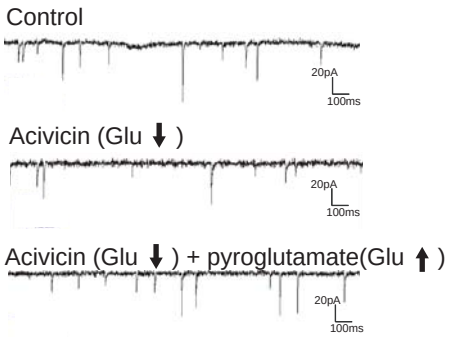

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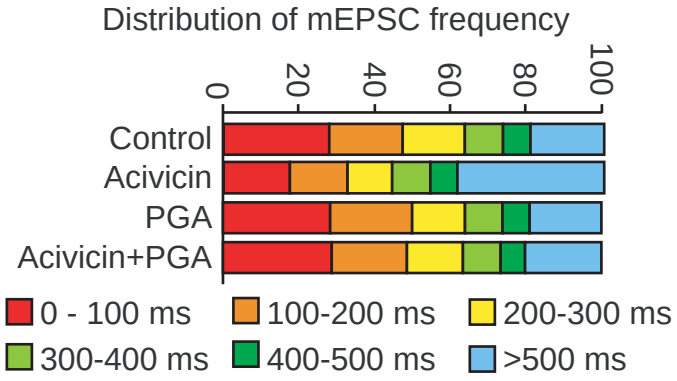

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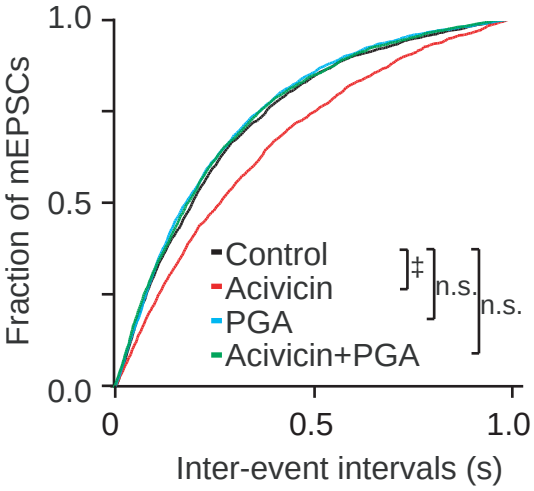

Figure 2

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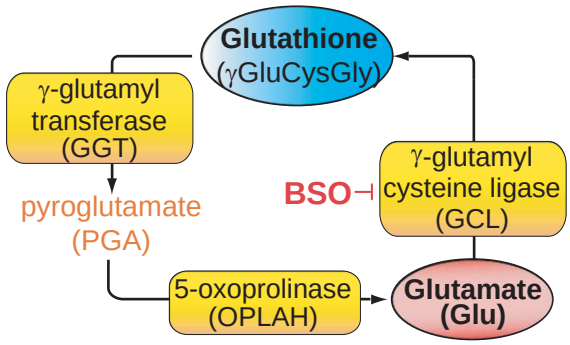

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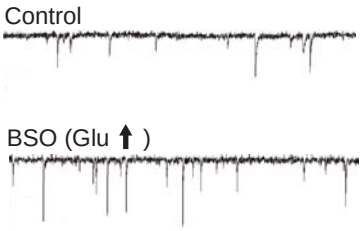

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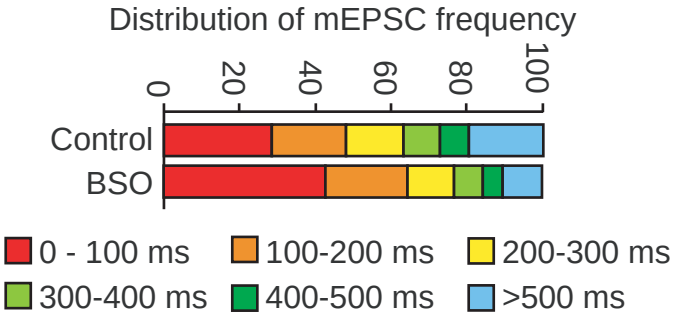

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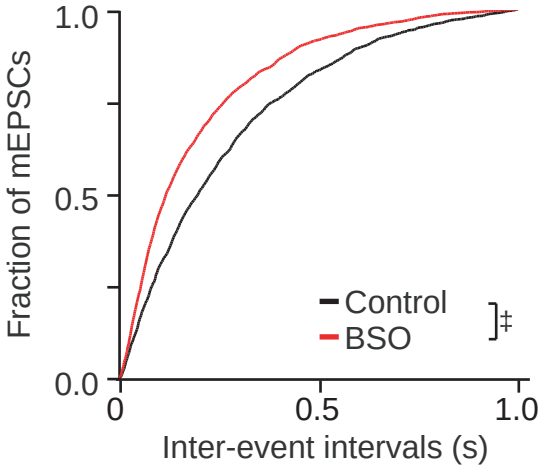

Figure 3

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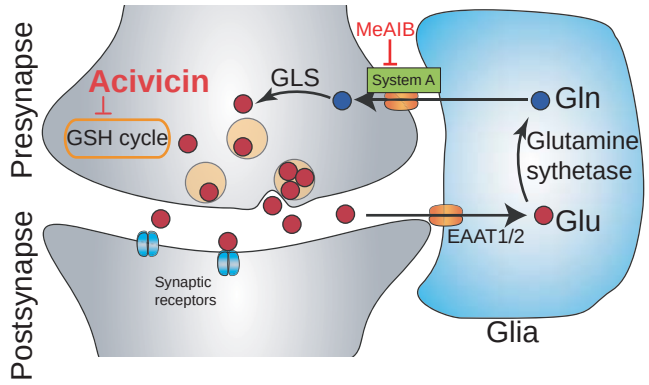

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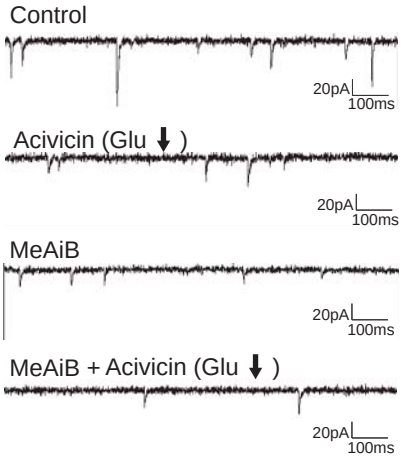

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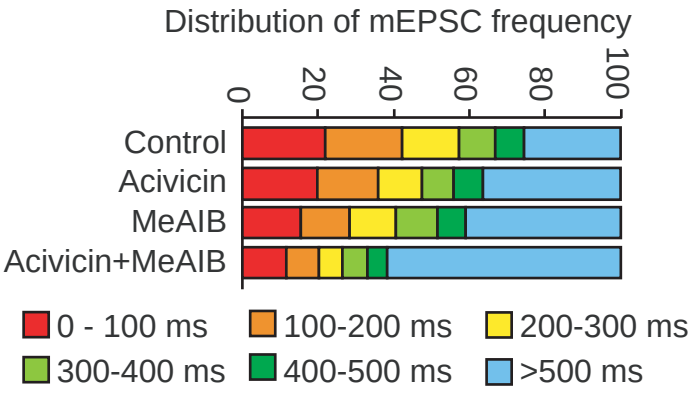

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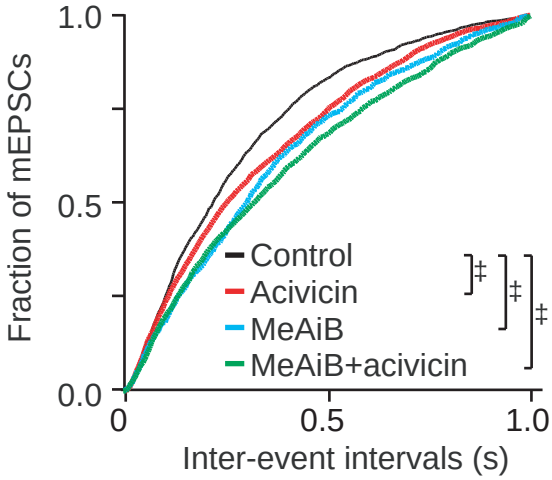

Figure 4

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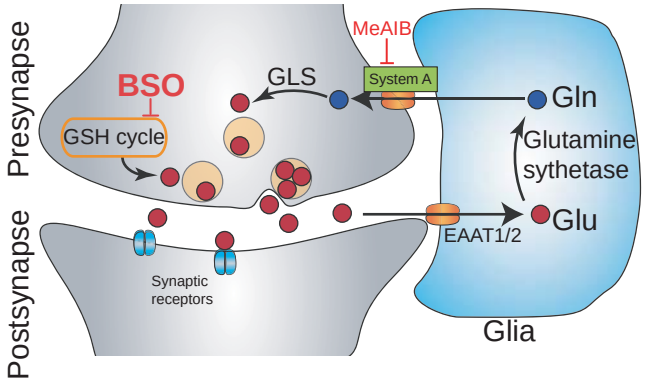

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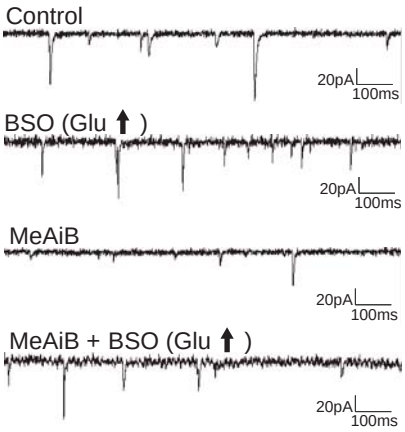

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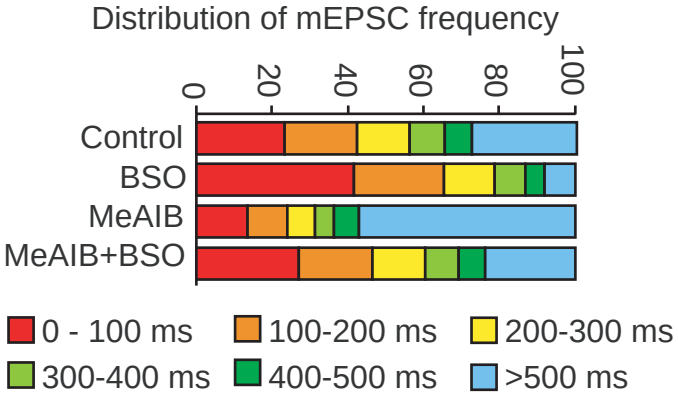

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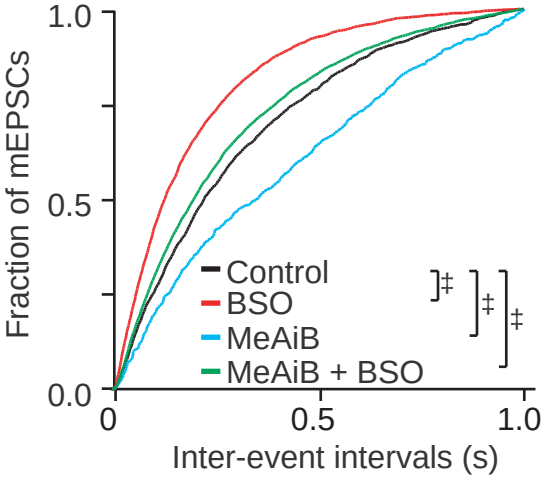


Supplementary Figure Legends

Supplementary Figure 1 | 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.

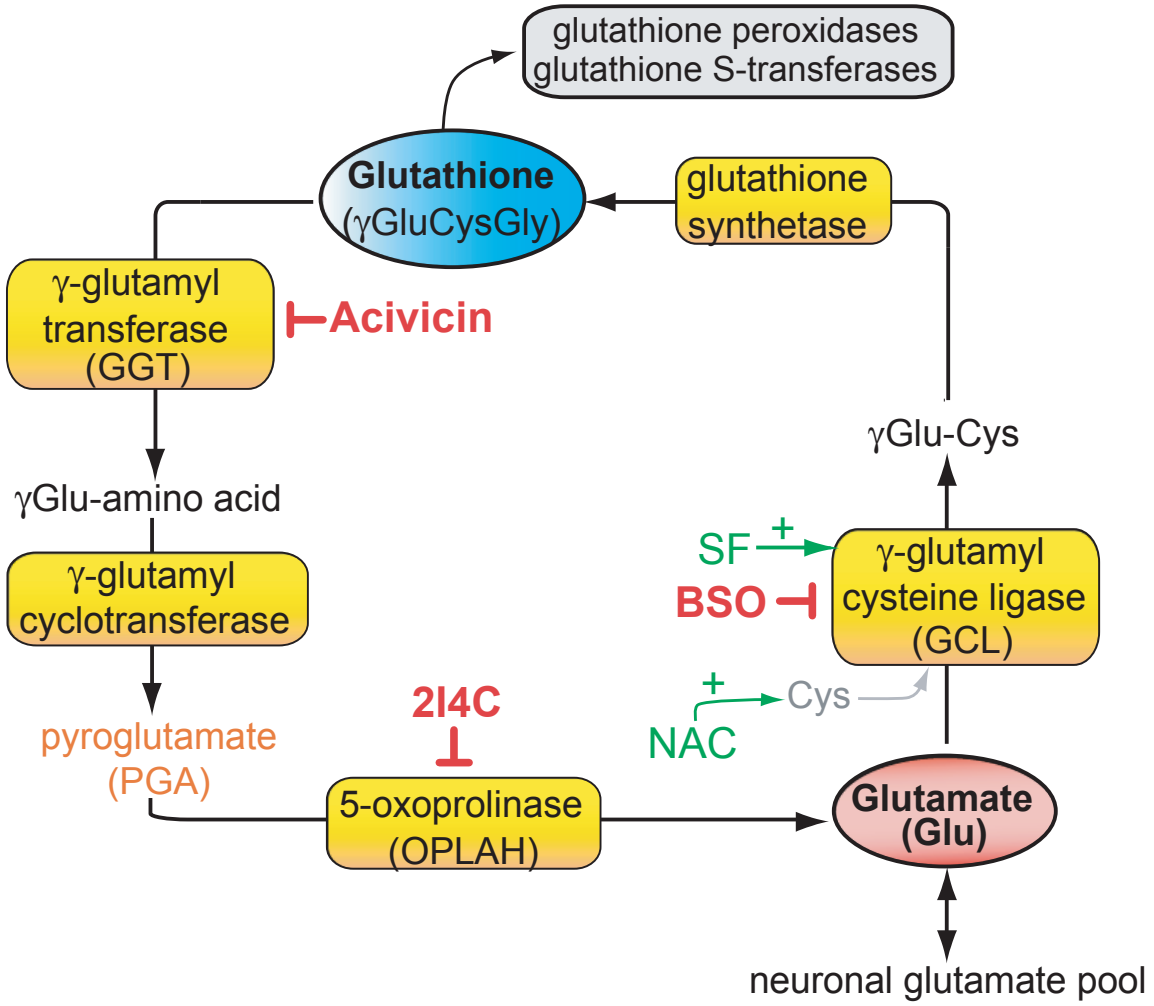
Supplementary Figure 2 | Inhibition of ¥-glutamyltransferase diminishes glutamate levels.
(a,b) Acivicin treatment of cortical neurons (25 uM, 24 h) decreases glutamate and glutathione levels. Co-administration of 5 uM pyroglutamate (PGA) selectively rescues the glutamate decrease. (c) shRNA targeting 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.

Supplementary Figure 3 | 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 uM 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 uM 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.

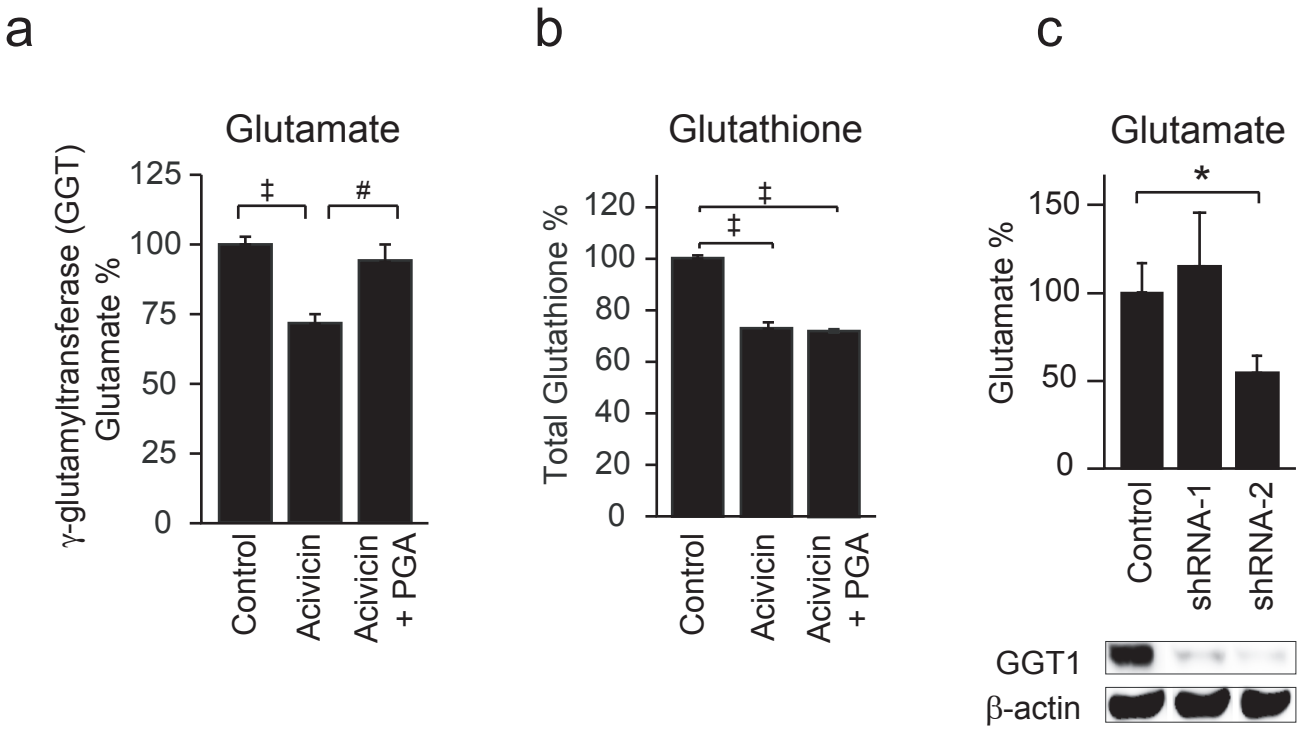
Supplementary Figure 4 | Glutamate flux into and out of the glutathione cycle impacts depolarization induced calcium flux.
(a) BSO, which decreases glutamate utilization by glutathione, augments depolarization associated calcium fluxes. (b) Acivicin which decreases glutamate liberation from the glutathione cycle, decreases depolarization associated calcium flux. Cortical neurons transfected with calcium indicator, GCaMP3, were depolarized with 100 mM KCl. Data represent average dF/F0 (thick lines) ± SE (N=3 per group for acivicin, N=5 per group for BSO). *, $p<0.05$ by 1-way ANOVA with Tukey-Kramer posthoc test.

Supplementary Figure 5 | 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 glutamine transporters that import glutamine.

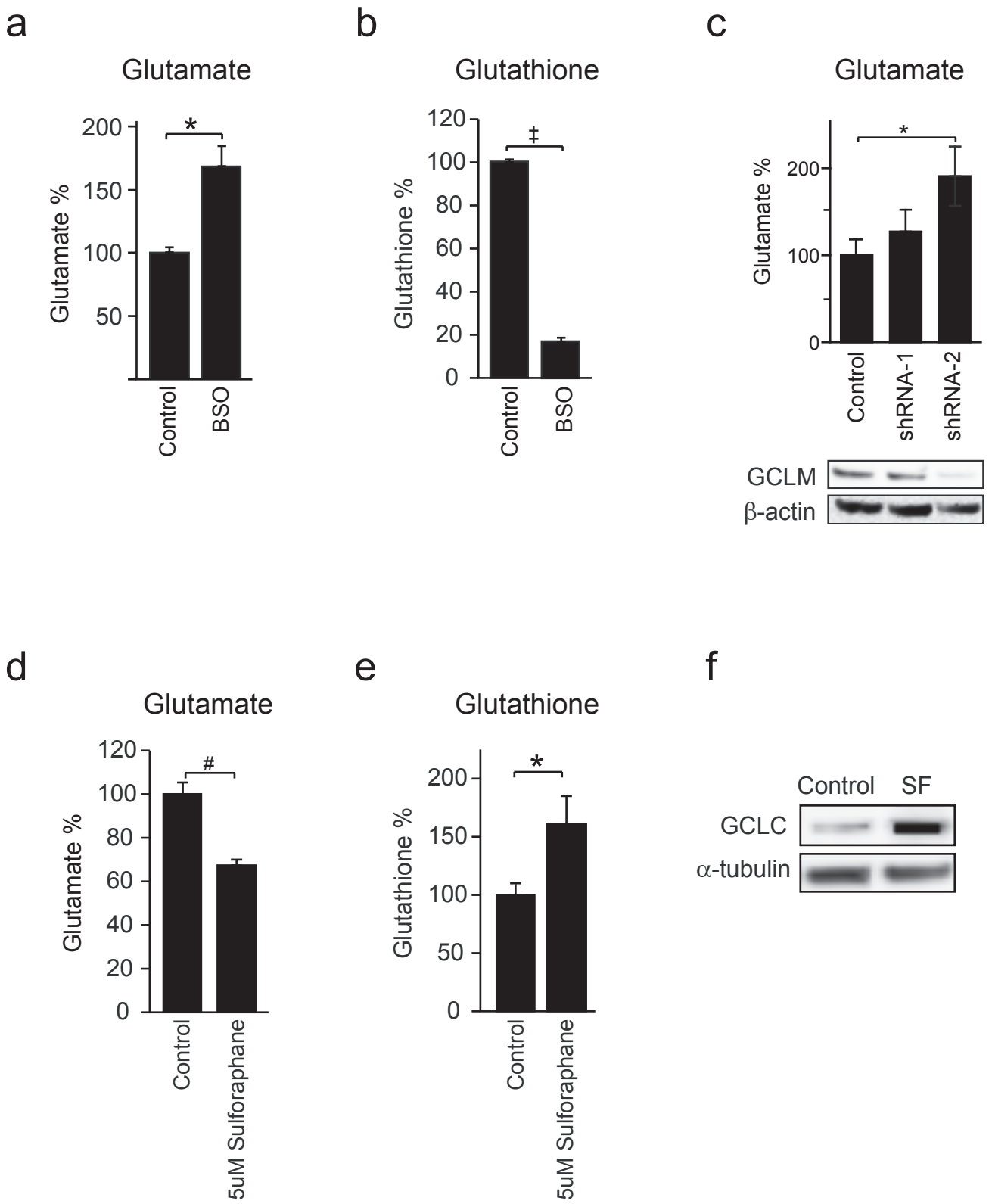
Supplementary Figure 1



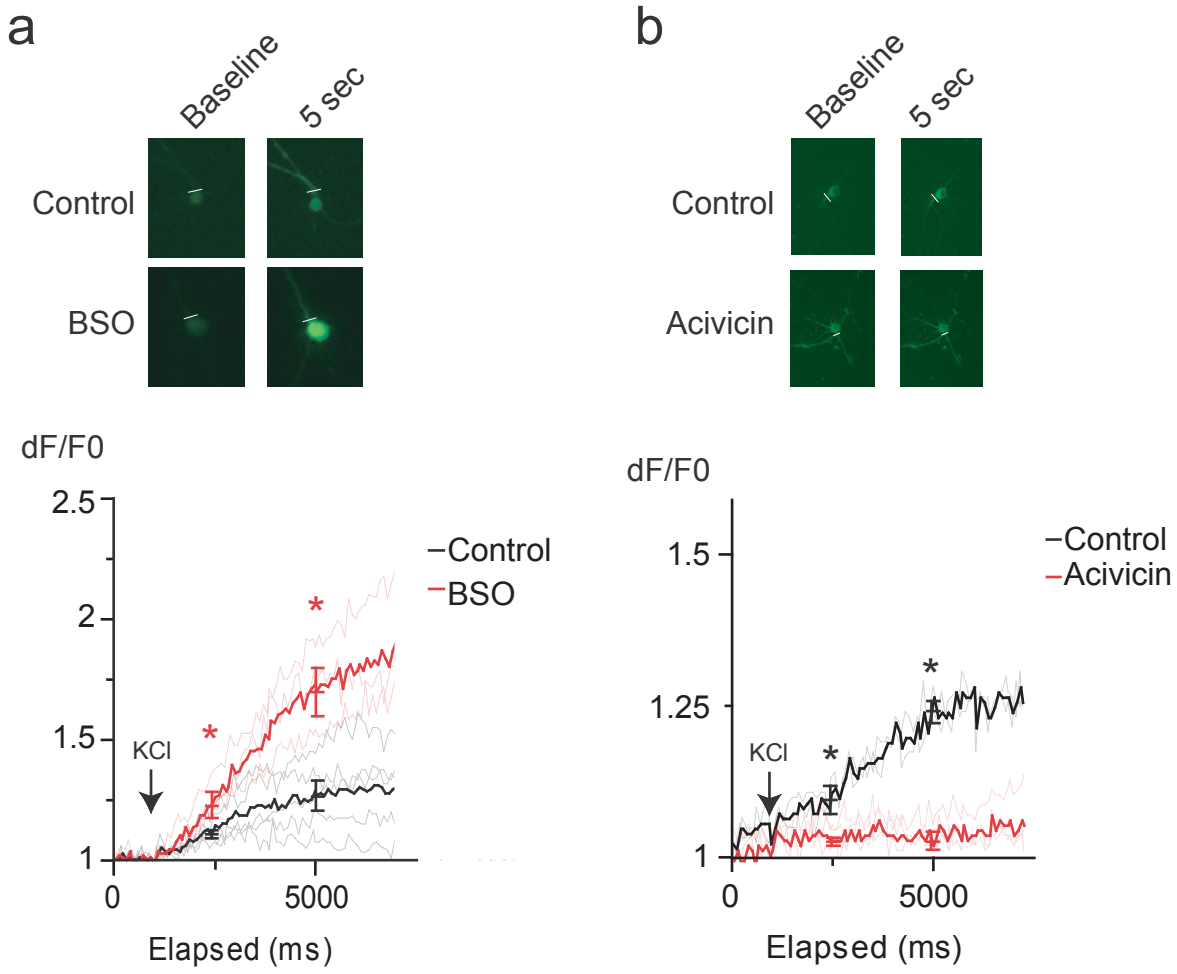
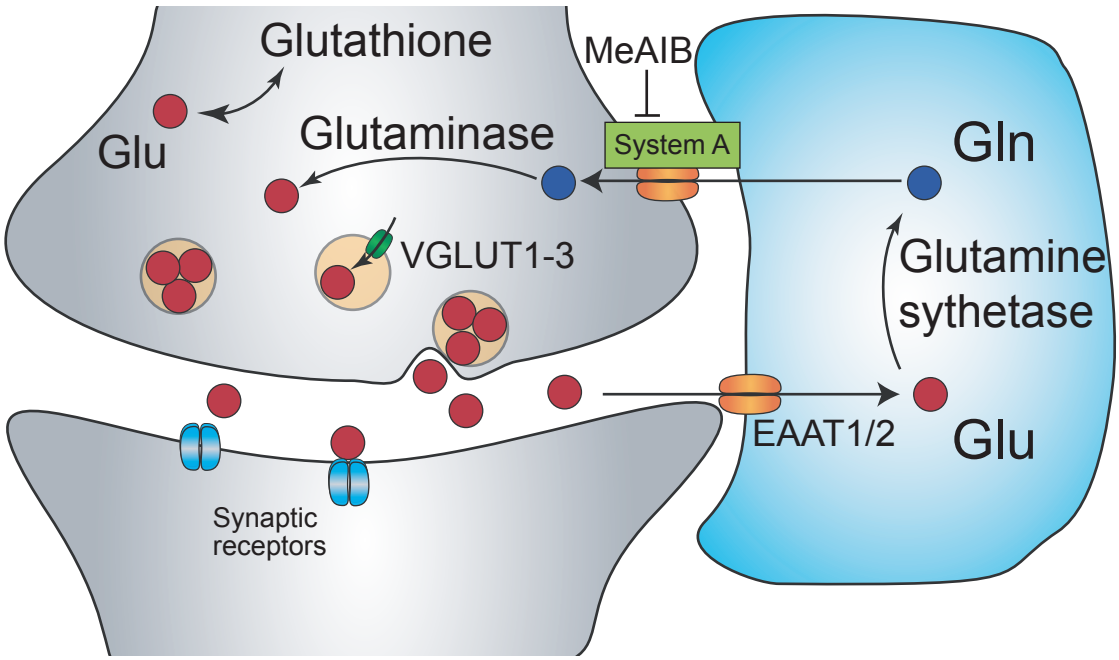
Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5