Activation of proline biosynthesis is critical to maintain glutamate homeostasis during acute methamphetamine exposure

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Methamphetamine (METH) is a highly addictive psychostimulant that causes long-lasting effects in the brain and increases the risk of developing neurodegenerative diseases. The cellular and molecular effects of METH in the brain are functionally linked to alterations in glutamate levels. Despite the well-documented effects of METH on glutamate neurotransmission, the underlying mechanism by which METH alters glutamate levels is not clearly understood. In this study, we report an essential role of proline biosynthesis in maintaining METH-induced glutamate homeostasis. We observed that acute METH exposure resulted in the induction of proline biosynthetic enzymes in both undifferentiated and differentiated neuronal cells. Proline level was also increased in these cells after METH exposure. Surprisingly, METH treatment did not increase glutamate levels nor caused neuronal excitotoxicity. However, METH exposure resulted in a significant upregulation of pyrroline-5-carboxylate synthase (P5CS), the key enzyme that catalyzes synthesis of proline from glutamate. Interestingly, depletion of P5CS by CRISPR/Cas9 resulted in a significant increase in glutamate levels upon METH exposure. METH exposure also increased glutamate levels in P5CS-deficient proline-auxotropic cells. Conversely, restoration of P5CS expression in P5CS-deficient cells abrogated the effect of METH on glutamate levels. Consistent with these findings, P5CS expression was significantly enhanced in the cortical brain region of mice administered with METH and in the slices of cortical brain tissues treated with METH. Collectively, these results uncover a key role of P5CS for the molecular effects of METH and highlight that excess glutamate can be sequestered for proline biosynthesis as a protective mechanism to maintain glutamate homeostasis during drug exposure.

Methamphetamine (METH) is a powerful and highly addictive psychostimulant1. It belongs to a larger group of drugs called Amphetamine-Type Stimulants (ATS)1–3. Use of ATS generate a sense of euphoria, increase energy and concentration as well as decrease appetite, induce weight loss, leading to a variety of emotional, cognitive, and physical effects4,5. Among the ATS, METH has the greatest potential for abuse because of easy availability, low cost, and longer duration of action1–3. Therefore, METH use and abuse continues to be a significant public health problem in the United States and all over the world1–3.

METH is a lipophilic compound that crosses the blood–brain barrier easily to cause long lasting effects in the brain4,5. Since METH’s chemical structure is similar to monoamines, it targets both dopaminergic (DA) and...
serotonergic (5HT) neurons in the brain. METH enters these neurons by binding to the membrane-bound DA transporter (DAT) and/or 5HT transporter. There is also evidence that METH can cross neuronal membranes by passive diffusion mechanism. Once inside the neuron, METH can alter the function of the vesicular monoamine transporter 2 (VMAT-2) and affect cytoplasmic monoamine concentrations and DA release. METH administration is also known to increase DAT internalization and affect 5HT transporter functionality. In addition, METH prevents degradation of neurotransmitters, reduces neurotransmitter reuptake, and causes an efflux of DAT and 5HT transporters. These biochemical and cellular effects result in an increased neurotransmitter availability in the synapse and cause continuous stimulation of neurons to manifest the euphoric effects among METH users.

In addition to its effects on DA and 5HT neurons, METH has been shown to affect glutamate neurotransmission. There is evidence that METH’s effect on glutamate neurotransmission is mediated by increasing levels of extracellular glutamate. However, the mechanism of glutamate homeostasis during METH exposure has not been clearly delineated. Glutamate is the principal excitatory neurotransmitter and is ubiquitously distributed in the brain. Due to its critical role in neuronal plasticity, glutamate is involved in a number of critical brain functions including learning and memory. It is important to note that glutamate is produced in the nerve terminals predominantly from two sources: (1) the tricarboxylic acid (TCA) cycle, and (2) glutamine produced by the glial cells. Glutamate produced in the nerve terminals is packaged into vesicles by the vesicular glutamate transporters (vGLUT) and is released to the extracellular space upon stimulus of an action potential. After release, the glial cells clear glutamate from the synapse via the glutamate transporters, GLAST and GLT-1, to maintain sub-neurotoxic levels of extracellular glutamate. Maintaining glutamate homeostasis is critical for proper neuronal function since excessive glutamate has been implicated in neuronal excitotoxicity.

The link between glutamate metabolism and carbohydrate/amino acid metabolism has been well established. Specifically, glutamate is channeled to the TCA cycle—a common pathway in carbohydrate and amino acid metabolism. However, the metabolic link between glutamate and proline remains largely understudied. Proline, unlike other amino acids, has its α-amino group within a pyrrolidine ring, and thus is the sole proteinogenic secondary (imino) amino acid. Proline is also unique since it has its own set of metabolic enzymes. It is known that glutamate can be converted to proline through Δ1-pyrroline-5-carboxylate (P5C) by the enzymatic activity of P5C synthase (P5CS) and P5C reductase (PYCR). Conversely, proline can also be converted to glutamate through the catabolic pathway catalyzed by proline oxidase/dehydrogenase (POX/PRODH) and P5C dehydrogenase (P5CDH). Given this important metabolic link, proline metabolism has been reported to play key roles in normal brain function and various neurological disorders.

Proline is abundantly found in the central nervous system (CNS). The presence of high affinity proline transporters has also been reported in a subset of glutamatergic neurons in rodent brain. In animal models, elevated proline levels affect glutamatergic transmission, depolarize neurons, elevate synaptic activity, alter cognitive tasks, sensorimotor gating, and locomotor activity. In humans, hyperprolinemia—a condition associated with abnormally elevated levels of proline—has been linked to epilepsy, seizures, and impaired cognitive function. Hyperprolinemia is also linked to schizophrenia disorders and schizophrenia. Even though these studies and others demonstrate key roles of proline in brain function and neurological disorders, the role of proline metabolism during drug exposure remains largely unknown.

In this study, we report a functional link between proline and glutamate metabolism during acute METH exposure. First, we utilized the SH-SYSY neuronal cell model, that has been widely used to study neuronal biology. SH-SYSY cells are positive for tyrosine hydroxylase (TH) and dopamine-β-hydroxylase characteristic of catecholaminergic neurons that are known to be affected by METH. In this model, we tested the functional contribution of proline and glutamate metabolism in METH-exposure mediated alterations in glutamate levels. Interestingly, acute treatment with METH markedly induced the enzymes of the proline biosynthetic pathway. Specifically, a marked induction in the expression of P5CS and PYCRs was observed in METH treated neuronal cells. This induction was accompanied by a concomitant increase in intracellular proline levels. Surprisingly, METH exposure neither increased extracellular glutamate levels or caused neuronal excitotoxicity. However, blocking the proline synthetic arm by knocking-out P5CS increased both intracellular and extracellular glutamate levels in METH treated cells. Furthermore, treatment of proline auxotrophic-Chinese hamster ovary (CHO-K1) cells with METH significantly enhanced extracellular glutamate levels. These results suggested a functional link between proline biosynthesis and glutamate during METH exposure. Finally, levels of proline biosynthetic enzymes, specifically, P5CS and PYCR2 were also significantly induced in the cortex of animals administered with METH and in the slices of cortical brain tissues exposed to METH. Collectively, our studies demonstrate activation of proline biosynthesis to sequester increased glutamate during acute METH exposure and imply an essential role of proline metabolism in limiting neuronal glutamate excitotoxicity.

Results
Acute METH exposure activates the proline synthetic pathway. METH exposure affects glutamate neurotransmission primarily by altering glutamate levels. While, glutamine serves as the major source of glutamate, proline metabolism is also closely linked to glutamate. (Fig. 1A). However, the functional link between proline and glutamate during drug exposure-induced glutamate neurotransmission is poorly understood. Therefore, we examined the effects of METH exposure on proline metabolism using the SH-SYSY neuronal cell model. First, we measured the levels of enzymes of the proline catabolic arm—POX and P5CDH—which sequentially convert proline to P5C and then to glutamate. (Fig. 1A). We treated SH-SYSY cells with METH in a dose dependent manner using concentrations of 1 mM and lower. Although higher concentrations of METH have been used in published studies, we chose concentrations below 1 mM since higher levels of the drug are rarely achieved in METH associated disorders. Lysates of METH-treated and control cells were analyzed...
by immunoblot to measure the levels of POX and P5CDH. These analysis revealed that SH-SY5Y cells endogenously express all the key metabolic enzymes of proline (Fig. 1A). Interestingly, METH exposure minimally altered the expression of POX and P5CDH when compared to the untreated controls (Fig. 1B–D). Even at 1 mM concentration of METH, expression of these two enzymes were not significantly altered (Fig. 1B–D), suggesting that acute METH exposure minimally alters the proline catabolic arm that converts proline to glutamate (Fig. 1A).

We next measured the expression of enzymes of the proline biosynthetic arm (Fig. 1A), P5CS synthase (P5CS) and P5C reductase (PYCR) that convert glutamate to proline by sequential catalysis26. Notably, the expression of P5CS was significantly increased in cells exposed to METH (Fig. 1E,F). Densitometry analysis illustrated a dose-dependent increase in P5CS expression in cells treated with METH relative to untreated cells (Fig. 1F). Then, we measured the levels of PYCR1, and PYCR2, which are involved in biosynthesis of proline from glutamate (n = 3) (Fig. S1). (C,D) Densitometry analysis of POX and P5CDH, respectively normalized to β-actin. (E–H) Effect of acute METH treatment on proline metabolic enzymes. (E) Representative immunoblot of enzymes, P5CS, PYCR1 and PYCR2, which are essential for proline biosynthesis from glutamate (n = 3). (F–H) Densitometry analysis of P5CS, PYCR1, and PYCR2, respectively normalized to β-actin. Data presented in panels (C,D,F–H) are mean values of three independent experiments with error bars representing SEM. *p < 0.05, **p < 0.005 represent statistical comparison of untreated vs METH-treated cells.

**Figure 1.** Acute METH exposure upregulates enzymes of proline synthetic pathway from glutamate. (A) Schematic depicting the metabolic link between glutamate and proline. Synthesis of proline begins with the conversion of glutamine to glutamate by the enzyme glutaminase (GLS). Glutamate is then converted by P5C synthase (P5CS) to glutamic-γ-semialdehyde that spontaneously cycles to Δ1-pyrroline-5-carboxylate (P5C). P5C is subsequently catalyzed to L-proline by P5C-reductase (PYCR). Conversely, catabolism of proline to glutamate is catalyzed in two consecutive steps by proline oxidase (POX) and P5C dehydrogenase (P5CDH). Glutamate is finally converted to glutamine by glutamine synthase (GS). (B–D) Effect of acute METH treatment on proline catabolic enzymes. SH-SY5Y cells were treated with increasing concentrations of METH for 24 h. Post-treatment cells were harvested and lysed. Cell lysates were subjected to immunoblot analyses. (B) Representative immunoblot of enzymes involved in catabolism of proline to glutamate (n = 3) (Fig. S1). (C,D) Densitometry analysis of POX and P5CDH, respectively normalized to β-actin. (E–H) Effect of acute METH treatment on proline metabolic enzymes. (E) Representative immunoblot of enzymes, P5CS, PYCR1 and PYCR2, which are essential for proline biosynthesis from glutamate (n = 3). (F–H) Densitometry analysis of P5CS, PYCR1, and PYCR2, respectively normalized to β-actin. Data presented in panels (C,D,F–H) are mean values of three independent experiments with error bars representing SEM. *p < 0.05, **p < 0.005 represent statistical comparison of untreated vs METH-treated cells.

**METH exposure upregulates key proline metabolic enzymes in differentiated neuronal cells and cortical brain slices.** Our results in Fig. 1 showed upregulation of proline synthetic enzymes by METH exposure in undifferentiated SH-SY5Y cells. Even though the SH-SY5Y neuroblastoma cells show some characteristics of neurons, we have previously shown that these cells acquire a neuronal phenotype by differentiation utilizing ATRA50. Therefore, we used differentiated SH-SY5Y cells and treated them with METH in a dose dependent manner. 24 h post treatment cellular extracts were prepared to measure the expression of P5CS and
Figure 2. METH exposure upregulates P5CS and PYCR2 in differentiated neuronal cells and slices of cortical brain. (A–C) Differentiated SH-SY5Y cells were treated with increasing concentrations of METH for 24 h. Post-treatment cellular lysates were subjected to immunoblot analyses. (A) Representative immunoblot of P5CS and PYCR2 (n = 3). (B,C) Densitometry analysis of P5CS and PYCR2, respectively normalized to β-actin. (D–F) Slices of the frontal cortex were prepared from whole brain and were incubated in 200 µM METH (n = 4) or vehicle (n = 2). Tissue lysates were subjected to western blot analysis. (D) Representative immunoblot of P5CS and PYCR2 (n = 3). (E,F) Densitometry analysis of P5CS and PYCR2, respectively normalized to β-actin. (G–I) SH-SY5Y cells were treated with increasing concentrations of AMPH for 24 h. After treatment, cellular lysates were analyzed by immunoblot analyses. (G) Representative immunoblot of P5CS and PYCR2 (n = 3). (HI) Densitometry analysis of P5CS and PYCR2, respectively normalized to β-actin. (J–L) Differentiated SH-SY5Y cells were treated with increasing concentrations of AMPH for 24 h and the cellular lysates were analyzed by immunoblot. (J) Representative immunoblot of P5CS and PYCR2 (n = 3). (K,L) Densitometry analysis of P5CS and PYCR2, respectively normalized to β-actin. (M–O) Brain slices of the frontal cortex were prepared from whole brain and were incubated in 200 µM AMPH (n = 4) or vehicle (n = 2). Lysates of the cortical tissues were analyzed by immunoblot. (M) Representative immunoblot of P5CS and PYCR2 (n = 3). (N–O) Densitometry analysis of P5CS and PYCR2, respectively normalized to β-actin. Data in panels (B,C,E,H,I,K,L,N,O) are presented as the mean ± SEM of at least three independent experiments. *p < 0.05, **p < 0.005 represent statistical comparison of untreated/saline-treated vs METH-treatment.

PYCR2, as representatives of proline synthetic enzymes. Immunoblot analysis showed that METH treatment resulted in a significant upregulation of both P5CS and PYCR2 when compared to untreated controls (Fig. 2A–C). These observations are similar to the results obtained with the undifferentiated cells (Fig. 1). To further probe the physiological relevance of these cell-based observations, we carried out METH exposure experiments using slices of cortical regions of rodent brain. Given the technical challenges associated with brain slice experiments, 200 µM METH was selected based on the significant upregulation of both P5CS and PYCR2 in neuronal cells at this concentration (Figs. 1E–H, 2A–C). Brain slices were incubated in METH or vehicle (saline) for a 6-h period. Then cortical enriched tissue was isolated and immunoblot analysis was carried out of the tissue lysates. Data from these studies illustrated that P5CS and PYCR2 levels were significantly upregulated in the cortical brain slices upon METH exposure (Fig. 2D–F). Taken together, results in Fig. 1 and 2A–F establish that acute METH exposure upregulates the proline metabolic enzymes in neuronal cells and cortical brain slices.

Next, we determined whether activation of proline synthetic arm is specific to METH exposure or a general mechanism utilized by other ALS. To test this, we first treated undifferentiated SHSY-5Y cells with increasing concentration of Amphetamine (AMPH). Immunoblot analysis of cellular lysates showed a minimal but non-significant increase in P5CS and PYCR2 protein expression by AMPH exposure when compared to untreated controls (Fig. 2G–I). Interestingly, AMPH exposure of differentiated SH-SY5Y cells illustrated a modest increase in the expression of P5CS at 1000 µM METH. However, PYCR2 expression was minimally changed in the differentiated cells exposed to increasing concentrations of AMPH (Fig. 2J–L). Finally, exposure of brain slices to AMPH (200 µM) showed a lack of significant increase in the expression of both P5CS and PYCR2 (Fig. 2M–O). Collectively, results described in Fig. 2 strongly suggested that acute METH exposure activates the proline catabolic arm, whereas AMPH exposure lacks such activation.

Acute METH exposure enhances proline levels but does not increase glutamate levels. Results in Figs. 1 and 2 demonstrated that METH exposure upregulated the expression of P5CS and PYCR2. Since these enzymes catalyze the synthesis of proline from glutamate, we measured intracellular proline levels in METH treated SH-SY5Y cells. We chose 200, 500 and 1000 µM of METH for this experiment since both P5CS and PYCR2 were significantly upregulated at these drug concentrations (Figs. 1, 2). To quantify proline levels, we utilized the acid-ninhydrin assay that specifically detects proline. Results from this assay illustrate that the levels of intracellular proline were significantly elevated in METH treated cells when compared to the untreated control cells (Fig. 3A). Specifically, treatment of cells with 200 µM METH resulted in a ~100 µM increase in proline levels relative to the untreated cells. Similarly, treatment with 500 µM METH further increased the proline levels up to ~130 µM and 1000 µM METH enhanced proline levels up to ~155 µM. These results indicate that acute METH exposure significantly enhances proline biosynthesis in accordance with the induction of proline synthetic enzymes.

A number of studies have shown that METH exposure alters glutamate levels. Therefore, we tested whether the increased proline levels were due to higher glutamate in METH-treated cells. We measured the levels of both intracellular and extracellular glutamate in SH-SY5Y cells. We treated these cells with 50–1000 µM METH for 24 h. Following that, we measured glutamate levels in the cellular lysates and supernatants by a colorimetric assay. As shown in Figs. 3B,C, treatment of SH-SY5Y cells with METH at concentrations of 0–1000 µM did not significantly increase either intracellular (Fig. 3B) or extracellular (Fig. 3C) glutamate levels. Surprisingly, there was a slight decrease in both intracellular and extracellular glutamate levels (~20–30%) in cells treated with METH at the concentrations 200–1000 µM. Collectively these results suggest that acute METH treatment at concentrations ≤ 1 mM minimally affects intracellular and extracellular glutamate levels.

A lack of increased glutamate in METH treated cells could be due to reduced glutamate synthesis from glutamine-the major source of glutamate in neurons. To test this hypothesis, we measured the levels of glutaminase (GLS) enzyme that converts glutamine to glutamate in neurons (22–24). Western blot analysis revealed that METH treatment did not reduce GLS levels in SH-SY5Y cells, (Fig. 3D,E). Interestingly, a modest increase in GLS expression was observed in METH treated cells (Fig. 2D,E). These observations suggested that
glutamate synthesis is not reduced in METH treated cells. Thus, a lack of increased glutamate during acute METH exposure was most likely not due to reduced synthesis from glutamine.

Extracellular glutamate levels are also dependent on packaging of intracellular glutamate by the vesicular glutamate transporters: vGLUT1, vGLUT2, and vGLUT3 (22–24). Among these transporters, the functional characteristics of vGLUT1 and vGLUT2 for glutamate transport is similar56. Interestingly, vGLUT3 has been reported to be found in cholinergic neurons but not in glutamatergic cell populations57. Therefore, we measured the levels of vGLUT1 as the selected marker of glutamate release in METH treated cells. Surprisingly, western blot analysis revealed a dose-dependent decrease in vGLUT1 expression with increasing concentration of METH (Fig. 3D,F). The reduction in vGLUT1 expression suggested that the packaging of glutamate was most likely reduced in METH treated cells. Interestingly, the reduction in vGLUT1 expression is correlated with the decrease in extracellular glutamate levels in cells treated with METH at concentrations of 200–1000 µM (Fig. 3C). Finally, we also measured neuronal cytotoxicity by LDH assay in the supernatants of METH-treated cells. METH treatment did not cause any cytotoxicity even at 1 mM concentration when compared to untreated control cells (Fig. 3G). Collectively, these results demonstrate that acute METH exposure at concentrations below 1 mM significantly enhances proline levels but minimally increases extracellular glutamate and lack the ability to cause neuronal cytotoxicity.

**P5CS is the key enzyme that regulates glutamate levels during acute METH exposure.** Our results in Fig. 3A demonstrated that acute METH exposure activates proline biosynthesis. However, a lack of alterations in glutamate levels in these cells (Fig. 3B,C), led us to hypothesize that activation of proline biosynthesis may aid in regulating glutamate levels during acute METH exposure. To test this, we created SH-SY5Y cells
that are deficient in proline biosynthesis from glutamate. We specifically depleted the P5CS enzyme since synthesis of proline from glutamate is regulated by P5CS (Fig. 1A)\(^26\) and METH significantly increased P5CS levels (Figs. 1, 2). Even though PYCR2 was also upregulated by METH, blocking the reductase step required depletion of both PYCR1 and PYCR2 due to the functional redundancy of these two enzymes. Therefore, we generated P5CS knockout (P5CS-KO) SH-SY5Y cells (P5CS-KO) using CRISPR/Cas9 approach\(^58\). We employed a lentiviral vector-based expression of nuclease-active Cas9 and P5CS gRNAs and selected single clones of P5CS-KO. Western blot analysis confirmed lack of P5CS expression in P5CS-KO cells when compared to the wild type cells (Fig. 4A). We then treated the P5CS-KO cells with METH in a dose-dependent manner for 24 h. Measurement of glutamate levels showed a significant increase in intracellular glutamate levels in P5CS-KO cells upon METH treatment (Fig. 4B). For instance, METH exposure at 50 µM resulted in ~1.8-fold increase in intracellular glutamate levels in P5CS-KO cells compared to the untreated control. This increase was further enhanced to ~2.6-fold with 1 mM METH treatment P5CS-KO cells (Fig. 4B). Interestingly, acute METH exposure also significantly elevated extracellular glutamate levels in the P5CS-KO cells (Fig. 4C). These results are in contrast to the minimal effect of METH exposure on glutamate levels in the wild type cells that express P5CS (Fig. 3B,C).

Figure 4. METH treatment of P5CS knockout (P5CS-KO) SH-SY5Y cells significantly increases glutamate levels. The P5CS-KO SH-SY5Y cells were generated from wild-type SH-SY5Y cells using CRISPR/Cas9 system. Following which the P5CS-KO cells were treated with METH for 24 h and cellular lysates and cell-free supernatants were collected. (A) Western blot showing a lack of P5CS expression in P5CS-KO vs. WT SH-SY5Y cells (n = 3). β-actin was used as a loading control. (B,C) Effect of METH on glutamate in P5CS-KO cells. (B) The cell extracts were used to measure intracellular glutamate while (C) the cell-free supernatants were used to measure extracellular glutamate (n = 3). A marked increase in levels of both intracellular and extracellular glutamate was obtained in P5CS-KO cells after METH treatment. (D,E) Effect of acute METH treatment on PYCR2 in P5CS-KO cells. Representative western blot of PYCR2 in (D) following acute METH treatment (n = 3). β-actin was used as a loading control. (E) Densitometric analyses of PYCR2 normalized to β-actin. (F–H) Effect of acute METH treatment on GLS and vGlut1 in P5CS-KO cells. (F) Representative western blot of GLS and vGLUT1 in METH-treated P5CS-KO SH-SY5Y cells (n = 3). β-actin was used as a loading control. (G,H) Densitometric analyses of GLS and vGLUT1 western blot normalized to β-actin. Data are presented as the mean ± SEM of at least three independent experiments. *p < 0.05, **p < 0.005 represents statistical comparison of untreated vs METH-treated cells.

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To further solidify the role of P5CS in regulating glutamate levels, we probed the expression of PYCR2 in the P5CS-KO cells, since METH treatment increased PYCR2 expression in wild type cells (Figs. 1, 2). Results presented in Fig. 4D,E clearly show that METH exposure minimally alters the expression of PYCR2 in the P5CS-KO cells. Moreover, in P5CS-KO cells treatment of METH minimally affected GLS expression (Fig. 4F,G), suggesting that depletion of P5CS does not affect glutamate synthesis from glutamine. Interestingly, a marginal increase in vGLUT1 expression was observed in the P5CS-KO cells upon METH treatment above 500 µM (Fig. 4H). This increase is consistent with the increased glutamate levels observed in the P5CS-KO cells after METH exposure. Collectively, these results establish that depletion of P5CS causes accumulation of glutamate and strongly suggest the involvement of proline biosynthetic arm in maintaining METH-induced glutamate homeostasis.

**METH exposure increases glutamate levels in proline-auxotrophic Chinese hamster ovary (CHO) cell line.** Our results demonstrated that P5CS expression is associated with alterations in glutamate levels.
levels during acute METH exposure (Fig. 4). To further probe a functional link between proline synthetic arm and glutamate, we exploited the proline auxotrophic nature of Chinese Hamster Ovary (CHO) cells. CHO cells lack the proline biosynthetic arm that is needed to catalyze proline from glutamate. Specifically, these cells do not express P5CS and PYCR2 as measured by western blot analysis (Fig. 5A). Therefore, to further strengthen the role of P5CS during glutamate homeostasis, CHO cells were treated with increasing doses of METH for 24 h. Then extracellular glutamate levels were measured in the culture supernatants of treated and untreated cells. As shown in Fig. 5C, a dose-dependent increase in glutamate levels was detected in the presence of increasing concentrations of METH.

Next, we tested whether the higher levels of glutamate in METH-treated CHO cells is a consequence of lack of proline synthesis from glutamate. We exogenously expressed P5CS in the CHO cells by transfecting a P5CS expression construct. Post-transfection, western blot analysis of the cellular lysates of these cells confirmed the expression of P5CS compared to the lack of P5CS expression in wild type cells (Fig. 5B). Then, the P5CS-expressing CHO cells were exposed to increasing concentrations of METH for 24 h and extracellular glutamate levels were measured in the supernatants. Results from these assays show that P5CS expression significantly abrogates the increase in extracellular glutamate upon METH exposure as compared to the P5CS-lacking wild type cells (Fig. 5C). Taken together, these observations provide strong evidence that P5CS regulates glutamate levels during acute METH exposure.

**Acute METH exposure activates proline biosynthetic pathway in the cortical regions of mice brain.** Our studies using neuronal cell model and proline auxotrophic cells (Figs. 1, 2, 3, 4, 5) demonstrated a key role of proline biosynthetic pathway in regulating glutamate levels during acute METH exposure. To determine the in vivo significance of these observations, we investigated the effects of METH exposure on the proline synthetic pathway in a rodent model. Mice were administered with either METH (2 mg/kg IP) or saline under acute conditions for 24 h. Then, the cortical regions of the brain were isolated and total protein from these brain tissues were extracted and subjected to western blot analyses. We focused on the cortical regions, since...
glutamatergic neuronal projections originating in the prefrontal cortex (PFC) extend to the striatum, nucleus accumbens (NAC), ventral tegmental area (VTA) and substantia nigra of the midbrain16,22, and METH has been shown to affect glutamate in the PFC of mice brain23. Data in Fig. 6A show that PSCS is constitutively expressed in the cortical region of mouse brain. Upon acute METH treatment of these mice, a significant increase in PSCS levels was detected when compared to the control animals (Fig. 6A,B), suggesting upregulation of PSCS during METH exposure. Furthermore, protein levels of the PYCR2 were also increased in the cortical tissues of METH administered animals (Fig. 6C,D). However, the level of increase in PYCR2 was lower when compared to the significant increase in PSCS levels. These data are in accordance with the results obtained with the neuronal cell model (Figs. 1, 2). Collectively, these observations support the activation of the proline synthetic arm in the cortical region of mice brain during acute METH exposure.

Discussion

Glutamate is the key excitatory neurotransmitter in the brain15–17. During glutamatergic neurotransmission, glutamate is packaged in presynaptic neurons and released into the synapse15–17. The released glutamate binds to ionotropic and metabotropic receptors on postsynaptic neurons to propagate the incoming signals15–17. Subsequently, high-affinity transporters present on the glial cells and neurons rapidly sequester the glutamate to facilitate a continuous cycle of neurotransmitter activity24. Given the central role of glutamate in neurotransmission, it is involved in most aspects of normal brain function including cognition, memory, and learning25. Glutamate also plays a critical role in drug reward, reinforcement, and relapse26–28. Importantly, recent studies suggest that in individuals with substance use disorder, glutamate is associated with synaptic plasticity65–68. Specifically, METH has been reported to affect glutamate neurotransmission by altering glutamate levels13. However, the mechanism by which METH exposure affects glutamate homeostasis is poorly understood.

METH has been shown to alter the extracellular concentrations of both glutamate and dopamine in the brain13. For example, chronic treatment with high doses of METH has been reported to increase glutamate efflux in the striatum24. Depletion of striatal dopamine levels with low dose METH has been shown to enhance glutamate release24. Similarly, a low dose of METH has been demonstrated to increase extracellular dopamine but not glutamate in PFC and striatum24. Additionally, repeated METH administrations were shown to enhance cortical glutamate efflux24. Interestingly, a single injection of a high dose of METH (30 mg/kg, i.p.), was reported to cause glutamate depletion70. These observations suggest that alterations in the levels of glutamate are dependent on the duration and dose of METH exposure. Accordingly, our results with a neuronal cell model demonstrated that acute METH exposure at concentrations below 1 mM minimally altered extracellular glutamate levels. It is noteworthy that majority of the published studies examined the effects of long-term exposure and relatively high doses of METH on glutamate71–73. Therefore, based on our results we speculate that a single dose of METH below 1 mM concentrations causes minimal changes in glutamate homeostasis.

Neuronal glutamate levels are regulated by a multi-prong mechanism that involves glutamate biosynthesis from glutamine and synaptic vesicle loading of intracellular glutamate. Glutamate synthesis is dependent on the glutamine–glutamate cycle between astrocytes and neurons16. In this metabolic cycle, GLS is primarily responsible for synthesizing glutamate from glutamine to continuously replenish the neurotransmitter pool (22–24). While, GLS regulates glutamate biosynthesis from glutamine (22–24), vGLUTs mediate the transport of glutamate into synaptic vesicles22–24 and vGLUT expression determines the amount of glutamate packaged into vesicles22–24. Specifically, in glutamatergic neurons the functionally similar vGLUT1 and vGLUT2 are responsible for glutamate transport22. Interestingly, our results demonstrated that expression of GLS remains mostly unaffected in METH treated cells (Fig. 3). Moreover, with acute METH treatment, a minimal to marginal reduction in vGLUT-1 expression was detected (Fig. 3). These observations strongly suggest that acute METH exposure minimal alters ongoing glutamate biosynthesis and vesicular packaging, consistent with the minimal effect of METH on glutamate levels under acute exposure conditions.

The glutamine–glutamate biosynthetic cycle is not stoichiometric, but is an open biochemical pathway wherein glutamate interfaces with several other metabolic pathways including carbohydrate and amino acid metabolism through the TCA cycle70. In this context, our results describing activation of proline synthetic arm by METH exposure highlights the novel role of proline biosynthesis during METH exposure. Glutamate can be channeled for proline synthesis through P5C. These biochemical reactions are sequentially catalyzed by PSCS and PYCR (Fig. 1A). Remarkably, our data showed that acute METH treatment resulted in a marked enhancement in both PSCS and PYCR2 expression (Figs. 1, 2). Concomitant increase in intracellular proline levels (Fig. 3A) indicated that induction of these two enzymes are most likely responsible for synthesizing proline from glutamate. Moreover, this increase was observed only with METH and not AMPH indicating the effect to be specifically mediated by METH. Previously, a genome-wide study, analyzed METH-induced mRNA expression in rodent brain to reveal the potential regulatory consequences in response to METH75. Pathways upregulated by METH included proline metabolism especially the proline synthetic enzyme PSCS (encoded by ALDH18A1) and PYCRs75. Similarly, a metabolomic study of brain tissues from METH affected animals uncovered neurochemical signatures related to the metabolism of amino acids including glutamate and proline76. Similar to METH, morphine is also known to affect glutamatergic neurotransmission77–79. In a proteomics analysis, PYCR2 was identified as one of the proteins that was induced by morphine in mouse hippocampal postsynaptic density–associated proteins (HPSD)79. These studies indicate an interplay between glutamate and proline metabolism. However, the functional relevance of proline metabolism during METH exposure is unknown. Therefore, our studies showing a functional association between glutamate and proline metabolism during METH exposure are highly significant.

Our results provide strong evidence of glutamate efflux to proline biosynthesis. Specifically, a marked induction in the expression of PSCS was observed during acute METH exposure not only in the SH-SYSY neuronal
cells but also in brain cortical region of mice. Interestingly, disruption in proline biosynthesis by knocking out of P5CS dramatically increased both intracellular and extracellular glutamate with METH treatment. The role of P5CS was further confirmed using a proline auxotrophic cell line (CHO-K1) that lacks the proline biosynthetic machinery. A marked enhancement in glutamate levels was also observed in these cells with METH treatment and this effect was abrogated by overexpression of P5CS. Collectively, these results demonstrated sequestration of neuronal glutamate pool towards proline biosynthesis. A lack of neuronal cell death by acute METH exposure even at 1 mM concentrations supports the hypothesis that sequestration of glutamate to proline is a critical mechanism activated to avert the accumulation of excess glutamate and prevent excitotoxicity.

Sequestration of glutamate to proline may have significant implications in the CNS. For instance, P5C formed during proline synthesis from glutamate can serve as the source for carbon exchange between the TCA and urea cycle. Proline can also be deposited in collagen, the most abundant protein by weight in the human body. Approximately, 25% of proline residues make up collagen, therefore, collagen has been suggested to be both a dump as well as a reservoir for proline. This may be very relevant in the context of the extracellular matrix (ECM) of neurons. For example, excess glutamate can be converted to proline and deposited into the ECM as collagen without dramatically altering extracellular proline levels. High levels of proline have been shown to have excitotoxic properties and studies have shown that addition of proline to hippocampal slices decreased glutamate uptake causing glutamate to accumulate. It is plausible that once the threshold for proline deposition is reached, especially with chronic METH, it may result in the accumulation of proline, which may also contribute to the glutamate excitotoxicity. Thus, the interplay between glutamate and proline may be an important regulator in METH-induced glutamate excitotoxicity.

Importantly, we envision that increased conversion of glutamate to proline offers a bioenergetic metabolic advantage. The bioenergetic cost of maintaining homeostatic levels of synaptic glutamate is expensive. Glucose via glycolysis provides the main source of energy for maintaining glutamate homeostasis in the brain. However, for efficient glycolysis a sustained supply of electron acceptor nicotinamide adenine dinucleotide (NAD⁺) is essential. Proline biosynthesis has been shown to augment glycolysis by affecting the levels of NAD⁺, especially in cancer metabolism. Therefore, our results demonstrating METH-induced proline biosynthesis may confer a metabolic bioenergetic advantage to neurons in response to increased neuronal stimulus by METH. In summary, our results identify a key role of P5CS during METH exposure and highlight that sequestering excess glutamate for proline biosynthesis is a key mechanism to maintain glutamate levels.

Materials and methods
All the methods used in this study are in accordance with relevant guidelines and regulations.

Figure 7. Proposed model demonstrating the key role of P5CS in maintaining glutamate homeostasis during acute METH exposure. The schematic model shows presynaptic and postsynaptic neurons along with astrocytes and highlights the pathways involved in glutamate homeostasis in the brain. The left panel depicts the sequestration of excess glutamate in the neuron for proline biosynthesis during acute METH exposure. Specifically, METH-induced enhancement of P5CS expression is associated with utilization of glutamate for proline synthesis, thus maintaining glutamate homeostasis. Whereas, the right panel describes that depletion of P5CS blocks the proline synthetic arm from glutamate and abrogates glutamate sequestration for proline biosynthesis. This metabolic disruption results in an increased levels of glutamate that could be detrimental for the neurons.
PCR and Sanger sequencing. Compatible ends of the pcDNA 3.1 plasmid (Addgene). The recombinant plasmids were confirmed by colony BamH1 and Xho1 restriction enzyme sites. The BamH1 and Xho1 digested and reverse: 5′-AAG TTT ACC-3′

SupT1 total RNA using P5CS-specific primers (forward: 5′-TTA TAG GAT CCG CCA CCA TGT TGA GTC of the isolated plasmids.

Transformation Kit (Zymo Research, USA) were transformed with an aliquot of the ligation reaction products as incubating at room temperature for 1 h. Subsequently, NEB-Stbl competent cells, prepared using the Mix&Go phosphorylated TLCV2 in a ligation mixture containing T4 ligation buffer (NEB) and T4 DNA ligase (NEB) and incubating at 37 °C for 30 min followed by incubation at 95 °C for 5 min and then ramping down to 25 °C at 5 °C/min. The annealed oligos were diluted 200 fold and then ligated to the BsmB1-digested and dephosphorylated TLCV2 in a ligation mixture containing T4 ligation buffer (NEB) and T4 DNA ligase (NEB) and incubating at room temperature for 1 h. Subsequently, NEB-Stbl competent cells, prepared using the Mix&Go Transformation Kit (Zymo Research, USA) were transformed with an aliquot of the ligation reaction products as per manufacturer-recommended protocol and the bacterial recombinants were confirmed by Sanger sequencing of the isolated plasmids.

To construct the P5CS overexpression plasmid, the P5CS ORF was obtained by RT-PCR (abm, Canada) from SupT1 total RNA using P5CS-specific primers (forward: 5′-TTA TAG GAT CCG CCA CCA TGT TGA GTC AAG TTT ACC-3′ and reverse: 5′-TGA CAC TCG AGT CAG TTG GTG TTT CTC TGA G-3′) containing BamH1 and Xho1 restriction enzyme sites. The BamH1 and Xho1 digested P5CS ORF amplicon was ligated to compatible ends of the pcDNA 3.1 plasmid (Addgene). The recombinant plasmids were confirmed by colony PCR and Sanger sequencing.

Lentivirus transduction. HEK293T cells (5 × 10^5) seeded per well in 6-well plates and cultured overnight were transfected with plasmid DNAs using the PEI transfection reagent (Polysciences Inc.). For transfecting cells in each well, a 4:3:1 ratio of lentiviral transfer plasmid (described above), packaging plasmid psPAX2, and pseudotyping envelope plasmid pMD2.G, and the PEI at a DNA:PEI ratio of 1:3 were used. After overnight cultivation, the culture medium was removed and the cells were replenishef with fresh medium. Forty-eight hours post transfection, the lentivirus-containing culture medium was collected, centrifuged at 5000 × g for 5 min at room temperature to remove cell debris, and the supernatant was filtered through 0.45 μm filter. Unused virus stocks were stored at −80 °C for later use.

P5CS CRISPR/Cas9 knockout in SH-SY5Y cells. For generation of stable cell lines, SH-SY5Y cells were inoculated with lentivirus for 24–48 h, then replenished with fresh culture medium containing the selection drug puromycin at 1 μg/mL. Cells were replenished with fresh culture medium containing puromycin every 2 days until majority of the uninfected control cells were eliminated (in ~ 7–10 days). To generate single cell clones, cells were treated with 1 μg/mL DOX for 24 h and the GFP positive cells were sorted out by flow cytometry. The GFP-positive single cells were then seeded into a 96-well plate and allowed to expand. The cell clones that were depleted of the target protein as analyzed by western blot were used in functional experiments.

Lactate dehydrogenase (LDH) assay. To determine cytotoxicity, LDH release was measured with the LDH Cytotoxicity Assay Kit (Pierce) according to the manufacturer’s protocol. 5 × 10^5 SH-SY5Y cells were plated into 6-well plates and maintained overnight before treatment. The cells were treated with METH at various concentrations for 24 h. After treatment, the cell supernatants were collected by centrifugation. Supernatants were assayed for LDH in triplicates as per the manufacturer’s protocol. Absorbances were measured at 490 nm and 680 nm using a spectrophotometer (Biotek). The percentage of cytotoxicity was calculated based on the percentage difference compared with the LDH-positive control provided with the kit.

Glutamate assay. 5 × 10^5 SH-SY5Y cells were plated in 6-well plate overnight and treated with METH for 24 h. Supernatants were collected, briefly centrifuged to remove cell debris, and analyzed by a Glutamate Col-
of p < 0.05 were considered to be statistically significant. Significance of differences between control and treated samples was determined by Student’s t test. Values and flash frozen until processing for immunoblot analysis.

30-min recovery period, slices were then incubated in either 200 µM amphetamine, 200 µM methamphetamine Leica Vibratome in oxygenated (95% O2; 5% CO2) ice-cold artificial cerebrospinal fluid (aCSF, in mM: 119 NaCl, which 300-µm coronal brain slices containing the frontal cortex were prepared from whole brain tissue using a

Animal studies. All protocols were approved by the University of Florida and Vanderbilt University Institutional Animal Care and Use Committee (IACUC) policies and adhered to the NIH guidelines. WT C57BL/6J male mice were obtained from Jackson Laboratories (Vanderbilt; Stock No. 000664) or from the University of Florida Animal Care Services and were maintained on a 12-h light/dark cycle with food and water available ad libitum in their home cages. The mice were injected with a single dose of METH at 2 mg/kg body weight (acute exposure). Control mice received saline injections. The animals were euthanized after 24 h and whole brain were extracted, rapidly rinsed with ice-cold PBS and immediately flash frozen. Punches from the cortical regions were taken for each sample and homogenized manually in a sucrose buffer system supplemented with protease inhibi-

Measurement of intracellular proline levels. 5 × 10⁵ SH-SY5Y cells were plated per well in 6-well plates overnight and treated with METH for 24 h. After treatment cells were harvested and lysed in cold PBS with 1% Triton X-100. The cellular debris were removed by centrifugation (10,000×g). The supernatants were transferred to a boiling water bath, and intracellular amino acids were extracted by boiling for 10 min. After centrifuga-

Western blot analyses. Cells were treated with various concentrations of METH for 24 h, after which cells were harvested and washed with PBS. Cell lysates were prepared as per our published method⁵⁰ and quantified according to standard BCA protein assay (Pierce, USA). Equal amounts of cell lysates (20 µg) were resolved on SDS–polyacrylamide gels and transferred to nitrocellulose membranes using a semi-dry blotter (Bio-Rad). Membranes were blocked using Tris-buffered saline with 5% nonfat milk (pH 8.0; Sigma). Blots were then probed with the appropriate primary antibody in blocking buffer overnight at 4 °C. Incubation with secondary anti-mouse or anti-rabbit IgG antibodies conjugated to horseradish peroxidase (1:2000) was performed at room temperature for 1 h. All blots were washed in Tris-buffered saline with Tween 20 (pH 8.0; Sigma) and developed using the enhanced chemiluminescence (ECL) procedure (BioRad). Blots were routinely stripped by Restore Plus Stripping Buffer (Pierce) and reprobed with anti-actin monoclonal antibody to serve as loading controls. The density of the band was evaluated by Bio-Rad imaging-lab 4.0 software (https://www.bio-rad.com/en-pl/product/image-lab-software?ID=KRE6P5E8Z).

Transfection of P5CS expression construct in CHO-K1 cells. CHO-K1 cells (1 × 10⁵) were seeded per well in 6-well plates for 24 h in complete F12K media. Prior to transfection, the cells were pretreated with METH for 1 h and then transfected with pcDNA control vector or P5CS expression vector. Transfections were performed with JetPrime (Polyplus) according to the manufacturer’s instructions. 24 h post transfection the cells were harvested by scraping. The cell supernatants and pellets were collected by centrifugation. Supernatants were assayed for glutamate and the cell pellets were analyzed by western blot as described before.

Animal studies. All protocols were approved by the University of Florida and Vanderbilt University Institutional Animal Care and Use Committee (IACUC) policies and adhered to the NIH guidelines. WT C57BL/6J male mice were obtained from Jackson Laboratories (Vanderbilt; Stock No. 000664) or from the University of Florida Animal Care Services and were maintained on a 12-h light/dark cycle with food and water available ad libitum in their home cages. The mice were injected with a single dose of METH at 2 mg/kg body weight (acute exposure). Control mice received saline injections. The animals were euthanized after 24 h and whole brain were extracted, rapidly rinsed with ice-cold PBS and immediately flash frozen. Punches from the cortical regions were taken for each sample and homogenized manually in a sucrose buffer system supplemented with protease inhibi-

Statistical analyses. Data are expressed as mean ± SEM obtained from at least three independent experiments. Significance of differences between control and treated samples was determined by Student’s t test. Values of p < 0.05 were considered to be statistically significant.

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Author contributions
BJ, JP, FV, BG, and CD conceptualized the study and designed the experiments. BJ, MB, AT, BG, CG and JP performed the experiments. BJ, MB, JP and CD analyzed the data. JL and HK designed the animal experiments and assisted in data analysis. BJ, JP and CD wrote the manuscript. All other authors reviewed the manuscript.

Competing interests
The authors declare no competing interests.

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