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D-serine suppresses one-carbon metabolism by competing with mitochondrial L-serine transport

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eLife Assessment

This work presents **valuable** new data on the role of D-serine and how it competes with its stereoisomer L-serine to influence metabolism. The work presents a variety of **convincing** experimental data combined with simulated results to investigate the mechanisms focused on one-carbon metabolism, which is relevant for several research fields. However, some claims are only partially supported by data, and critical areas comparing L- vs D-serine and further mechanistic studies are required. Furthermore, while the work has potential for various fields, the work has only been studied in a limited cell type and context.

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Abstract

L-serine serves as a central metabolic node that integrates glycolytic flux, lipid metabolism, and one-carbon metabolism. In the mature central nervous system, L-serine is actively stereo-converted to D-serine, which functions as a neurotransmitter. However, the role of D-serine in cellular metabolism remains unclear. Here, we show that D-serine competes with mitochondrial L-serine transport, thereby suppressing one-carbon metabolism. Metabolomic analysis revealed that D-serine reduces intracellular glycine and formate levels, indicating inhibition of the initial step of the one-carbon pathway. Molecular dynamics simulations and enzymatic assays revealed that D-serine has low affinity for serine hydroxymethyltransferase 2 (Shmt2), which catalyzes the first step in mitochondrial one-carbon metabolism, and does not directly inhibit its activity. Instead, membrane transport assays demonstrated that D-serine competes with mitochondrial L-serine transport, depleting the substrate of Shmt2. Functionally, under L-serine poor conditions *in vitro* and *ex vivo*, D-serine inhibited the proliferation of immature and undifferentiated neural cells including glioblastoma stem cells, which depend highly on one-carbon metabolism. Notably,

endogenous D-serine levels were low during early neurodevelopment, but increased with maturation, coinciding with a shift in the transcriptional profiles of serine metabolic enzymes at the cellular level. Given that L-serine supports neurodevelopment and D-serine modulates neurotransmission, this developmental shift in serine enantiomer metabolism appears to align with the functional transitions of the maturing nervous system. Thus, our findings reveal that serine chirality can influence mitochondrial substrate availability and one-carbon flux, offering previously unappreciated insight into the stereoselective regulation of cellular metabolism.

Introduction

Serine is a unique amino acid, both L- and D-enantiomers of which are synthesized *de novo* in the central nervous system. L-serine is primarily synthesized from a glycolytic intermediate through the phosphorylated pathway, and further stereo-converted to D-serine. Serine enantiomers and their metabolites are critically involved in neural development and neurophysiology in three major ways. First, L-serine is a crucial carbon donor with the cofactor, folate for the one-carbon metabolism, which is a universal metabolic process for nucleic acid synthesis, methylation, and reductive metabolism. As these pathways essentially support proliferative cells, undifferentiated or cancer cells are particularly susceptible to deprivation of L-serine or inhibition of *de novo* L-serine synthesis (Geeraerts et al., 2021). Second, biosynthesis of membrane lipids requires L-serine as an integral component of sphingolipids and phosphatidylserine. The nervous system includes highly polarized neurons and glia and exhibits the highest lipid abundance and complexity. Among membrane lipids, sphingolipids are particularly abundant in the nervous system and are essential for neuronal differentiation, polarization, synapse formation, and myelination, which are required for development and functional integrity of the nervous system (Hirabayashi and Furuya, 2008). Third, L-serine serves as a precursor for neurotransmitters, such as glycine and D-serine. L-serine is converted to glycine by serine hydroxymethyltransferase (Shmt) in the one-carbon metabolic pathway, and also into D-serine by serine racemase (Srr). Both glycine and D-serine bind *N*-methyl-D-aspartate (NMDA) receptor subunits with distinct affinities and regulate excitatory neurotransmission essential for neural development and function (Chatterton et al., 2002; Nancy and Dingledine, 1988). In addition, glycine also binds to inhibitory glycine receptors and controls early embryonic development as well as a variety of motor and sensory functions (Lynch, 2004).

Defects of L-serine synthesis due to single nucleotide polymorphisms in the genes encoding 3-phosphoglycerate dehydrogenase (PHGDH), the rate-limiting enzyme in the phosphorylated pathway, or the downstream enzyme phosphoserine aminotransferase (PSAT1) are associated with congenital microcephaly and hypomyelination, and exhibit psychomotor retardation and seizures in humans (Jaeken et al., 1997; KONING et al., 2002). Phgdh deficient mice die in embryo after 13.5 days post-coitum and show systemic growth defects, especially severe hypoplasia of the central nervous system, with marked reductions of L-serine and sphingolipids (Yoshida et al., 2004). Indeed, proliferative-marker-positive cells and mature neurons are almost entirely absent in these mice, indicating that *de novo* biosynthesis of L-serine is critical for neuronal proliferation and differentiation. Given its contribution to one-carbon metabolism, lipid synthesis, and production of glycine and D-serine (Yang et al., 2010), L-serine likely serves multiple functions in neural development. Of note, serine synthesis shows drastic changes after neurogenesis. Neural progenitor cells (NPCs) express PHGDH, whereas mature neurons lack its expression and lose the ability to synthesize L-serine. In mature cerebral cortex and hippocampus, on the other hand, astrocytes take over L-serine synthesis, and supply neurons with L-serine for synthesis of neurotransmitter D-serine (Wolosker, 2011). This metabolic compartmentalization suggests distinct functions of D- and L-serine across neural cell types and developmental stages. However, how D-serine contributes to cellular metabolism beyond neurotransmission has not been fully elucidated. In this study, we used a metabolomics-based approach to examine how D-serine influences cellular metabolism, identified its primary target pathway in vitro, and assessed its functional consequences in neural cells both *in vitro* and *ex vivo*.

Main

D-serine impacts metabolites in one-carbon metabolism

To test whether serine chirality influences cellular metabolism in cortical neurons, we compared metabolomic profiles of primary cultured neurons (PCNs) treated with or without L- or D-serine for 48 h starting at DIV1. Serine enantiomers markedly altered the levels of metabolites such as glycine, cysteine, hypotaurine, taurine, glutathione, AMP, uracil, and polyamines (Fig. 1A and Fig. S1A and B). These metabolites are components of the one-carbon metabolic network, including the folate cycle and synthetic pathways for purine, glutathione, taurine, and polyamine (Fig. 1B), all of which are initiated by one-carbon donation from L-serine. Compared to D-serine, L-serine affected a broader range of metabolic pathways, including several amino acid metabolism (Fig. 1C and Fig. S1C). Notably, however, D-serine uniquely caused a marked reduction in glycine levels (Fig. 1A), which was further confirmed using a two-dimensional HPLC system (2D HPLC), a highly sensitive tool to quantify enantiomers, to occur independently of any change in L-serine levels (Fig. 1E and F). Metabolites that showed similar reduction patterns to glycine were primarily polyamines and their intermediates (Fig. 1D). Given that glycine is synthesized in the initial step of the one-carbon pathway, and that polyamines are produced in a downstream branch of the one-carbon network (Fig. 1B), these results suggest that D-serine broadly suppresses one-carbon metabolism. In support of our view, D-serine treatment on PCNs further reduced production of formate, an intermediate of the folate cycle in one-carbon metabolism, whereas L-serine enhanced it (Fig. 1G). Therefore, these findings indicate that D-serine exerts an inhibitory effect on the one-carbon metabolism, in contrast to the supportive role of L-serine.

D-serine suppresses one-carbon flux by competing with L-serine transport to mitochondria

In the central nervous system, glycine is synthesized from L-serine by serine hydroxymethyltransferase (Shmt) 1 and 2 (Fig. 2A), which performs the first transfer of a carbon unit from L-serine to folate in one-carbon metabolism (Pan et al., 2020). In the presence of mitochondrial Shmt2, transfer of a carbon unit from L-serine to tetrahydrofolate (THF) and glycine production depends primarily on mitochondrial enzyme, but not cytoplasmic Shmt1 (Fig. 2A)(Ducker et al., 2016). Therefore, the molecular target of D-serine in its inhibition of the initial step of one-carbon metabolism is likely mitochondrial Shmt2 or supply of its substrates into mitochondria (Fig. 2A). To study whether D-serine impacts catalytic activity of Shmt2, we compared homotetrameric assembly of human SHMT2 combined with either L- or D-serine in the presence of THF and pyridoxal phosphate (PLP) (Fig. S2). When L- or D-serine was placed in the binding pocket of Shmt2, the eliminating carbon in D-serine was more distant from phosphorus (P) and oxygen (O) in PLP by 1.50 Å (C-P) and 1.10 Å (C-O) than that in L-serine (Fig. 2B and Fig. S2A and B), suggesting that D-serine is not a good substrate for SHMT2. To investigate the stability of serine enantiomers in the binding pocket, we further performed molecular dynamic (MD) simulation of the serine-bound SHMT2 complex systems. MD simulation indicated that binding of D-serine did not affect the overall structure of SHMT2 compared to that of L-serine (Fig. S2C). On the other hand, molecular interactions of D-serine with SHMT2 were unstable and D-serine could not stay in the serine-binding pocket of SHMT2 (Fig. 2C and Supplementary movie 1), which is supported by a recent report that SHMT does not use D-serine as a substrate in the canonical hydroxymethyl-transferase reaction in one-carbon metabolism (*K*m of SHMT2 = 0.07 mM for L-serine and 8.5 mM for D-serine) (Miyamoto et al., 2024) pocket. In contrast, L-serine remained placed stably in the pocket (Fig. 2C and Supplementary movie 2). Therefore, D-serine was unlikely to compete with L-serine binding to SHMT2. Indeed, an *in vitro* experiment using recombinant SHMT2 showed that D-serine did not affect conversion of L-serine to glycine (Fig. 2D).

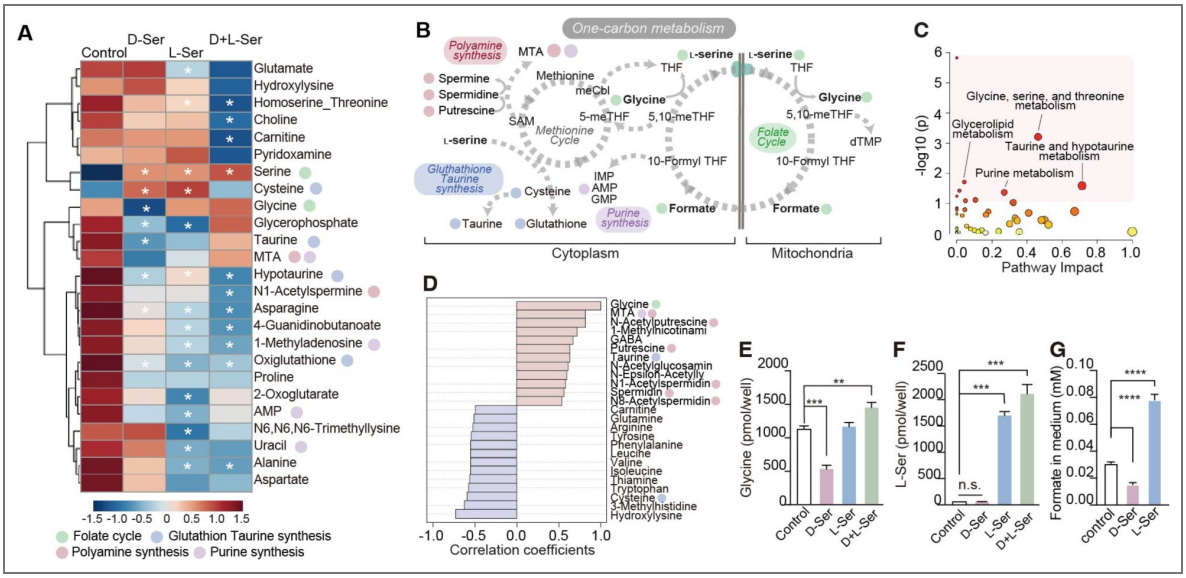

Fig. 1. D-serine inhibits one-carbon metabolism

A. A heatmap shows top 25 metabolites in PCNs altered by treatment with D- and/or L-serine in the metabolomic analysis (n = 3). Color code represents z-score of the relative amount among conditions. Metabolites related to folate cycle (green), glutathione taurine synthesis (blue), polyamine synthesis (pink), and purine synthesis (purple) are indicated with colored circles. *$p < 0.05$ One-way ANOVA, compared to control. **B**. Major metabolites and pathways in the one-carbon metabolism. **C.** KEGG metabolic pathways enriched in D-serine treated cells compared to vehicle-control treated cells. The Pathway impact score is displayed on the X-axis and the -log10 transformed p-value on the Y-axis. **D.** Pearson's correlation coefficients between glycine and other metabolites. Top 25 metabolites positively (red) and negatively (blue) correlated with glycine are shown. The colored circles indicate the relevant pathways, as in Fig. 1A. **E, F.** Concentrations of glycine (**E**) and L-serine (**F**) in PCNs treated with D- and/or L-serine for 48 hours, were quantified using HPLC (n = 4). **G.** Concentrations of formate in the culture media of PCNs treated with 2 mM D- or L-serine for 48 hours were measured using an enzymatic assay (n = 4). Data are presented as the mean ± s.e.m. (**E, F,** and **G**). Statistical significance was evaluated by one-way ANOVA followed by Dunnett's post hoc-test (**E, F,** and **G**). *$p < 0.05$ (**A**). Data are the representative of two independent experiments.

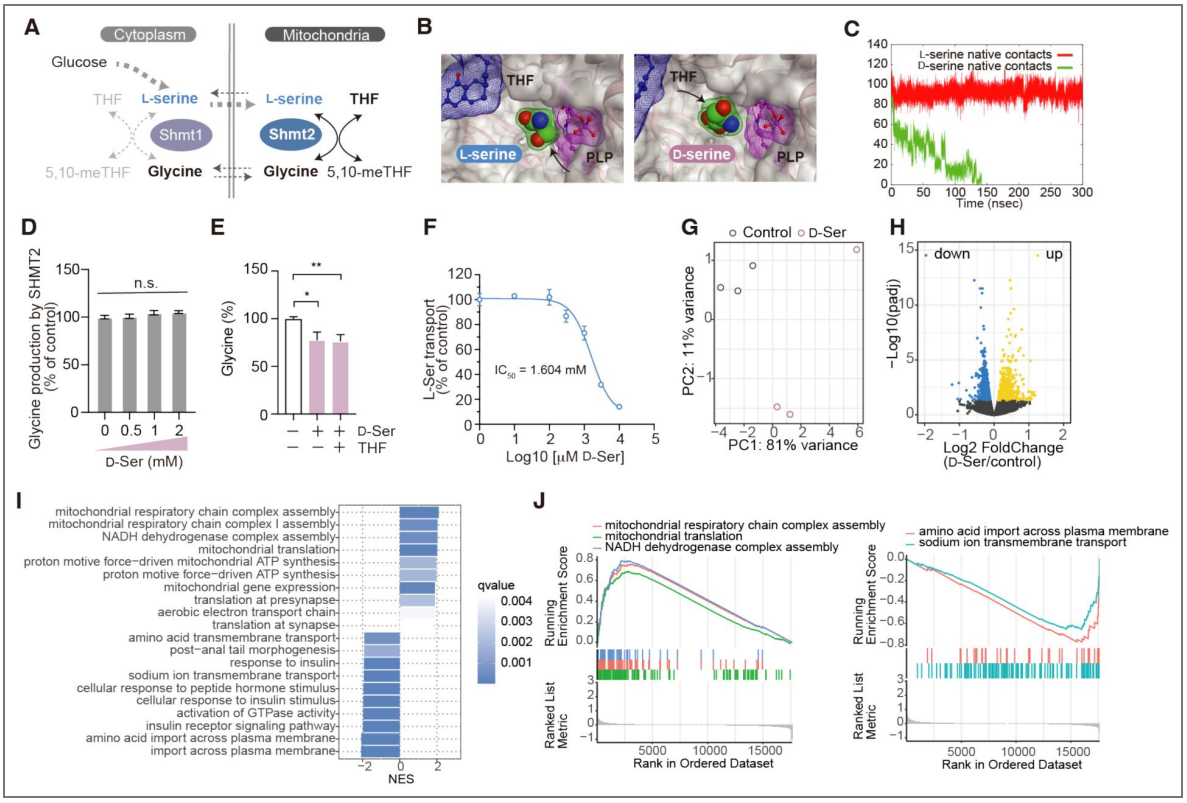

Fig. 2. D-serine inhibits cytoplasmic L-serine transport to mitochondria

A. L-serine/glycine transport and conversion across cytoplasm and mitochondria in one-carbon metabolism. **B.** Binding sites of D- (right) or L-serine (left), THF, and pyridoxal phosphate (PLP) in the ligand-bound SHMT2 complex systems. Arrows indicate carbon atoms on serine enantiomers that transfer to THF. **C.** Time series variation of the number of contact atoms within the distance cutoff of 7.0 Å from D-serine (green) or L-serine (red) observed at the initial structure. **D.** SHMT2 enzymatic activity was determined. Glycine produced by purified SHMT2 from L-serine in the presence or absence of D-serine, was measured using HPLC. The result is shown as percent of control (with no D-serine) (n = 3). **E.** Relative concentrations of glycine in NPCs treated with or without 2mM D-serine and/or 50 μM THF were measured using HPLC. **F**. Inhibition of mitochondrial L-serine transport by D-serine was examined in semi-permeabilized cells (n = 4). The x-axis and y-axis represent the D-serine concentration used (log10-transformed), and the L-serine transport rate compared to the untreated control, respectively. **G.** A PCA plot shows RNA-seq results from cells treated with D-serine (pink) or control (gray). **H**. A volcano plot indicates differentially expressed genes in cells treated with D-serine vs controls. Gene expressions with adjusted *p*-values < 0.05 are highlighted in yellow (up) or blue (down). **I**. Bar plots show Normalized Enrichment Score (NES) for the top 10 up or down-regulated GSEA-enriched categories. The color code indicates the q-value. **J**. GSEA plots for a core subset of gene ontologies are displayed. The left panel shows pathways of mitochondrial respiratory chain complex assembly (red), mitochondrial translation (green), and NADH dehydrogenase complex assembly (blue). The right panel shows pathways of amino acid import across plasma membrane (red) and sodium ion transmembrane transport (cyan). Data are plotted as the mean ± s.e.m. (**D, E,** and **F**). Statistical significance was evaluated by one-way ANOVA followed by Tukey's post hoc-test (**D**) or Dunnett's post hoc-test (**E**).

Next, we wondered whether D-serine inhibits supply of Shmt2 substrate(s), L-serine or THF, to mitochondria. Since reduction of glycine caused by D-serine in PCNs was compensated by addition of L-serine, but not of THF (Fig. 1E, 2E), we assumed that L-serine supply to mitochondria is disturbed by D-serine. Moreover, in PCNs, endogenous L-serine synthesis was not disturbed by addition of D-serine (Fig. 1F), suggesting that D-serine affects L-serine transport to mitochondria, but not its synthesis. To confirm that D-serine impacts L-serine transport to mitochondria, we semi-permeabilized a neuroblastoma cell line, Neuro2a, to loosen cell membranes and monitored [H^3]L-serine transport into mitochondria and other organelles in the presence or absence of D-serine. Intriguingly, D-serine competitively inhibited transport of L-serine in a dose-dependent manner (IC_{50}, 1.604 mM) (Fig. 2F). These results suggested that D-serine impairs one-carbon metabolism by competing with L-serine transport to mitochondria, but not by direct inhibition of Shmt2 activity. Sideroflexin 1 (Sfxn1), a multipass inner mitochondrial membrane protein, has been identified as a mitochondrial L-serine transporter (Kory et al., 2018) To assess whether Sfxn1 mediates the observed effect, we performed siRNA-mediated knockdown of Sfxn1. However, suppression of Sfxn1 alone did not significantly reduce L-serine transport, and D-serine retained its inhibitory effect under these conditions (Fig. S3). Given that Sfxn homologs (Sfxn1-Sfxn5) are proposed to function redundantly in mitochondrial serine transport (Kory *et al*, 2018), these results suggest that D-serine interferes with mitochondrial L-serine transport through multiple Sfxn family members rather than Sfxn1 alone.

To further understand the overall cellular responses to the D-serine burden, we performed RNA-sequencing using Neuro2a cells treated with D-serine, as these proliferative neural cells share metabolic characteristics with PCNs at early stages of differentiation. D-serine mildly, but significantly altered the transcriptome profile compared with vehicle control (Fig. 2G and H). There were 685 up-regulated genes (log_2 fold-change > 0, adjusted p-value < 0.05) and 905 down-regulated genes (log_2-fold change < 0, adjusted p-value < 0.05) (Fig. 2H). To determine whether genes with significant alterations are clustered in specific functional gene sets, we performed gene set enrichment analysis. Notably, D-serine enhanced expressions of genes associated with mitochondrial functions, such as respiratory chain complex assembly, ATP synthesis driven by proton motive force, and mitochondrial gene expression / translation. On the other hand, gene sets linked to amino acid import across the plasma membrane, cellular growth, and neuron projection extension were down-regulated (Fig. 2I and J and Fig. S4). These dynamic transcriptional changes in the neuroblastoma cell line indicate that D-serine can impact polarization and growth of immature neurons negatively and further indicate that immature neurons resist D-serine-induced cellular stress by enhancing mitochondrial function, including energy synthesis.

D-serine inhibits proliferation of tumor cells

One-carbon metabolism provides essential metabolites for nucleotide synthesis, methylation, and reductive metabolism, and supports cellular proliferation. Tumor cells are highly proliferative and are particularly susceptible to deprivation of one-carbon units by L-serine restriction or inhibition of *de novo* serine synthesis (Newman and Maddocks, 2017). Given that D-serine inhibits one-carbon flux by competing with L-serine in PCNs and the neuroblastoma cell line, we wondered whether D-serine influences proliferation of neural tumor cells. To test this idea, we treated several human/rodent neuroblastoma cell lines cultured in the regular medium containing sub-millimolar L-serine and fetal bovine serum with D-serine. Consistent with our findings that D-serine competes with L-serine transport (Fig. 2F), high concentrations of D-serine were required to suppress proliferation of neuroblastoma cell lines cultured in regular medium (Fig. S5A). In contrast, culturing in L-serine/glycine-free medium with dialyzed fetal bovine serum reduced the proliferation of Neuro2a cells with much lower D-serine concentrations (IC_{50} = 1.94 mM) (Fig. S5B-D), which corresponds to the efficacy of D-serine on inhibition of L-serine transport (IC_{50} = 1.604 mM) (Fig. 2F). Indeed, with L-serine/glycine-free medium, D-serine significantly inhibited proliferation of all neuroblastoma cell lines tested, at low millimolar concentrations (Fig. 3A). In accordance with its effect on PCNs (Fig. 1E), D-serine inhibited production of glycine and suppressed proliferation of Neuro2a cells, which was rescued by supplementation with L-serine (Fig. 3B and Fig. S5E). Moreover, supplementations with glycine and downstream metabolites of

the folate cycle in the one-carbon pathway (Fig. 1B), such as formate, inosine monophosphate (IMP), or 5,10-meTHF, phenocopied the rescue effect by L-serine (Fig. S5E-I). On the other hand, combinations of glycine and metabolites of the methionine cycle (methionine or methylcobalamin (meCbl)) as well as other metabolites associated with one-carbon metabolism (thymidine monophosphate (dTMP) or glutathione) (Fig. 1B) failed to liberate cells (Fig. S5J and K). These results suggest that D-serine impacts the folate cycle rather than the methionine cycle in one-carbon metabolism to prevent growth of neuroblastoma cells.

As neurons and astroglia share a common developmental origin, we further tested whether glial tumor cells also show vulnerability to D-serine. Glioblastoma is the most common malignant brain tumor in adults, whose survival rate is low despite advances in surgical and medical neuro-oncology(Bent et al., 2023; Delgado-López and Corrales-García, 2016). Among human glioblastoma cell lines (U87, U251, SF126), U251 cells were the most sensitive to D-serine treatment (Fig. 3C). Supplementation with glycine and formate restored proliferation of U251 (Fig. 3D), and 5,10-meTHF recovered the vulnerability to D-serine, albeit very mildly (Fig. 3E), supporting the significance of one-carbon metabolism in growth of glioblastoma cell lines too. To further test the anti-proliferative effect of D-serine in the tumor microenvironment, we made an organotypic brain slice culture from mice with orthotopic xenografts of glioblastoma cells expressing Venus fluorescence(Tamura et al., 2019). Brain slices of mice implanted with U87 cells in the striatum were cultured *ex vivo* for 7 days in serine/glycine-free media containing D-serine or vehicle. D-serine strikingly suppressed expansion of U87 cells with Venus fluorescence compared to the control treatment, even in *ex vivo* tumor culture (Fig. 3F and G). Since glioma stem cells (GSCs) in the glioblastoma have self-renewal and tumor-initiating capacity and cause cancer recurrence(Gimple et al., 2022; Tang et al., 2021), we wondered whether GSCs also have similar vulnerability to D-serine. *In vitro*, D-serine reduced viability of cell lines for GSCs (hG008 and hG020), established from human glioblastoma specimens[25], in a dose-dependent manner with greater efficacy to the hG008 line (Fig. 3H). This growth defect in hG008 cells induced by D-serine was rescued by supplementation with glycine and formate, as in neuroblastoma and glioblastoma cells (Fig. S5L). hG008 cells have a sphere-forming capacity[23], and we also observed D-serine-induced reduction of sphere diameters of hG008 cells cultured under the sphere-forming conditions (Fig. 3I). Moreover, in brain slices of mice with orthotopic xenografts of hG008 cells expressing Venus fluorescence, D-serine significantly inhibited growth and invasiveness of hG008 cells (Fig. 3J and K). Thus, brain tumor cells showed consistent vulnerability to D-serine via inhibition of one-carbon metabolism.

D-serine induces apoptosis by interfering with one-carbon metabolism in neuroprogenitor cells

Neural stem cells have similar characteristics to neural tumor cells with high proliferative capacity(Reya et al., 2001). To test whether D-serine also affects immature neurons, we made a primary culture of neuroprogenitor cells (NPCs) from telencephalon in E14 mice in serine-free Neurobasal medium, treated with D- or L-serine from DIV1. At DIV7, L-serine treatment significantly increased the number of neurons with a condensed neurites compared to vehicle-treated neurons. In contrast, D-serine-treated culture had fewer number of neurons with sparse neurites, suggesting that D-serine has a negative impact on maturation of NPCs (Fig. 4A). Furthermore, D-serine activated cleavage of caspase-3 dose-dependently in Nestin-positive NPCs and Tubb3+ developing neurons 48 h after treatment, whereas L-serine had no effect (Fig. 4B-D, Fig. S6A-C). In contrast, vesicular glutamate transporter 2 (vGlut2)-positive mature excitatory neurons, glutamate decarboxylase-67 (Gad67)-positive mature inhibitory neurons, and glial fibrillary acidic protein (Gfap)-positive glial cells did not undergo apoptosis under D- or L-serine treatment (Fig. 4B and Fig. S6D and E), suggesting that mature neurons and astrocytes are resistant to D-serine. Consistently, D-serine treatment at DIV7 did not activate cleavage of caspase-3 or reduce proliferation of neurons (Fig. 4E and F). Susceptibility of NPCs was pronounced in the presence of D-serine, but was not caused by other D-amino acids, such as D-aspartate, D-alanine, or D-proline (Fig. S7A), which can be detected in mammalian intestinal bacteria(Gonda et al.,

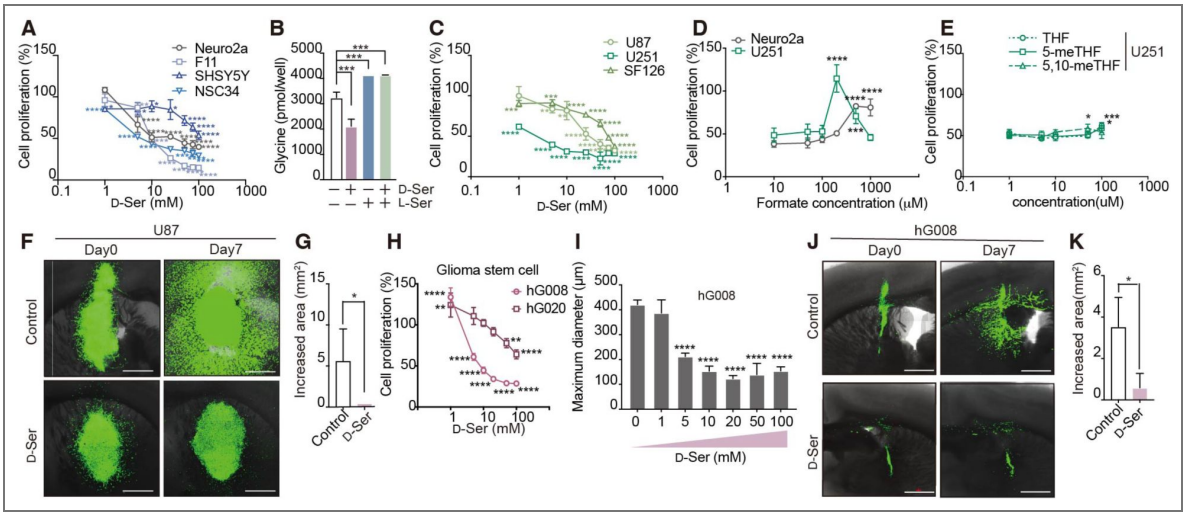

Fig. 3. **D-serine attenuates proliferation of neural tumor cells A, C,**

H. Relative proliferation of neuroblastoma cell lines (**A**), glioblastoma lines (**C**), and glioma stem cells (**H**) treated with D-serine at indicated doses were analyzed 48 h after treatment (n = 4, each). Cells with the vehicle treatment were used as 100% proliferation controls. **B**. Glycine concentration in Neuro2a cells was quantified by HPLC at 48 h after treatment with D- and/or L-serine (n = 3). **D, E**. Cell proliferation was assessed at 48 h after treatment with 5 mM D-serine and formate/folates at indicated doses (n = 4). Cell proliferation was compared to that of cells treated with D-serine alone. **F, G, J, K.** *Ex vivo* slice cultures of mouse brain transplanted with U87 (**F, G**) or hG008 cells (**J, K**) expressing Venus fluorescence were treated with D-serine. Representative images of the xenograft at 0 and 7 days after treatment are shown (**F, J**). Increased area of fluorescence after 7 days of culture was measured (**G, K**). **I**. Sphere diameters of glioma stem cells (hG008) were measured at 7 days after treatment with D-serine at indicated doses. Data are presented as the mean ± s.e.m. (**A-E, G-I,** and **K**). Statistical significance was evaluated by one-way ANOVA followed by Dunnett's post-hoc test (**B, D,** and **E**) or by Student's t-test (**G** and **K**).

2023). As D-serine is a coagonist of NMDA receptors(Mothet et al., 2000; Wolosker and Balu, 2020), we further tested whether D-serine neurotoxicity involves NMDA receptors(McNamara and Dingledine, 1990). Blockade of Ca^{2+} influx through NMDA receptors by a non-competitive NMDA antagonist, MK-801, or of D-serine-binding to the receptors by 5,7-dichlorokynurenic acid (DCKA) did not attenuate D-serine neurotoxicity (Fig. S7B and C). Also, inhibition of the receptor's downstream signal by *N*-nitro-L-arginine methyl ester (L-NAME) or an antioxidant (glutathione) did not affect enhanced cleavage of caspase-3 by D-serine (Fig. S7D and E), although D-serine decreased cellular glutathione (Fig. S7F). Thus, our findings suggest that apoptosis triggered by D-serine in NPCs does not mediate NMDA receptors. To test whether apoptosis triggered by D-serine involves amino acid metabolism, we supplemented NPCs treated with D-serine from DIV1 with various L-amino acids or glycine. As expected, among L-amino acids and glycine, L-serine specifically and dose-dependently inhibited cleavage of caspase-3 caused by D-serine (Fig. 4G and H), which is consistent with competitive mitochondrial transport of serine enantiomers (Fig. 2F). Moreover, similar to the observation that 5,10-methylene THF rescued neural tumor cell growth (Fig. S5I), 5,10-methylene THF restored D-serine-induced cleavage of caspase-3 in NPCs (Fig. 4I), suggesting that D-serine interferes with L-serine functions, such as its role in one-carbon metabolism.

To further rule out alternative mechanisms, we examined whether D-serine disrupts L-serine-mediated lipid synthesis. During the neuronal development, NPCs require L-serine to synthesize membrane sphingolipids and phospholipids that support cellular expansion and neurite elongation (Fig. S8A). A comparative lipidomic study using NPCs revealed that D-serine treatment increased phosphatidylserine, but not sphingolipids or other phospholipids (Fig. S8B and C). To test whether D-serine is incorporated into phosphatidylserine, we extracted membrane lipids, hydrolyzed phosphatidylserine using phospholipase D (PLD) to release serine, and quantified serine enantiomers using 2D HPLC (Fig. S8D and E). Phosphatidylserine extracted from NPCs cultured without D-serine harbored only L-serine, but supplementation with D-serine significantly increased phosphatidyl-D-serine and decreased phosphatidyl-L-serine (Fig. S8F). This effect was abolished by co-supplementation with L-serine (Fig. S8F). However, exogenous phosphatidyl-L-serine failed to rescue D-serine-induced apoptosis (Fig. S8G), indicating that composition of membrane phosphatidyL-serine is not the primary cause of cell death. Together, these results demonstrate that D-serine impairs the survival of immature neural cells not by modulating neurotransmission or lipid metabolism, but by disrupting one-carbon metabolism through competition with L-serine.

Enantiomeric shift of serine metabolism during neural development

Serine metabolism is fundamental to multiple cellular functions and crucial for neural development. To gain a functional overview of how serine chirality contributes to these processes, we monitored serine enantiomers and glycine using the 2D HPLC system. L-serine levels in the mouse cerebral cortex showed a gradual increase during embryonic development, dropped at birth, and remained at a concentration above 500 nmol/g thereafter (Fig. 4J). As glycine synthesis depends in part on L-serine, glycine showed a similar trend to L-serine in embryo and maintained levels above 800 nmol/g after birth (Fig.4J). On the other hand, D-serine was trace during the embryonic stage, but increased gradually after birth. Therefore, the D/L-serine ratio exhibited a steady increase after birth and reached 0.4 in mature mouse brain (Fig. 4J). The dynamics of serine enantiomers during the brain development is corroborated by transcriptional profiles associated with serine chiral metabolism. Bulk RNA-seq data of developing neurons derived from mouse embryonic stem cells (ESC)(Hubbard et al., 2013) or human inducible pluripotent stem cells (iPSC)(Burke et al., 2020) in public databases showed that diverse transcriptions found in the phosphorylated pathway of L-serine synthesis (PHGDH, PSAT1, and PSPH), one-carbon metabolism (SHMT1/2, MTHFD1/2, and MTHFD1/2L), and nucleic acid synthetic pathway (DHFR, TYMS, MTHFS) decrease along with the neuronal maturation (Fig. S9A and B). In contrast, D-serine-synthetic enzyme SRR increases after days *in vitro* 7 (DIV7) in ESC-derived

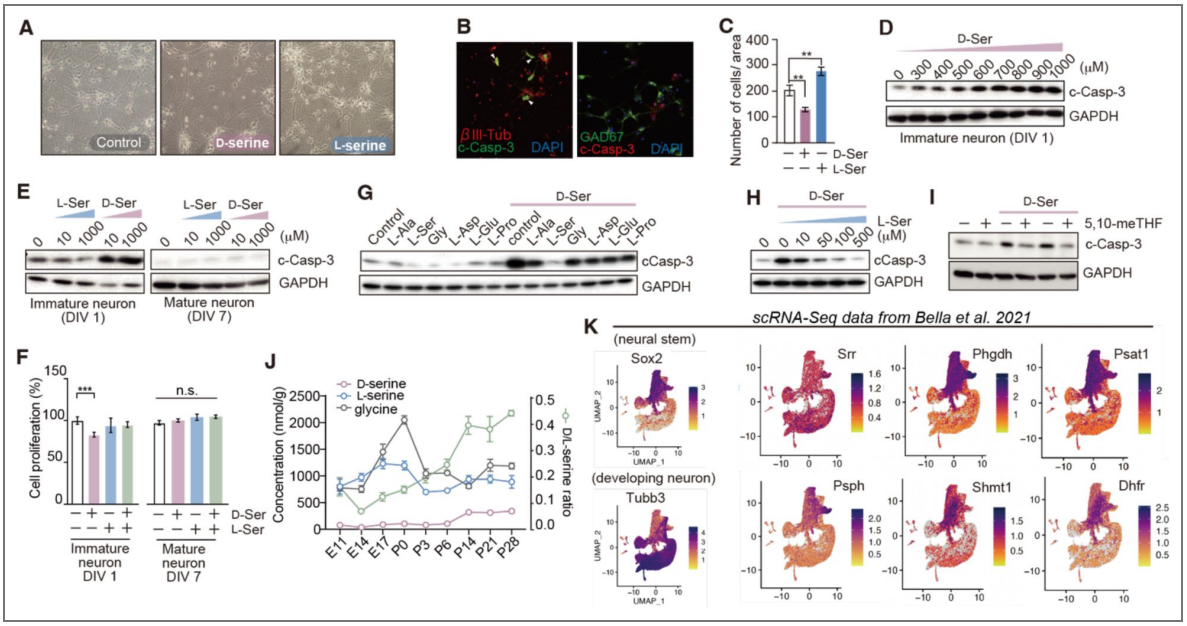

Fig. 4. Developmental transition of serine enantiomer synthesis and enantio-selective effects on immature and mature neurons

A. Light microscopic view showing primary cultured NPCs treated with 1mM D- or L-serine for 7 days in serine-free media. **B.** Immunofluorescent images are NPCs treated with 1 mM D-serine for 48 hours. The left panel shows the co-staining with antibodies against cleaved caspase-3 (c-Casp-3, green), βIII Tubulin (red), and DAPI (blue). The right panel shows the co-staining with antibodies against c-Casp-3 (red), GAD67 (green), and DAPI (blue). **C**. Numbers of NPCs per area at DIV7 in (**A**) were counted (n = 4). **D.** Western blots indicate cleavage of caspase-3 in cultured neurons at 48 h after treatment of D-serine with indicated doses. **E.** Western blots indicate cleavage of caspase-3 in cultured neurons at 48 h after treatment of serine enantiomers with indicated doses starting at DIV1 (immature) or DIV7 (mature neurons). **F.** Relative cell proliferation of NPCs after 48 h treatment of 1mM serine enantiomers was analyzed (n = 4). **G. H.** Cleavage of caspase-3 in NPCs induced by 1mM D-serine was observed in the presence of various L-amino acids (1 mM each) (**G**) or L-serine at indicated doses (**H**). **I**. Cleavage of caspase-3 in NPCs triggered by 2 mM D-serine was observed in the presence or absence of 40 μM 5,10-meTHF. **J**. Concentrations of serine enantiomers and glycine and the ratio of serine enantiomer concentrations in the cerebrum during development (n= 3-4). **K**. Single-cell transcriptome profiles of enzymes involved in serine metabolic pathways during mouse brain development. Original data were from Bella et al (Bella et al., 2021). Data are plotted as the mean ± s.e.m. Statistical significance was evaluated by One-way ANOVA followed by Dunnett's post hoc test (**C**). All *in vitro* data are the representative of at least two independent experiments.

neurons and NPC/Rosetta cells (DIV14-21) in human iPSC-derived neurons. Subunits of NMDA receptors, including Grin1/GluN1, to which D-serine or glycine binds, are expressed in matured neurons after DIV16 of mouse ESC-derived cells (Fig. S9A) or those at DIV49 of human iPSC-derived cells (Fig. S9B). Alterations of serine metabolism during development were further supported by single-cell RNA-seq performed by Bella et al.(Bella et al., 2021), which provides crucial information about the trajectory of differentiating cells and cell type-specific transcriptional profiles during mouse brain development *in vivo*. Uniform manifold approximation and projection (UMAP) illustrates that Sox2+ neural progenitor cells (cluster 1, 7, 9, and 11) express L-serine synthetic enzymes (Phgdh, Psat1, Psph) and folate cycle enzymes (Tyms, Mthfd1, Dhfr) as well as a proliferation marker gene Mki67 (Fig. 4K and Fig. S9C and D). Apoe+ and Aldh1l1+ astrocytes (cluster 7) also express L-serine synthetic pathways, whereas Tubb3+ mature neurons (cluster 0, 2, 3, 4, 5, 8, 12, 13, 15, 16, 19, 22) do not express serine synthetic enzymes (Fig. 4K and Fig. S9C and D). Tubb3+ neurons weakly express Srr at P4 but not E10 (Fig. S9E). These transcriptional profiles suggest that reduction of L-serine biosynthesis during neural development accompanies inactivation of the folate cycle and one-carbon metabolism, and that mature neurons start to synthesize D-serine. Given that the folate cycle and one-carbon metabolism, essential for nucleic acid biosynthesis, are the metabolic signature of cell proliferation, NPCs lose their metabolic activity for proliferation in the embryonic stage but instead develop the capacity for neurotransmission during the postnatal development. Although we found that D-serine inhibits L-serine-dependent one-carbon metabolism, its endogenous production is minimal in neural stem/progenitor cells and increases only after the onset of neuronal maturation, coinciding with the upregulation of Srr. Thus, D-serine is unlikely to play a direct role in regulating proliferative metabolism during early neurodevelopment. Rather, its selective synthesis in post mitotic neurons appears metabolically rational, aligning with their shift from proliferation to neurotransmission.

Discussion

We found that D-serine competes with mitochondrial transport of L-serine, thereby limiting substrate availability for Shmt2 and suppressing one-carbon metabolic flux. This metabolic interference reduces glycine and formate levels and alters downstream pathways including polyamine synthesis. Functionally, under L-serine limited conditions, D-serine suppresses the proliferation and survival of immature neural cells, independent of NMDA receptor activation. Moreover, we demonstrated that endogenous D-serine production increases postnatally in tandem with neuronal maturation, whereas early progenitor cells remain devoid of D-serine synthetic enzyme. In addition to the known role of D-serine in the functional maturation of neurons, our findings identify a previously unrecognized stereoselective role of D-serine in cellular metabolism, and provide a rationale for its selective synthesis in mature neurons where proliferative metabolic activity is no longer required.

In the central nervous system, L-serine increases during fetal development and declines rapidly after birth, while D-serine increases only after birth and reaches the mature levels within a few weeks (Fig. 4J). This postnatal increase of D-serine is consistent with a previous report in rats (Hashimoto et al., 1993) and can be explained by a gradual increase of Srr after birth in mouse glutamatergic neurons (Fig. S9A and E) or forebrain tissue (Folorunso et al., 2021; Miya et al., 2008; Wang and Zhu, 2003). In the mature brain, the majority of Srr is restricted to neurons (Balu et al., 2014; Miya et al., 2008), which is consistent with the idea that D-serine synthesis accompanies neuronal maturation. In contrast, PHGDH is not expressed in neurons, but is confined to astrocytes in mature brain (Yang et al., 2010). This astrocyte-specific expression of the L-serine synthetic enzyme accords with our findings that a set of genes encoding enzymes for L-serine synthesis and one-carbon metabolism are downregulated in mature neurons, but confined to a cell population with mature astrocyte markers (Fig. 4K and S9). Given that astrocytes require one-carbon metabolism for proliferation and that neurons prioritize neurotransmission, such cell-type specific and enantio-selective serine metabolism in the matured brain appears to be a reasonable metabolic compartmentalization. Indeed, loss of D-serine synthesis does not trigger developmental abnormalities (Miya et al., 2008), but causes functional

or structural abnormalities in mature neurons (Balu et al., 2012; Basu et al., 2009; Horn et al., 2017; Sultan et al., 2015). Furthermore, supplementation with D-serine in the neonatal brain enhances functional development of forebrain neurons (Nomura et al., 2016). These findings suggest that D-serine primarily contributes to functional maturation rather than differentiation of NPCs.

While D-serine is important for functional maturation of neurons as well as excitatory neurotransmission through NMDA receptors(Basu et al., 2009; Horn et al., 2017; Mothet et al., 2000), D-serine has an inhibitory effect on proliferation and triggers apoptosis in NPCs and other immature cells (Fig. 3 and 4). Extracellular D-serine is a classic trigger of excitotoxicity in neurons (Patel et al., 1990; Shleper et al., 2005), whereas it induces intracellularly necrosis/apoptosis in renal tubular cells at high doses (Kaltenbach et al., 1979; Williams and Lock, 2004). Excitotoxicity of D-serine has been reported in mature neurons in ischemic or neurodegenerative diseases and mediates NMDA receptors (Inoue et al., 2008; Mustafa et al., 2010; Sasabe et al., 2007; Shleper et al., 2005). In contrast, the D-serine toxicity, which we observed in neural culture, occurs only in immature neurons, and can be rescued by L-serine or intermediates of the one-carbon metabolism, but not by inhibitors of NMDA receptors or their downstream signals (Fig. 4G-I and S7). The latter toxicity reported in kidneys mediates hydrogen peroxide generated through degradation of D-serine by a D-serine catabolic enzyme, D-amino acid oxidase, plentiful in kidneys (Maekawa et al., 2005). Nonetheless, D-amino acid oxidase is not expressed in neurons or in the forebrain (Gonda et al., 2022). Furthermore, as we could not find protective effects by an antioxidant against D-serine toxicity in immature neurons (Fig. S7E), involvement of oxidative stress by hydrogen peroxide is unlikely. Thus, D-serine toxicity observed here appears mechanistically distinct from previously reported forms. Importantly, Okada et al., suggests that renal tubular toxicity by D-serine can be ameliorated by L-serine (Okada et al., 2017). Although the authors did not demonstrate involvement of one-carbon metabolism in D-serine toxicity in the kidneys, their observation could be associated with our findings that D-serine functionally competes with L-serine (Fig. 4H).

L-serine can fuel one-carbon metabolism through both cytoplasmic Shmt1 and mitochondrial Shmt2; however, the relative contribution of these pathways is likely to depend on cell type, differentiation state, and proliferative demand. Consistent with this notion, the differential sensitivity of immature and mature primary cortical neurons to D-serine (Fig. 4E) may reflect differences in compartmentalized one-carbon metabolism. On the other hand, we demonstrated that D-serine does not directly inhibit Shmt2 activity (Fig. 2D). Given that both Shmt1 and Shmt2 exhibit strong stereoselectivity for L-serine and do not utilize D-serine as a substrate (Miyamoto et al., 2024), the suppression of one-carbon metabolism observed in this study is more likely explained by altered mitochondrial L-serine transport rather than direct inhibition of Shmt reactions. Future isotope tracing studies using labeled L-serine will be valuable to dissect how D-serine influences the balance between cytosolic and mitochondrial one-carbon fluxes. Notably, the mitochondrial branch of one-carbon metabolism plays a critical role during embryonic development (Ducker and Rabinowitz, 2017; MacFarlane et al., 2008), highlighting mitochondrial L-serine transport as a key regulatory step for cellular proliferation during development. A seminal study by Kory et al. demonstrated that SFXN1 and its homologs mediate L-serine transport to mitochondria(Kory et al., 2018), and further showed *in vitro* that D-serine competes with L-serine transport through SFXN1. Together with our *in vivo* L-serine competition assays performed in the presence and absence of Sfxn1 (Fig. 2F and S3), these findings support a model in which D-serine suppresses mitochondrial one-carbon metabolism by competing with mitochondrial L-serine transport mediated by multiple members of the Sfxn family rather than Sfxn1 alone.

We show that D-serine attenuates cell proliferation by inhibiting one-carbon metabolism (Fig. 3). The concentration of D-serine required to inhibit proliferation in our system exceeds reported physiological concentration (~300 μM). However, local intracellular concentrations of D-serine may be substantially higher than those estimated from homogenized cerebral tissue, particularly because Srr is localized within neuronal cytoplasm(Miya et al., 2008) (Miya et al, 2008).

Moreover, elevated D-serine levels have been reported in adult-onset neurodegenerative disorders including amyotrophic lateral sclerosis and Alzheimer's disease, in which D-serine degradation is impaired (Madeira et al., 2015; Mitchell et al., 2010; Sasabe et al., 2007). Adult neurogenesis is reduced in these pathological conditions (Cao et al., 2024; Galán et al., 2017; Moreno-Jiménez et al., 2019; Sasabe et al., 2012), raising the possibility that dysregulated D-serine metabolism contributes to impaired neurogenesis (Sultan et al., 2013; Zhao et al., 2019). Nevertheless, proliferating neural progenitor cells express little Srr, making it unlikely that physiological D-serine concentrations alone determine cell fate during normal development. Rather, our findings suggest that the progressive increase of D-serine during brain maturation may contribute to a developmental metabolic shift that interferes with efficient L-serine-dependent one-carbon metabolism in postmitotic neurons. One-carbon metabolism is essential for nucleotide synthesis and methylation in proliferative tissues(Ducker and Rabinowitz, 2017), and immature neural cells appear particularly susceptible to D-serine-mediated metabolic perturbation (Fig. 4). Consistent with the importance of this pathway during development, deficiencies of either L-serine or folate lead to congenital abnormalities of the central nervous system in humans(Acuna-Hidalgo et al., 2014; Beaudin and Stover, 2009). Furthermore, *de novo* serine synthesis and mitochondrial one-carbon pathway are required for the proliferative phenotype in a large variety of human cancers (Jain et al., 2012; Labuschagne et al., 2014; Lee et al., 2014; Lewis et al., 2014; Nilsson et al., 2014). The broad anti-proliferative effects of D-serine on neural tumor cells (Fig. 3) are consistent with the conclusion that D-serine suppresses mitochondrial one-carbon metabolism. Although we tested only neural tumor models, the underlying mechanism may extend to wide range of cancers with varying dependence on mitochondrial one-carbon metabolism. Since cancer genetics indicate two pathways not targeted by existing therapies, de novo serine synthesis and mitochondrial one-carbon metabolism (Ducker and Rabinowitz, 2017; Newman and Maddocks, 2017), inhibition of mitochondrial L-serine transport to disconnect these two pathways may represent a potential therapeutic strategy. However, since D-serine has neuro-modulatory function through the NMDA receptor and potential nephrotoxicity (Guercio and Panizzutti, 2018; Meftah et al., 2021; Wolosker and Balu, 2020), direct administration of D-serine itself may have limited therapeutic applicability. Future studies may therefore explore optimized D-serine derivatives or delivery strategies that preserve metabolic effects while reducing NMDA receptor-mediated activity.

The role of D-amino acids in cellular metabolism and neural development is only beginning to be understood. In addition to D-serine, Srr is capable of producing other D-amino acids, including D-cysteine, raising the possibility that multiple amino acid enantiomers contribute to developmental and metabolic regulation. Recent studies suggest both overlapping and distinct functions of D-serine and D-cysteine. For example, neural deletion of Srr reduces adult neurogenesis and alters lipid metabolism in the subventricular zone (Roychaudhuri et al., 2023) whereas embryonic Srr knockout increases $Sox2^{+}$ neural progenitor cells, a phenotype proposed to involve D-cysteine rather than D-serine. Consistent with this idea, D-cysteine levels peak during early embryogenesis and decline during development, in contrast to the postnatal increase in D-serine (Semenza et al., 2021). Beyond neural development, D-cysteine has also been implicated in broader metabolic regulation, including tumor suppression through inhibition of cysteine desulfurase (NFS1) (Zangari et al., 2025) and modulation of insulin secretion via DNA methylation (Roychaudhuri et al., 2024). Given the close connection between L-serine metabolism, transsulfuration pathways, and S-adenosyl-methionine (SAM)-dependent methylation reactions, D-serine-mediated inhibition of mitochondrial L-serine transport may also influence cysteine and methylation metabolism. However, supplementation with SAM, methionine, or glutathione did not rescue D-serine-induced growth inhibition in our system (Fig. S5J), suggesting that these pathways are not the primary drivers of the observed phenotype. Together, these findings suggest that developmental-stage-dependent production of D-serine and D-cysteine by Srr may contribute to coordination of proliferation, differentiation, and metabolic state during neural development and disease.

Neuronal metabolism exhibits the unique property of serine isomerization to adapt to functional maturation (Fig. 4). Consequently, mature cerebrum contains sub-millimolar levels of D-serine, which account for a quarter of total serine in the cerebrum and are about 100-times higher than

blood levels(Miyoshi et al., 2009). This active stereo-conversion of serine in the cerebrum is unique to mammals among vertebrates(Nagata et al., 1994), implying that neurophysiological functions of D-serine are inseparable from the functional evolution of the cerebrum in higher vertebrates. Indeed, whereas either D-serine or glycine are required to activate NMDA receptors, D-serine binds to the GluN1 subunit of NMDA receptors with higher affinity than does glycine(Furukawa and Gouaux, 2003) and also has an inhibitory effect on the GluN3 subunit by competing with glycine(Chatterton et al., 2002). Therefore, extracellular D-serine appears to be evolutionarily important as a neurotransmitter, with different properties than glycine. On the other hand, the inhibitory function of intracellular D-serine against mitochondrial one-carbon metabolism (Fig. 1 and 2) may be an evolutionarily inherited trait from the biological ancestors of mitochondria, bacteria(Archibald, 2015). Notably, bacteria depend on one-carbon metabolism for their growth, and D-serine attenuates bacterial growth by inhibiting L-serine metabolism(Cosloy and McFall, 1973). Therefore, bacterial sensitivity to D-serine may reveal a common pathway for inhibition of mitochondrial one-carbon metabolism by D-serine. Given that neurons do not express enzymes for mitochondrial one-carbon metabolism upon maturation (Fig. S9), evolutionary acquisition of neurophysiological functions of D-serine without cytotoxicity may be due to the fact that mature neurons are not proliferative and do not depend on this conserved metabolic feature of mitochondria. While complete understanding of the significance of serine stereo-conversion in the developmental brain will require further studies to illuminate neurogenesis under spatio-temporally controlled D-serine, our findings provide a basis to understand the significance of maintaining serine enantiomer balance in the central nervous system.

Materials and Methods

Animals

All animal experiments were approved by the institutional Animal Experiment Committee and conducted in accordance with Institutional Guidelines on Animal Experimentation at Keio University. C57BL/6Jjcl mice were purchased from CLEA Japan (Tokyo, Japan). ICR mice were from Charles River Laboratories (Wilmington, MA, USA). Female BALB/c nude mice (BALB/cSlc-nu/nu) were from Japan SLC (Shizuoka, Japan). Mice were raised in 12 h light and dark cycle with free access to food (CE-2, CLEA Japan) and water in the specific pathogen-free environment.

Antibodies

Rabbit polyclonal antibodies to cleaved caspase-3 and to GAPDH were purchased from Cell Signaling Technologies (Danvers, MA, USA). Mouse monoclonal antibodies to GFAP, vGLUT2, and GAD67 were from Agilent Technologies (Santa Clara, CA, USA), Abcam (Cambridge, UK), and Merck Millipore (Darmstadt, Germany), respectively.

Cell culture and proliferation assay

NPCs and primary cultured astrocytes (PCAs) were prepared as follows. Telencephalon tissues from ICR mice were dissected at the embryonic day 14, treated with papain, and disassembled by pipetting. The cell suspension was passed through a 40-μm filter, centrifuged, and resuspended in a Neurobasal medium without serine (NB-S). NB-S was manufactured by Research Institute for the Functional Peptide (Yamagata, Japan) based on the concentration of each chemicals in Neurobasal media (ThermoFisher: 21103049) described in the technical resources at ThermoFisher. NPCs were cultured in NB-S supplemented with 2% B-27 supplement (ThermoFisher, Waltham, MA, USA), 1% Penicillin-streptomycin solution (ThermoFisher), and 1% GlutaMAX supplement (ThermoFisher). For PCAs, NPCs were differentiated into astrocytes within 7 days by changing the medium to D-MEM (high glucose) (Fujifilm Wako, Tokyo, Japan) supplemented with 10% FBS (A5256701, ThermoFisher) and 1% Penicillin-Streptomycin at 24 h *in vitro*.

Neuroblastoma (Neuro2a, F11, NSC34, and SHSY5Y) and glioma (U87, U251, SF126) cell lines were cultured in MEM supplemented with 10% dialyzed FBS (S-FBS-NL-065, SERENA Europe GmbH, Brandenburg, Germany), 5 mM D-glucose, 65 μM sodium pyruvate, 1 × MEM vitamin solution (ThermoFisher), 2 mM L-glutamine, 0.15 mM L-proline, 0.15 mM L-alanine, 0.15 mM L-aspartic acid, 0.15 mM L-glutamic acid, and 0.34 mM L-asparagine (-SG media) as described in Tajan et al. (Tajan et al., 2021), unless otherwise noted. Proliferation of NPCs, PCAs, and cancer cell lines was analyzed with Cell Counting Kit-8 in accordance with the manufacture's protocol (Dojindo, Kumamoto, Japan).

Western blot

NPCs were harvested in a lysis buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 20 mM ethylenediaminetetraacetate (EDTA), 1% TritonX-100, protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA)]. Protein concentration was analyzed with a BCA assay kit (ThermoFisher), and 20-80 μg of protein were subjected to SDS-PAGE. Then, proteins were transferred to the PVDF membranes and blocked with 10% skim milk in PBST. Proteins were detected with anti-cleaved caspase-3 antibody (clone: 5A1E) (Cell Signaling Technology, MA, USA), anti-Sfxn1 antibody (HPA019543) (Sigma-Aldrich), or ant-GAPDH antibody (clone: 14C10) (Cell Signaling Technology), and HRP-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, PA, USA).

Metabolomics

Cells were washed twice with 10 mL of PBS(-). The PBS(-) was aspirated, and 1 mL of MeOH containing internal standards was added to the dish. The dish was left for 10 min, and a sample solution was obtained and transferred to a tube. For metabolite extraction, the tube was vortexed and subsequently centrifuged at 20,380×g for 10 minutes at 4 °C (MDX-310, TOMY Seiko, Tokyo, Japan). The 150 μL of supernatant was transferred to another tube and dried by centrifugation at 1,600 rpm (366×g) for 90 min at room temperature (VC-96W, Taitec, Saitama, Japan). Then, 10 μL of 90% MeOH were added, 30 μL of H_2O were added and mixed, and then the tube was centrifuged at 20,380×g for 10 minutes at 4 °C. The 20 μL of supernatant were transferred to a vial and injected into the LC-MS system.

The LC-MS instrument has previously been described in detail(Fuse et al., 2020). Briefly, an Agilent Technologies 1290 Infinity LC system and a G6230B time-of-flight MS (TOF–MS) (Agilent Technologies, Santa Clara, CA, USA) were used. Each sample was analyzed in positive and negative modes. Conditions for the analysis in positive mode were set as described previously, with slight modification(Tomita et al., 2018). The temperature of the LC columns was set at 40 °C. For negative mode, the chromatographic separation was performed using an ACQUITY HSS T3 column (2.1 i.d. × 50 mm, 1.8 μm; Waters, Milford, MA, USA) at 30 °C. The mobile phase, consisting of solvent A (0.1% formic acid in water) and solvent B (acetonitrile), was delivered at a flow rate of 0.3 mL/min. In this study, 50–1,200 m/z was used for the MS setting in negative mode.

Data processing from raw LC-MS data to produce a data matrix including metabolite concentration (sample × metabolite) was described previously(Pang et al., 2021). Data analyses were performed using MetaboAnalyst (ver. 5.0, *https://www.metaboanalyst.ca/*) (Pang et al., 2021) to produce volcano plots and to conduct pathway analysis and pattern searches. Overall data profiles were visualized using MeV TM4 (ver. 4.9.0, *https://sourceforge.net/projects/mev-tm4/*).

Quantification of glycine and serine enantiomers

Glycine and serine enantiomers in cell-cultured media or brain tissues were measured using two-dimensional HPLC, as previously described(Gonda et al., 2023; Ishii et al., 2018). Briefly, samples were mixed with 9 volumes of MeOH and centrifuged to remove protein depositions. Supernatants were spin-dried and resuspended in Milli-Q water. Resuspensions were resuspended in 200 mM sodium borate, and then derivatized with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F). NBD-conjugated amino acids were injected into a two-dimensional HPLC system (NANOSPACE SI-2 series, Shiseido, Tokyo, Japan), separated on an octadecylsilyl column (Singularity RP18, 1.0 mm inner diameter (ID) × 250 mm) (designed by Kyushu University and KAGAMI Co. Ltd., Osaka,

Japan), and further separated into enantiomers on a Pirkle-type enantioselective column (Singularity CSP-001S, 1.5 mm ID × 250 mm) (designed by Kyushu University and KAGAMI). Fluorescence of NBD-amino acids was detected at 530 nm with excitation at 470 nm. D- and L-serine peak in a chromatogram were detected and standard curve was drawn by the peak height of those standards. The absolute amount of D- and L-serine were calculated by standard curve method.

Quantification of formate in the culture media

Formate concentration in the culture media was measured using Enzychrom Formate assay kit (BioAssay Systems) according to the manufacturer's protocol.

SHMT2 activity assays

The SHMT2 gene was cloned from cDNA of HeLa cells using primers 5'-GCCCATATGGCCATTCGGGC TCAGCAC-3' (forward) and 5'-GCCCTCGAGATGCTCATCAAAACCAGGCA-3' (reverse) and subcloned into the pET41a vector (Novagen, WI, USA) at restriction enzyme sites of NdeI and XhoI. C-terminally hexahistidine (His_6)-tagged human SHMT2 was purified as follows. *E. coli* BL21 (DE3) pLysS-competent cells were transformed with pET41-SHMT2, cultured at 37 °C in Luria-Bertani medium containing 20 μg/mL kanamycin, and grown until the OD600 reached 0.6-0.7. Isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM, and culturing was continued for an additional 24 h at 20°C. Harvested cells were resuspended in 20 mM sodium phosphate buffer (pH7.4) containing 500 mM NaCl and protease inhibitors (Nacalai Tesque, Kyoto, Japan), and sonicated using a Sonifier 250 instrument (Branson, CT, USA). Lysed cells were centrifuged at 20,000 × g for 10 min at 4 °C, and imidazole was added to the supernatant to a final concentration of 100 mM. The supernatant was applied to a His SpinTrap column (Cytiva, Tokyo, Japan). The column was washed with 20 mM sodium phosphate buffer (pH7.4) containing 500 mM NaCl and 100 mM imidazole. and recombinant protein was eluted with 20 mM sodium phosphate buffer (pH7.4) containing 500 mM NaCl and 500 mM imidazole. Protein fractions were buffer-exchanged into 50 mM Tris-HCl (pH8.0) containing 100 mM NaCl and 10% glycerol using an Amicon Ultra-0.5 centrifugal filter 10K device (Merck Millipore, Darmstadt, Germany).

SHMT2 activity was determined by measuring the production of glycine. The reaction mixture (150 μL) contained 50 mM HEPES-NaOH (pH8.0), 0.2 mM L-serine, 50 μM PLP, 50 μM THF, 1 mM dithiothreitol, and recombinant SHMT2 (2 μg). D-serine was added at concentrations varying from 0.1 to 0.4 mM. The reaction mixture was incubated at 37 °C for 10 min, and then 600 μL of MeOH were added to terminate the reaction. Glycine was derivatized with ortho-phthaldialdehyde (OPA) and *N*-tert-butyloxycarbonyl-L-cysteine (Boc-L-Cys), and an aliquot (10 μL) was injected into an LC-4000 Series HPLC system (Jasco Corp., Tokyo, Japan) equipped with a Mightysil RP-18GP column (150 × 4.6 mm i.d.; Kanto Chemical Co., Tokyo, Japan). A gradient of solvent A (50 mM sodium acetate buffer, pH 6.0) and solvent B (acetonitrile) was applied at a flow rate of 1 mL/min. A linear gradient was applied for 35 min from 10% to 21% solvent B for glycine analysis. Excitation and emission wavelengths for fluorescence detection were 344 nm and 443 nm, respectively.

L-serine transport assay in semi-permeabilized cells

Neuro2a cells were seeded on poly-D-lysine coated 24-well-plates and transfected with either Sfxn1-targeting siRNA (ON-TARGETplus Mouse Sfxn1 (14057) siRNA SMARTpool, Dharmacon) or non-targeting control siRNA (ON-TARGETplus Non-targeting siRNA#1, Dharmacon) using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions. Cells were cultured for 3 days after transfection and then subjected to the transport assay. Transport of L-[^{3}H]serine was examined in semi-permeabilized cells. For plasma membrane permeabilization, cells were treated with saponin, an agent that permeabilizes the plasma membrane but leaves membranous organelles intact(Klepinin et al., 2016; Kuznetsov et al., 2008). The optimized saponin concentration and treatment time were verified by optical microscopy. Transport assays were performed as described previously(Lee et al., 2022; Wiriyasermkul et al., 2012) with some modifications. After washing the cells with 37°C pre-warmed Dulbecco's Phosphate Buffered

Saline (DPBS) with 1 mM $MgCl_2$ and 0.25 mM $CaCl_2$, they were incubated for 6 min in the same buffer containing 40 μg/mL saponin (Fujifilm) for plasma membrane permeabilization. Transport of 10 μM L-[^{3}H]serine (100 Ci/mol; Moravek Biochemicals, Brea, CA, USA) with or without D-serine (at indicated concentrations) was measured for 10 min in the same buffer including saponin. After terminating the reaction and cell lysis, an aliquot of the lysate was used to measure protein concentration by BCA protein assay (Takara Bio). The lysate was mixed with OptiPhase HiSafe 3 (PerkinElmer), and radioisotope activity was monitored by LSC-8000 β-scintillation (Hitachi, Tokyo, Japan). Data shown in the figures were those subtracted from uptake values at 0 min. Kinetics of inhibition was fitted to the Nonlinear Regression: dose-response – inhibition model of GraphPad Prism 8.4.

RNA-sequence and analysis

Neuro2a cells were cultured in D-MEM with 10% FBS. The medium was changed to D-MEM(-SG) including 10% dialyzed FBS with or without 5 mM D-serine. Cells were harvested in Trizol after 18 h of incubation at 37 °C in a CO_2 incubator. RNA was extracted from cells according to the manufacture's protocol. mRNA libraries of each sample were prepared using a TruSeq Stranded mRNA library Kit (Illumina, San Diego, CA) according to the manufacturer's protocol and paired end sequences were read by NovaSeq (Illumina). Adapter sequences were trimmed from raw sequences with Trim Galore (*https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/*) and trimmed sequences were mapped to the mouse genome (GRCm38/mm10) using HISAT2 (*https://daehwankimlab.github.io/hisat2/*). Aligned sequences were counted using FeatureCounts (*https://subread.sourceforge.net/*). Differentially expressed genes were identified by DESeq2 (*https://bioconductor.org/packages/release/bioc/html/DESeq2.html*). Gene Set Enrichment analysis was performed to find enriched biological pathways using ClusterProfiler (*https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html*) with the following settings: pAdjustMethod = fd, pvalueCutoff = 0.05, minGSSize = 10, maxGSSize = 400.

Glioma stem cell culture

hG008 and hG020 cells, established from human glioblastoma specimens(Fukaya et al., 2016), were cultured in Ultra-Low attachment cell culture flasks (Corning, Kennebunk, ME, USA) in a medium, in which ingredients were manually mixed in MEM to make DMEM/Ham's F-12 with HEPES without serine and glycine (D/H-SG). The D/H-SG medium was supplemented with 2% B-27 (ThermoFisher), 20 ng/mL recombinant human fibroblast growth factor-basic (PeproTech, Rocky Hill, NJ, USA), 20 ng/mL recombinant human epidermal growth factor (PeproTech), 1000 units/mL recombinant human leukemia inhibitory factor (Nacalai Tesque, Kyoto, Japan), and 1 unit/mL heparin. Sphere formation of hG008 cells (1×10^4 cells/well in 96-well plates with ultra-low attachment surface, Corning) was observed using EVOS M5000 (ThemoFisher) and the maximum diameter of spheres in each well was measured after 7 days of culture.

Ex vivo cancer growth assay

U87 and hG008 cells were transduced with the lentiviral vector CSII-EF-ffLuc, and single-cell suspensions were cultured in 96-well plates. Xenografts were implanted in the brain of female BALB/c nude mice as described previously[25]. Briefly, mice were implanted with Venus fluorescence-labeled U87 (5×10^5 cells/2 μL in PBS) or hG008 (1×10^5 cells/2 μL in PBS) under anesthesia on a stereotaxic apparatus (Narishige Scientific Instrument Lab, Tokyo, Japan). Mice were sacrificed after 6 days (U87) or 35 days (hG008) of implantation. Mouse brains were dissected, cut into a 200-μm slices, and cultured on the Millicell cell culture inserts (Millipore-Sigma, Burlington, MA) placed on a 35 mm glass-bottom dish filled with D/H-SG medium with or without 100 mM D-serine. Fluorescence images were taken with a FV3000 (Olympus, Tokyo, Japan) on days 0 and 7 and analyzed with Image J software (*https://imagej.nih.gov/ij/index.html*).

Statistics

No statistical methods were used to predetermine sample size. Blinding was not performed. No randomization was used. Prism 10 (GraphPad Software) was used for data plotting and statistical analyses. Statistical significance was determined with unpaired t-tests to compare two groups, or one-way analysis of variance (ANOVA) for multiple comparisons when data were normally distributed and had equal variance. ANOVA was followed by Dunnett's post-hoc test (comparison to control) or Tukey's post-hoc test (comparisons among samples).

Data availability

RNA-seq data are available at Gene Expression Omnibus (GEO) with accession number GSE268106

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Additional files

Supplementary movie 1.

Supplementary movie 2.

Supplementary materials.

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Peer reviews

Reviewer #1 (Public review):

Summary:

The authors demonstrate the stereoselective role of D-serine in 1C metabolism showing that D-serine competes with L-serine and inhibits mitochondrial L-serine transport. They observe expression of 1C metabolites in their metabolomics approach in primary cortical neurons treated with L-serine, D-serine and mixture of both. Their conclusions are based on the reduction in levels of glycine, polyamines and their intermediates and formate. Single cell RNA sequencing of N2a cells showed that cells treated with D-serine enhanced expression of genes associated with mitochondrial functions such as respiratory chain complex assembly and mitochondrial functions with downregulation of genes related to amino acid transport, cellular growth and neuron projection extension. Their work demonstrates that D-serine inhibits tumor cell proliferation and induces apoptosis in neural progenitor cells highlighting the importance of D-serine in neurodevelopment.

Strengths:

D-amino acids do not merely function as ligands at receptors but have underlying roles in signaling and metabolism. These roles are just beginning to be uncovered. The authors elucidate the metabolic role of D-serine in the context of neuronal maturation by its suppression of mitochondrial L-serine availability for SHMT2 and 1C flux. This is the strength of the manuscript. The implications for the metabolic role of D-serine in neurons is a highlight and underlines its roles in neuronal metabolism.

Weaknesses:

These are some minor issues that come up on critical assessment of the manuscript and is only intended to strengthen the manuscript. The comments below are based on the revisions made by the authors including the justification of their approach and rebuttal.

(1) Kinetic assessment of D-serine versus L-serine: The authors have made reference to prior work by Miyamoto et al. and justify their rationale. This is acceptable.

(2) Molecular Dynamics simulations while a good first step in modeling interactions at the active site, relies on force fields. The authors state that any elaborate study into longer simulations is beyond the scope and their simulations data are supported by other experimental work. This is justified.

(3) The use of N2a cell line is also justified to reflect the proliferative nature of immature neurons.

(4) With regards to caspase 3 comment, the whole blot is convincing and shows cleaved caspase-3 band at approx. 15 kDa.

(5) Scale Bars are clearly visible and Fig S6 which was earlier S5 is legible. If possible, the authors can include an magnified inset in the merged image to show the clear activation of caspase-3.

(6) Issue of phosphatidyl serine standard in LC-MS is justified by the use of L-serine standard due to lack of availability.

(7) The authors mention about enantiomeric shift of serine metabolism during neural development which appears to be a discussion of prior published data from Hubbard et al 2013, Burk et al 2020, and Bella et al 2021 in Supplementary Figure panels 8 A-E.

The authors justify by citing references to the work which may be acceptable and also the current norms of publication. This reviewer felt contrary to the fact, however it is left to the editors to make a decision on this.

(8) The discussion section has been substantially revised and now reads well.

(9) The relevant references have been cited. In doing so, the work integrates and elucidates a mechanistic and functional role of D-serine in neurons.

(10) Figure S7A in the revised manuscript shows the specificity of D-serine in the cleaved caspase-3 assay which is informative.

Comments on revised version.

This reviewer is satisfied by the effort made by the authors based on the prior comments raised.

https://doi.org/10.7554/eLife.108562.2.sa3

Reviewer #2 (Public review):

Summary:

This study by Suzuki et al. reports an interesting stereo-selective role of D-serine in regulating one-carbon metabolism during neurodevelopment to adapt the functional transition, probably through the competition with mitochondrial transport of L-serine. The authors provide a multi-layered set of evidence, including metabolomics, enzyme assays, mitochondrial transport competition and functional assays in immature/neural progenitor cells, to build up a conceptual integration of D-serine as both a neurotransmitter and a

metabolic regulator in central neural system, which raises a broad potential interest to the neuroscience and metabolism communities.

Strengths:

This work provides a conceptual advance that D-serine is not only serves as a traditional neurotransmitter in central neural system but also critically contributes to metabolic regulation of neural cells. The authors performed solid metabolomic assays to validate the suppressive effect of D-serine on one-carbon metabolic pathway, providing some evidence that D-serine competitively inhibits mitochondrial serine transport, but not directly impairs SHMT2 enzymatic activity. All these data indicate a critical role of D-serine synthesis during neural maturation and suggest a potential translational strategy for targeting serine metabolism in neural tumors.

Comments on revised version.

My previous concerns have been appropriately addressed or discussed in this revised version of manuscript. I have to say that, at this stage, I have no further questions.

https://doi.org/10.7554/eLife.108562.2.sa2

Reviewer #3 (Public review):

Summary:

This manuscript presents a comprehensive and well-executed investigation into the metabolic role of D-serine in the central nervous system. The authors provide solid evidence that D-serine competitively inhibits mitochondrial L-serine transport, thereby impairing one-carbon metabolism. This stereoselective mechanism reduces glycine and formate production, suppresses cellular proliferation, and induces apoptosis in immature neural cells and glioblastoma stem cells. Developmental analyses further reveal a physiological enantiomeric shift in serine metabolism during neurogenesis, aligning with the transition from proliferation to maturation. Overall, the study bridges developmental neurobiology, cancer metabolism, and amino acid transport, uncovering a previously unrecognized metabolic function of D-serine beyond its role in neurotransmission.

Strengths:

(1) The discovery that D-serine inhibits one-carbon metabolism by competing for mitochondrial L-serine transport-rather than through enzymatic inhibition or receptor-mediated signaling-represents a significant and previously underappreciated mechanism. This finding has broad implications for understanding metabolic regulation during neurodevelopment and offers potential relevance for targeting metabolic vulnerabilities in cancer.

(2) The authors integrate metabolomics, mitochondrial transport assays, molecular dynamics simulations, genetic and pharmacologic perturbations, transcriptomics, and both in vitro and ex vivo models. The breadth of experimental approaches, combined with the coherence of the findings across systems, provides strong support for the central conclusions and enhances the overall impact of the study.

(3) The temporal shift in D-/L-serine levels during neurodevelopment is elegantly linked to the transition from proliferative to mature neuronal states. The selective vulnerability of neural progenitors and tumor cells-contrasted with the resistance of mature neurons-highlights a biologically meaningful and potentially targetable metabolic distinction.

Weaknesses:

(1) While the authors attribute D-serine's metabolic effects to competition with mitochondrial L-serine transport, the specific identity of the transporter(s) mediating this process remains undefined. This represents a meaningful mechanistic gap, as the central conclusion depends on D-serine limiting mitochondrial L-serine availability to inhibit one-carbon metabolism.

(2) The effective concentrations of D-serine used in vitro (IC_{50} ≈ 1-2 mM) exceed typical brain levels (~0.3 mM). While the authors acknowledge this, a more focused discussion on whether higher local D-serine concentrations could arise in specific microenvironments-such as synaptic compartments, tumor niches, or pathological states-would help contextualize the in vitro findings and strengthen their physiological relevance. For example, disruptions in D-serine clearance or altered expression of serine racemase and transporters in disease contexts could lead to localized accumulation. Moreover, differences between extracellular and intracellular D-serine pools-and the mechanisms governing their regulation-may further influence its metabolic impact in vivo.

(3) While the manuscript focuses on neural stem/progenitor cells and neural tumors, it remains unclear whether the anti-proliferative effects of D-serine are specific to neural lineages or extend to other highly proliferative non-neural cell types. A brief discussion addressing this point would help clarify the scope of D-serine's metabolic impact and whether its mechanism of action reflects a unique vulnerability in neural cells or a more general feature of proliferative metabolism. This distinction is particularly relevant for assessing the broader therapeutic potential of targeting mitochondrial L-serine transport.

https://doi.org/10.7554/eLife.108562.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *The authors demonstrate the stereoselective role of D-serine in 1C metabolism, showing that D-serine competes with L-serine and inhibits mitochondrial L-serine transport. They observe expression of 1C metabolites in their metabolomics approach in primary cortical neurons treated with L-serine, D-serine, and a mixture of both. Their conclusions are based on the reduction in levels of glycine, polyamines, and their intermediates and formate. Single-cell RNA sequencing of N2a cells showed that cells treated with D-serine enhanced expression of genes associated with mitochondrial functions, such as respiratory chain complex assembly, and mitochondrial functions, with downregulation of genes related to amino acid transport, cellular growth, and neuron projection extension. Their work demonstrates that D-serine inhibits tumor cell proliferation and induces apoptosis in neural progenitor cells, highlighting the importance of D-serine in neurodevelopment.*
>
> *Strengths:*
>
> *D-amino acids are a marvel of nature. It is fascinating that nature decided to make two versions of the same molecule, in this case, an amino acid. While the L-stereoisomer plays well-known roles in biology, the D-stereoisomer seems to function in obscurity. Research into these novel signaling molecules is gathering momentum, with newer stereoisomers being discovered. D-serine has been the most well-studied among the different stereoisomers, and we still continue to learn about this novel neurotransmitter. The roles of these molecules in the context of metabolism is not well studied. The authors*

> *aim to elucidate the metabolic role of D-serine in the context of neuronal maturation with implications for 1C metabolism and in cell proliferation. The metabolic role of these molecules is just beginning to be uncovered, especially in the context of mammalian biology. This is the strength of the manuscript. The authors have done important work in prior publications elucidating the role of D-amino acids. The advancement of the field of D-amino acids in mammalian biology is significant, as not much is known. The presentation of RNA seq data is a valuable resource to the community, however, with caveats as mentioned below.*
>
> *Weaknesses:*
>
> *The following are some of the issues that come out in a critical reading of the manuscript. Addressing these would only strengthen and clarify the work.*
>
> *(1) Kinetic assessment of D-serine versus L-serine: While the authors mention that D-serine is not a good substrate for SHMT2 compared to L-serine, the kinetic data are presented for only D-serine. In a substrate comparison with an enzyme, data must be presented for L-serine as well to make the conclusion about substrate specificity and affinity. Since the authors talk about one versus another substrate, there needs to be a kinetic comparison of both with Km (affinity). (Ref Figure 2 panel).*

We agree with the reviewer that kinetic parameters for L-serine are important for evaluating the substrate specificity of SHMT2. The hydroxymethyl-transferase activity of SHMT2 toward L-serine has been previously characterized by our co-author Tetsuya Miyamoto (Miyamoto et al., FEBS Journal, 2024; PMID: 37700610), which is appropriately cited in the manuscript (line 143). In that study, the kinetic parameters for L-serine were determined, with a *K*m of 0.07 ± 0.009 mM and a *K*cat of 33.5 ± 0.9 min^{-1}. The strong chiral selectivity of SHMT2 for the l-enantiomer in the hydroxymethyl-transferase reaction was also demonstrated in that work. On the other hand, the primary aim of the present study is different from characterizing D-serine as a catalytic substrate for SHMT2. Rather, our goal was to determine whether D-serine interferes with the hydroxymethyl-transferase reaction of SHMT2 by interacting with the L-serine binding site. Accordingly, the analyses shown in Fig. 2B-D were designed to evaluate whether D-serine could structurally occupy or interfere with the L-serine binding pocket of SHMT2. Therefore, our experiments focused on assessing the potential inhibitory effect of D-serine rather than performing a full kinetic comparison of D-serine and L-serine as substrates.

> *(2) Molecular Dynamics simulations, while a good first step in modeling interactions at the active site, rely on force fields. These force fields are approximations and do not represent all interactions occurring in the natural world. Setting up the initial conditions in the simulations can impact the final results in non-equilibrium scenarios. The basic question here is this: Is the simulated trajectory long enough so that the system reaches thermodynamic equilibrium and the measured properties converge? Prior studies have shown mixed results with the conclusion that properties of biological systems tend to converge in multi-second trajectories (not nanosecond scales as reported by the authors) and transition rates to low probability conformations require more time. (Ref Figure 2C).*

We thank the reviewer for raising the important point regarding the limitations of molecular dynamics (MD) simulations, including the dependence on force fields and the potential effects of simulation length and initial conditions. We agree that MD simulations represent approximations of molecular behavior and that longer trajectories may be required to fully explore rare conformational states in biological systems.

In the present study, however, the MD simulations were not intended to provide a comprehensive thermodynamic description of SHMT2 conformational dynamics. Rather, they were used as a structural assessment to evaluate whether D-serine could plausibly occupy the canonical L-serine binding site of SHMT2. As shown in Fig. 2BC and supplementary movie 1,

the simulations did not support stable occupation of the L-serine binding pocket by D-serine. Importantly, this structural observation is consistent with our biochemical data showing that D-serine does not inhibit the hydroxymethyl-transferase activity of SHMT2 when L-serine is used as the substrate (Fig. 2D). Together, these results indicate that the inhibitory effect of D-serine on one-carbon metabolism is unlikely to be mediated through direct inhibition of SHMT2.

As the reviewer correctly notes, it remains possible that D-serine interacts with SHMT2 at sites distinct from the canonical L-serine binding pocket and could exert potential allosteric effects. Indeed, previous work by Miyamoto et al. (FEBS Journal, 2024) demonstrated that SHMT2 exhibits dehydratase activity toward D-serine. However, this reaction is not directly linked to mitochondrial one-carbon metabolism. Therefore, further extensive simulations exploring alternative conformational states or potential allosteric interactions would extend beyond the scope of the present study.

Importantly, the key conclusions of this study do not rely solely on MD simulations but are supported by multiple independent experimental approaches, including metabolomics, enzymatic assays, and mitochondrial transport analyses.

> *(3) The authors use N2a cell line to demonstrate D-serine burden on primary cortical neurons. N2a is an immortalized cell line, and its properties are very different from primary neurons. The authors need to mention a rationale for the use of an immortalized cell line versus primary neurons. The transcriptomic profile of an immortalized cell line is different compared to a primary cell. Hence, the response to D-serine may vary between the two different cell types.*

We thank the reviewer for raising this important point regarding the differences between immortalized cell lines and primary neurons. As the reviewer notes, N2a cells and primary cortical neurons (PCNs) differ in several aspects, including their degree of differentiation and proliferative capacity. We appreciate the opportunity to clarify the rationale for using both systems in this study.

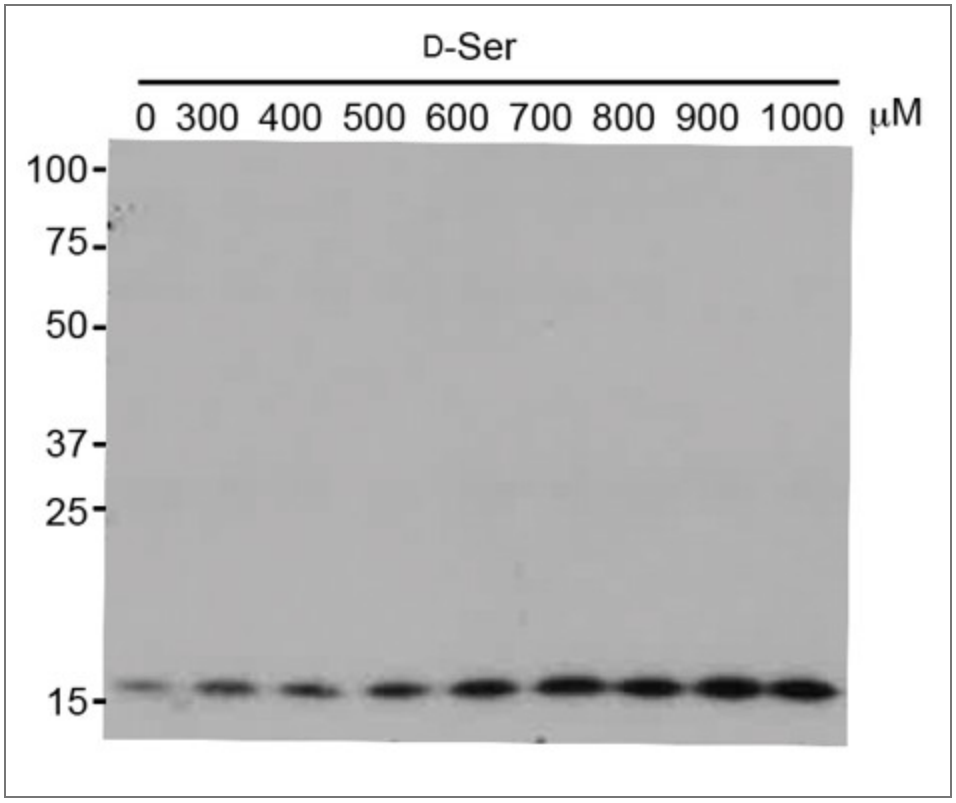
One-carbon metabolism is known to be particularly active in highly proliferative or relatively undifferentiated cells. Primary cortical neurons are initially obtained as immature neuronal populations and gradually undergo maturation during culture (Fig. S6). In our experiments, we observed that sensitivity to D-serine and dependence on one-carbon metabolism were primarily evident in immature neuronal states rather than in fully mature neurons (Fig. 4EF).

In this context, immature PCNs share certain metabolic characteristics with proliferative neural cell lines such as N2a cells. Consistent with this idea, the inhibitory effects of D-serine on one-carbon metabolism and cell proliferation were observed in both immature PCNs and N2a cells (Fig. 1E–G, Fig. 2E, Fig. 3AB, and Fig. 4F). Thus, the use of N2a cells provides a complementary experimental model for studying the metabolic vulnerability of immature neural cells that depend on one-carbon metabolism. Importantly, the key findings were consistently reproduced in primary cortical neurons, supporting the physiological relevance of the observations made in N2a cells. For clarity, we added descriptions in lines 107-108 and 167-168 in our revised manuscript.

> *(4) In Figure 4D, the authors mention that D-serine activates the cleavage of caspase 3. Figure 4D shows only cleaved caspase 3 as a single band. They need to show the full blot that contains the cleaved fragments along with the major caspase 3 band.*

In our experiments, we used an antibody that specifically recognizes cleaved caspase-3 and does not recognize full-length caspase-3 (Cell Signaling Technology, anti-cleaved caspase-3 antibody, clone 5A1E). Therefore, the Western blot detects only the cleaved caspase-3 fragment at approximately 17 kDa, which appears as a single band in the blot (please see

Author response image 1). For clarity, we have also included the antibody information in the Western blot section in the revised Materials and Methods (lines 501-502).



Author response image 1. An original image of western blot for Fig. 4D. An arrow indicates the bands of cleaved caspase-3 (17 kDa).

> *(5) In Figure panel 4, the authors use neural progenitor cells (NPCs). They need to demonstrate that the population they are working with is NPCs and not primary neurons. There must be a figure panel staining for NPC markers like SOX2 and PAX6. Also, Figure S5 needs to be properly labeled. It is confusing from the legend what panels B-E refer to? Also, scale bars are not indicated.*

We thank the reviewer for pointing out these issues. First, we have relabeled and rearranged the figure panels in revised Figure S6B-E, and revised the figure legend to provide a clearer description of each panel. We have also added scale bars to the revised images.

To confirm the identity of neural progenitor cells (NPCs), we performed immunostaining for Nestin, a well-established marker for NPCs, instead of Sox2 and Pax6 (Fig. S6B). Nestin is widely used as a marker for NPCs(Bernal and Arranz, 2018; Bott et al., 2019; Lendahl et al., 1990), and is known to be co-expressed with Sox2 in the mouse embryonic brain(Graham et al., 2003). In addition, Nestin has been identified as a Pax6-bound gene associated with the transcriptional program regulating neural progenitor identity (Thakurela et al., 2016). Consistent with these observations, analysis of published scRNA-seq data from the developing mouse brain (Bella et al., 2021) shows that the RNA expression profile of Nestin (*Nes*) closely parallels those of Sox2 and Pax6 (new Fig. S9C). Based on these lines of evidence, we consider Nestin-positive cells in our cultures to represent NPCs. Importantly, Nestin-positive cells were co-stained with cleaved caspase-3, suggesting that the apoptotic population corresponds to NPCs (Fig. S6B). Together, these observations support that the apoptotic cells observe in our culture correspond to NPCs rather than differentiated neurons.

> *(6) In Supplementary Figure panel 7F, the authors mention phosphatidyl L-serine and phosphatidyl D-serine. A chromatogram of the two species would clarify their presence as they used 2D-HPLC. On an MS platform, these 2 species are not distinguishable. Including a chromatogram of the 2 species would be helpful to the readers.*

We thank the reviewer for this helpful suggestion. As the reviewer correctly noted, mass spectrometry alone cannot distinguish phosphatidyl-D-serine and phosphatidyl-L-serine. To quantify these species separately, lipids were first extracted from cells using the Bligh and Dyer method, followed by phospholipase D treatment to cleave the serine moiety from phosphatidylserine (a schematic of the procedure is shown in Fig. S8D). The released serine was then derivatized with NBD-F and analyzed by 2D-HPLC for enantioselective separation and quantification of d- and L-serine, as described in the Materials and Methods section ("Quantification of glycine and serine enantiomers"). Phosphatidyl-L-serine (Sigma-Aldrich: P0474) was used to generate a standard curve for quantification (Fig. S8E). Because phosphatidyl-D-serine is not commercially available, and because the peak height of free D-serine is equivalent to that of free L-serine in the chromatograms of our 2D-HPLC system, the same standard curve was used to estimate phosphatidyl-D-serine levels.

As requested by the reviewer, we have now added representative chromatograms of d- and L-serine derived from phosphatidyL-serine in NPC samples (new Fig. S8F).

> *(7) The authors mention about enantiomeric shift of serine metabolism during neural development, which appears to be a discussion of prior published data from Hubbard et al, 2013, Burk et al, 2020, and Bella et al, 2021, in Supplementary Figure panels 8 A-E. This should not be presented as a figure panel, as it gives the false impression that the authors have performed the experiment, which is clearly not the case. However, its discussion can well serve as part of the manuscript in the discussion section.*

We appreciate this helpful suggestion. The datasets used in Fig. 4K and Fig. S9 were derived from previously published transcriptomic studies (Hubbard et al, 2013; Burk et al, 2020; Bella et al, 2021). While these studies reported transcriptomic profiles during neuronal development *in vitro* or *in vivo*, they did not specifically analyze serine metabolism, one-carbon metabolism, or D-serine biosynthesis, which are the focus of the present study. Therefore, we reanalyzed these publicly available datasets from a metabolic perspective, focusing on genes involved in serine metabolism and one-carbon metabolism. This re-analysis allowed us to examine a developmental shift in serine enantiomer metabolism, we presented the results as figure panels rather than simply citing the datasets in the Discussion. Re-analysis of publicly available transcriptomic datasets to address new biological questions has become a common approach in genomics and transcriptomics studies.

To avoid the impression that these experiments were performed in this study, we have clearly indicated the original references and clarified this point in the figure legends (Fig. 4K and Fig. S9). These panels are intended to provide supportive evidence for the developmental shift in serine enantiomer metabolism discussed in this study.

> *(8) The entire presentation of the section on enantiomeric shift of serine metabolism during neural development (lines 274-312) is a discussion and should be part of the discussion section and not in the results section. This is misleading.*

We thank the reviewer for this comment. This point overlaps with the concern raised in comment 7. Please see our response to comment 7 for a detailed explanation and the revisions made in the manuscript.

> *(9) The discussion section is not well written. There is no mention of recent work related to D-serine that has a direct bearing on its metabolic properties. In the discussion section, paragraph 1, the authors mention that their work demonstrates the selective*

> *synthesis of D-serine in mature neurons as opposed to neural progenitor cells. This concept has been referred to in prior publications:*
>
> *(a) Spatiotemporal relationships among D-serine, serine racemase, and D-amino acid oxidase during mouse postnatal development. PMID:14531937.*
>
> *(b) D-cysteine is an endogenous regulator of neural progenitor cell dynamics in the mammalian brain. PMID:34556581.*

We thank the reviewer for this helpful suggestion and for drawing our attention to these studies. We have revised the Discussion to better place our findings in the context of previous work related to D-serine.

Specifically, we have added references describing the spatiotemporal relationship between D-serine and serine racemase (Srr) during brain development (PMIDs 14531937 and 33592203) (line 333). These studies highlighted the tissue-level (PMID 14531937) and cellular-level (PMID: 33592203) relationship between Srr expression and development. These studies highlighted the role of D-serine in supporting the functional maturation of neurons during postnatal development. In contrast, our study addresses a complementary question of why D-serine is NOT present during embryonic and early postnatal stages of brain development, when proliferative metabolic activity is high. This question is fundamentally different from the previous reports. Our point is that D-serine is not favorable because it interferes with one-carbon metabolism, which is essential for cell proliferation. Therefore, this concept has not been referred to in prior publications. To clarify this point, we added the following sentence to the Discussion (line 325-328): “In addition to the known role of D-serine in the functional maturation of differentiated neurons, our findings highlight a previously unrecognized, stereoselective regulation of cellular metabolism by D-serine, and provide a rationale for its selective synthesis in mature neurons where proliferative metabolic activity is no longer required’.

We also appreciate the reviewer bringing our attention to the study describing d-cysteine as a regulator of neural progenitor cell dynamics (PMID: 34556581). We have added the description regarding the overlapping and distinct functions of d-cysteine and D-serine to the Discussion (lines 418-436).

> *(10) In the abstract, in lines 101 and 102, the authors mention "how D-serine contributes to cellular metabolism beyond neurotransmission remains largely unknown". In 2023, a paper in Stem Cell Reports by Roychaudhuri et al (PMID:37352848) showed that d and L-serine availability impacts lipid metabolism in the subventricular zone in mice, affecting proliferative properties of stem-cell derived neurons using a comprehensive lipidomics approach. There is no mention of this work even in the discussion section, as it bears directly on l and D-serine availability in neurons, which the authors are investigating. In the discussion section in lines 410-411, the authors mention the role of D-serine in neurogenesis, but surprisingly don't refer to the above reference. The role of D-serine in neurogenesis has been demonstrated in the Sultan et al (lines 855-857) and Roychaudhuri et al references.*

We thank the reviewer for highlighting these relevant studies. We have revised the statement in line 99 to avoid overgeneralization and to reflect that the metabolic roles of D-serine are incompletely understood rather than largely unknown. In addition, we have incorporated discussion of previous works, including the work by Roychaudhuri et al. (2023), into the Discussion section (lines 418-436) of our revised manuscript.

> *(11) Both D-serine and the structurally similar stereoisomer D-cysteine (sulfur versus oxygen atom) have a bearing on 1C metabolism and the folate cycle. With reference to the folate cycle, Roychaudhuri et al in 2024 (PMID:39368613) have shown in rescue*

> *experiments in mice that supplementing a higher methionine diet provides folate cycle precursors to rescue the high insulin phenotype in SR-deficient mice. Since 1C metabolism is being discussed in this manuscript, the authors seem to overlook prior work in the field and not include it in their discussion, even when it is the same enzyme (SR) that synthesizes both serine and cysteine. Since the field of D-amino acid research is in its infancy, the authors must make it a point to include prior work related to D-serine at least, and not claim that it is not known. The known D-stereoisomers are not many, hence any progress in the area must include at least a discussion of the other structurally related stereoisomers.*

We are grateful to the reviewer for drawing our attention to this relevant study.

We have now incorporated the findings from Roychaudhuri et al. 2024 into the Discussion. In that study, Srr-/- mice exhibited reduced levels of DNMT1 and DNMT3A, resulting in reduced DNA methylation activity. Notably, supplementation with a methyl-donor diet (containing choline, betaine, and methionine) restored the aberrant insulin phenotype in Srr-/- mice. These findings are relevant to our study, as DNA methylation depends on S-adenosyl-methionine (SAM), which is generated through one-carbon metabolism.

We have expanded the Discussion to include the relationship between D-serine and d-cysteine, both of which are synthesized by Srr in the revised manuscript (lines 418-436).

> *(12) Racemases (serine and aspartate) in general are promiscuous enzymes and known to synthesize other stereoisomers in addition to D-serine, D-cysteine, and D-aspartate. A few controls, like D-aspartate, D-cysteine, or even D-alanine must be included in their study to demonstrate the specific actions of D-serine, especially in the N2a cell treatment experiments. Cysteine and Serine are almost identical in structure (sulfur versus oxygen atom), and both are synthesized by serine racemase (published). Cysteine has also been very recently shown to inhibit tumor growth and neural progenitor cell proliferation. (PMIDs: 40797101 and 34556581). How the authors' work relates to the existing findings must be discussed, and this would put things in perspective for the reader.*

We thank the reviewer for this comment regarding the need to demonstrate the specificity of D-serine. As shown in Fig. S7A, D-serine, but not other d-amino acids commonly detected in mammals (Gonda et al., 2023), including d-aspartate, d-alanine, and d-proline, induced the cleavage of caspase-3 under the same experimental conditions, supporting the specific effect of D-serine in our system. We agree that d-cysteine shares structural similarity with D-serine and is also synthesized by Srr, suggesting potential functional overlap with D-serine. To place our findings in this context, we have added a paragraph in the Discussion (lines 418-436) describing the similarities of D-serine and d-cysteine. While both molecules may exert anti-proliferative effects, their underlying mechanisms appear to differ. Notably, supplementation with SAM, methionine, or glutathione did not rescue D-serine-induced growth inhibition in our system (Fig. S5J), suggesting that its effects are not primarily mediated through methylation or sulfur metabolic pathways. Instead, D-serine suppresses cellular proliferation by limiting mitochondrial L-serine availability and one-carbon metabolism. These observations highlight mechanistic divergence between D-serine and d-cysteine.

> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *This study by Suzuki et al. reports an interesting stereo-selective role of D-serine in regulating one-carbon metabolism during neurodevelopment to adapt the functional transition, probably through the competition with mitochondrial transport of L-serine. The authors provide a multi-layered set of evidence, including metabolomics, enzyme assays, mitochondrial transport competition, and functional assays in immature/neural*

> *progenitor cells, to build up a conceptual integration of D-serine as both a neurotransmitter and a metabolic regulator in the central neural system, which raises a broad potential interest to the neuroscience and metabolism communities.*
>
> *Strengths:*
>
> *This work provides a conceptual advance that D-serine not only serves as a traditional neurotransmitter in the central neural system but also critically contributes to metabolic regulation of neural cells. The authors performed solid metabolomic assays to validate the suppressive effect of D-serine on the one-carbon metabolic pathway, providing some evidence that D-serine competitively inhibits mitochondrial serine transport, but not directly impairs SHMT2 enzymatic activity. All these data indicate a critical role of D-serine synthesis during neural maturation and suggest a potential translational strategy for targeting serine metabolism in neural tumors.*
>
> *Weaknesses:*
>
> *(1) The detailed mechanism by which D-serine competes with L-serine for its mitochondrial transport is not investigated. For example, although the authors made some discussion, they did not provide direct genetic or biochemical evidence linking these effects to the specific transporters, such as SFXN1.*

We thank the reviewer for this important comment regarding the mitochondrial L-serine transport mechanism. To address this point, we performed additional experiments using N2a cells in which Sfxn1 was knocked down by siRNA. Under semi-permeabilized cell conditions, we newly examined the effect of D-serine on mitochondrial L-serine transport.

Interestingly, even under conditions where Sfxn1 expression was markedly suppressed, D-serine still inhibited mitochondrial D-serine transport. Given that SFXN1 is known to function redundantly with its paralogs (SFXN2–SFXN5) in mitochondrial serine transport (Kory et al., 2018), these findings suggest that D-serine may interfere with L-serine transport not only through SFXN1 but potentially through multiple members of the SFXN transporter family. These new data have been added as Fig. S3, and the corresponding results and discussion have been incorporated into the revised manuscript (lines 158–165 and 379-385).

> *(2) Unlike tumor cells, where SHMT2 usually plays a predominant role in catalyzing serine/THF-derived one-carbon metabolism, normal cells may employ both SHMT1 and SHMT2 to do the work. Even under certain conditions that SHMT2-mediated one-carbon metabolism is suppressed, the activity of SHMT1 could be elevated for compensation. Thus, it is important to investigate whether D-serine affects SHMT1 activity or changes the balance between SHMT1- and SHMT2-mediated one-carbon metabolism. To this aim, the authors are strongly encouraged to perform a metabolic flux assay (MFA) by using 13C-labeled L-serine in the model cells in the presence and absence of D-serine.*

We thank the reviewer for this thoughtful comment regarding the potential contribution of cytosolic SHMT1 to one-carbon metabolism. As the reviewer notes, while mitochondrial SHMT2 is generally considered the predominant enzyme supporting one-carbon metabolism in proliferating or tumor cells, SHMT1 in the cytosol may function in a complementary manner in normal cells. In our experiments using primary cortical neurons, we indeed observed that the sensitivity to D-serine differed between immature and mature neuronal states (Fig. 4E). This observation suggests that the relative contribution of SHMT1- and SHMT2-mediated one-carbon metabolism may vary depending on the differentiation status of the cells.

However, the primary focus of the present study was to investigate the mechanism underlying the anti-proliferative effect of D-serine in proliferative or undifferentiated neural cells. Our data demonstrate that D-serine inhibits one-carbon metabolism primarily by

limiting mitochondrial L-serine availability through inhibition of mitochondrial L-serine transport. Therefore, a detailed analysis of the compensatory balance between SHMT1 and SHMT2 after disruption of mitochondrial serine transport falls beyond the central scope of the present study. Importantly, previous work by Miyamoto et al. (FEBS Journal, 2024) demonstrated that both SHMT1 and SHMT2 exhibit strong stereoselectivity for L-serine in their hydroxymethyl-transferase activity and do not utilize D-serine as a substrate. These findings make a direct effect of D-serine on hydroxymethyl-transferase activity of SHMT1 unlikely.

Nevertheless, we agree that differences in the anti-proliferative effects of D-serine across cell types or differentiation states could reflect variations in cellular dependence on one-carbon metabolism or potential compensation by SHMT1. To address this point, we have expanded the Discussion section to clarify the possible contribution of SHMT1 and the limitations of the present study (lines 367–376). While isotope tracing analysis would be valuable for further dissecting compartmentalized one-carbon fluxes, such analyses would primarily address the relative contributions of SHMT1 and SHMT2 rather than the mitochondrial serine transport step that constitutes the central mechanism identified in this study.

> *(3) A defect in serine-derived one-carbon metabolism may cause multiple cellular stress responses. It is valuable to detect whether cellular NADPH/NADH, GSH, or ROS is altered before and after D-serine treatment.*

We appreciate this insightful comment regarding potential cellular stress responses associated with impaired one-carbon metabolism. Consistent with the reviewer's suggestion, we examined markers related to redox status. Transcriptomic analysis revealed compensatory changes in genes involved in mitochondrial metabolic function, including components of the NADH dehydrogenase complex (Fig. 2IJ and Fig. S4A), suggesting metabolic adaptation to D-serine treatment. In response to the reviewer's comment, we measured GSH levels in NPCs and found that D-serine treatment led to a reduction of GSH (new Fig. S7F), indicating altered redox balance. However, supplementation with exogenous GSH did not rescue D-serine-induced cell death (Fig. S7E). These results suggest that while D-serine induces changes in cellular redox status, including GSH depletion, redox imbalance alone is unlikely to be the primary driver of cell death in this context.

> *(4) The physiological relevance between D-serine and neural cell maturation/death should be further tested and discussed, since the dosage of D-serine used in the in vitro assay is much higher than that in physiological conditions.*

We thank the reviewer for this comment regarding the physiological relevance of the D-serine concentrations used in our study. We agree that the concentrations of D-serine required to compete with L-serine for mitochondrial transport are higher than those typically observed under physiological conditions, which we acknowledged in the Discussion of our manuscript (lines 386-388). Importantly, however, *in vivo*, D-serine levels are tightly regulated in a spatiotemporal manner during brain development, with low levels during embryonic and early postnatal stages and increased levels upon neural maturation. This temporal regulation coincides with the transition from proliferative neural progenitor states to differentiated neurons, thereby limiting the potential for D-serine to interfere with one-carbon metabolism during periods of active cell proliferation. Thus, while the concentrations used *in vitro* may exceed physiological levels, they allow us to uncover a latent metabolic effect of D-serine that may become relevant under specific cellular or developmental contexts. To clarify these points, we have revised the Discussion (lines 388-395) in the revised manuscript to more explicitly address the relationship between D-serine dosage and physiological relevance.

> ***Reviewer #3 (Public review):***
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> *Summary:*

> *This manuscript presents a comprehensive and well-executed investigation into the metabolic role of D-serine in the central nervous system. The authors provide solid evidence that D-serine competitively inhibits mitochondrial L-serine transport, thereby impairing one-carbon metabolism. This stereoselective mechanism reduces glycine and formate production, suppresses cellular proliferation, and induces apoptosis in immature neural cells and glioblastoma stem cells. Developmental analyses further reveal a physiological enantiomeric shift in serine metabolism during neurogenesis, aligning with the transition from proliferation to maturation. Overall, the study bridges developmental neurobiology, cancer metabolism, and amino acid transport, uncovering a previously unrecognized metabolic function of D-serine beyond its role in neurotransmission.*
>
> *Strengths:*
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> *(1) The discovery that D-serine inhibits one-carbon metabolism by competing for mitochondrial L-serine transport-rather than through enzymatic inhibition or receptor-mediated signaling-represents a significant and previously underappreciated mechanism. This finding has broad implications for understanding metabolic regulation during neurodevelopment and offers potential relevance for targeting metabolic vulnerabilities in cancer.*
>
> *(2) The authors integrate metabolomics, mitochondrial transport assays, molecular dynamics simulations, genetic and pharmacologic perturbations, transcriptomics, and both in vitro and ex vivo models. The breadth of experimental approaches, combined with the coherence of the findings across systems, provides strong support for the central conclusions and enhances the overall impact of the study.*
>
> *(3) The temporal shift in D-/L-serine levels during neurodevelopment is elegantly linked to the transition from proliferative to mature neuronal states. The selective vulnerability of neural progenitors and tumor cells-contrasted with the resistance of mature neurons-highlights a biologically meaningful and potentially targetable metabolic distinction.*
>
> *Weaknesses:*
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> *(1) While the authors attribute D-serine's metabolic effects to competition with mitochondrial L-serine transport, the specific identity of the transporter(s) mediating this process remains undefined. This represents a meaningful mechanistic gap, as the central conclusion depends on D-serine limiting mitochondrial L-serine availability to inhibit one-carbon metabolism.*

We thank the reviewer for this insightful comment regarding the identity of the mitochondrial L-serine transporter. As this concern overlaps with the point raised by Reviewer 2 (Weakness 1), we refer the reviewer to our response there for a detailed description of the additional experiments performed. Briefly, we conducted new experiments using siRNA-mediated knockdown of Sfxn1 in N2a cells and examined mitochondrial L-serine transport under semi-permeabilized conditions. Notably, even with marked suppression of Sfxn1 expression, D-serine continued to inhibit mitochondrial L-serine transport. Given that SFXN family members (SFXN1–SFXN5) are reported to function redundantly in mitochondrial serine transport (Kory et al., 2018), these findings suggest that D-serine may interfere with L-serine transport not only via SFXN1 but potentially across multiple SFXN paralogs. These results have been incorporated into the revised manuscript and are presented in Fig. S3, with the corresponding discussion added to the Results and Discussion sections (lines 158–164 and 379-385).

> *(2) The effective concentrations of D-serine used in vitro (IC_{50} ≈ 1-2 mM) exceed typical brain levels (~0.3 mM). While the authors acknowledge this, a more focused discussion*

> *on whether higher local D-serine concentrations could arise in specific microenvironments - such as synaptic compartments, tumor niches, or pathological states-would help contextualize the in vitro findings and strengthen their physiological relevance. For example, disruptions in D-serine clearance or altered expression of serine racemase and transporters in disease contexts could lead to localized accumulation. Moreover, differences between extracellular and intracellular D-serine pools - and the mechanisms governing their regulation - may further influence its metabolic impact in vivo.*

We appreciate this insightful comment regarding the physiological relevance of the D-serine concentrations used *in vitro*. We agree that the effective concentrations observed in our assays exceed typical bulk brain levels. To address this point, we have expanded the Discussion (lines 386-399) to consider conditions under which locally elevated D-serine concentrations may arise *in vivo*. These additions provide a more nuanced interpretation of the relationship between the concentrations used *in vitro* and the potential physiological contexts in which D-serine may exert metabolic effects.

> *(3) While the manuscript focuses on neural stem/progenitor cells and neural tumors, it remains unclear whether the anti-proliferative effects of D-serine are specific to neural lineages or extend to other highly proliferative non-neural cell types. A brief discussion addressing this point would help clarify the scope of D-serine's metabolic impact and whether its mechanism of action reflects a unique vulnerability in neural cells or a more general feature of proliferative metabolism. This distinction is particularly relevant for assessing the broader therapeutic potential of targeting mitochondrial L-serine transport.*

We thank the reviewer for this comment regarding the potential generality of the anti-proliferative effects of D-serine. We agree that it is important to clarify whether the observed effects are specific to neural lineages or reflect a broader vulnerability of proliferative cells. In our study, we focused on neural progenitor cells and neural tumour models. However, the mechanism identified here, namely limitation of mitochondrial L-serine availability, targets a fundamental metabolic pathway that supports cell proliferation. Therefore, it is possible that similar effects may extend to other highly proliferative cell types beyond the neural lineage. To address this point, we have expanded the Discussion (lines 408-410) to clarify that the observed effects of D-serine may reflect a general metabolic vulnerability associated with proliferative states, while also noting that the degree of sensitivity is likely to depend on cell-type-specific reliance on mitochondrial one-carbon metabolism.

> ***Recommendations for the authors:***
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> ***Reviewer #1 (Recommendations for the authors):***
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> *Minor issues:*
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> *The authors mention terms like neural tissue (line 109) and neural tumor cells (line 182). Neural tissue can mean anything under the sun. They need to mention the specific tissue being studied or investigated.*

We have changed the terms.

> ***Reviewer #2 (Recommendations for the authors):***
>
> *(1) The figure items were not well organized, and the legends were difficult to read since they apparently lacked key information. For example, in Figure 2D, did the author intend to show SHMT2 activity? It is not clear how this assay was performed (in vitro or in vivo experiment?). Additionally, more detailed information should be provided in the Methods section.*

We have improved the organization of figures and revised figure legends for better readability.

> *(2) The writing of the manuscript should be significantly improved, using a professional editing service, in order to increase the readability.*

We appreciate the reviewer's comment. The manuscript was professionally edited prior to submission. Nevertheless, we have made targeted revisions to the Introduction and Discussion sections to improve overall readability. We hope that these revisions have improved the clarity of the manuscript.

> ***Reviewer #3 (Recommendations for the authors):***
>
> *(1) The core mechanism centers on competition for mitochondrial L-serine transport, yet the identity of the transporter(s) involved remains speculative. While Kory et al. (2018) identified SFXN1 as a mitochondrial L-serine transporter, this connection is not directly addressed in the current study. It would strengthen the manuscript to clarify whether SFXN1 or related isoforms are expressed in the neural cell models used and whether their expression patterns correspond with the observed D-serine sensitivity. Even if functional validation is beyond the current scope, a more detailed discussion of potential transporter candidates and the limitations of existing data would provide important mechanistic context and help frame future directions.*

We thank the reviewer for this insightful comment regarding the mitochondrial L-serine transporter and the potential involvement of SFXN family members. As this concern overlaps with the point raised by Reviewer 2 (Weakness 1), we refer the reviewer to our response there for a detailed description of the additional experiments performed.

Briefly, we performed siRNA-mediated knockdown of Sfxn1 in N2a cells and examined mitochondrial L-serine transport under semi-permeabilized conditions. Even under conditions of marked suppression of Sfxn1 expression, D-serine continued to inhibit mitochondrial L-serine transport. Given that members of the SFXN family have been reported to function redundantly in mitochondrial serine transport (e.g., Kory et al., 2018), these findings suggest that D-serine may affect L-serine transport not only through SFXN1 but potentially across multiple SFXN paralogs.

These new results have been incorporated into the revised manuscript (Fig. S3), and the relevant discussion has been expanded to clarify the potential roles of SFXN family transporters and the current limitations in defining the exact transporter responsible (lines 158–165 and 379-385).

> *(2) The authors use ex vivo brain slice cultures with tumor xenografts to demonstrate tissue-level relevance, which is a valuable strength of the study. However, additional context would enhance its translational significance. It would be helpful to discuss whether in vivo D-serine administration (e.g., ICV or systemic) is feasible and safe, especially given the high concentrations required in vitro. Briefly addressing whether genetic models, such as Srr knockout mice, support a role for D-serine in tumor progression or neurodevelopment would also strengthen the interpretation.*

We thank the reviewer for this important comment regarding the translational relevance of D-serine administration *in vivo*. To address this point, we performed additional exploratory experiments using a subcutaneous tumour xenograft model in nude mice. Because the inhibitory effect of D-serine on one-carbon metabolism becomes evident under L-serine–limited conditions, tumor-bearing mice were fed an L-serine/glycine–deficient diet and administered D-serine in drinking water.

However, when D-serine was provided at high concentrations (≥ 500 mM) in drinking water, the mice exhibited marked behavioral abnormalities, including increased aggression and other abnormal behaviors. Due to these adverse effects, the experiment was ethically terminated. While our study focuses on the inhibitory effect of D-serine on one-carbon metabolism, D-serine is also a physiological co-agonist of the NMDA receptors, and therefore high systemic concentrations may influence neuronal excitability *in vivo*. These observations suggest that the concentrations required to achieve antitumor effects may be associated with significant neurological side effects.

Based on these findings, we consider that direct administration of D-serine itself may have limited therapeutic applicability as an antitumor reagent. Instead, future development of derivatives or strategies that retain the metabolic inhibitory effect while minimizing NMDA receptor–mediated effects may be required.

Regarding serine racemase (Srr) knockout models, xenograft tumour experiments would require an immunodeficient background, which makes the generation and use of such compound models technically challenging. Therefore, we did not pursue this approach in the present study. We have incorporated these considerations into the revised Discussion (lines 413-417) to clarify the translational implications and current limitations of our findings.

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