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Extracellular glycine is necessary for optimal hemoglobinization of erythroid cells

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ABSTRACT

Vertebrate heme synthesis requires three substrates: succinyl-CoA, which regenerates in the tricarboxylic acid cycle, iron and glycine. For each heme molecule synthesized, one atom of iron and eight molecules of glycine are needed. Inadequate delivery of iron to immature erythroid cells leads to a decreased production of heme, but virtually nothing is known about the consequence of an insufficient supply of extracellular glycine on the process of hemoglobinization. To address this issue, we exploited mice in which the gene encoding glycine transporter 1 (GlyT1) was disrupted. Primary erythroid cells isolated from fetal livers of GlyT1 knockout (GlyT1^{-/-}) and GlyT1-haplodeficient (GlyT1^{+/-}) embryos had decreased cellular uptake of [2-¹⁴C]glycine and heme synthesis as revealed by a considerable decrease in [2-¹⁴C]glycine and ⁵⁹Fe incorporation into heme. Since GlyT1^{-/-} mice die during the first postnatal day, we analyzed blood parameters of newborn pups and found that GlyT1^{-/-} animals develop hypochromic microcytic anemia. Our finding that Glyt1-deficiency causes decreased heme synthesis in erythroblasts is unexpected, since glycine is a non-essential amino acid. It also suggests that GlyT1 represents a limiting step in heme and, consequently, hemoglobin production.

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Introduction

In vertebrates, the enzyme 5-aminolevulinic acid synthase (ALAS; localized in mitochondria) catalyzes the first step of the heme synthesis pathway, namely a condensation reaction between glycine and succinyl-CoA resulting in 5-aminolevulinic acid (ALA).¹ In the subsequent enzymatic steps a total of eight molecules of ALA are used to assemble one tetrapyrrole macrocycle. In the final step, which takes place in mitochondria, the enzyme ferrochelatase inserts a ferrous ion (Fe²⁺) into the ring structure of protoporphyrin IX to produce heme. Shemin and co-workers found that eight of the porphyrin carbon atoms came from the α -carbon atom of each glycine and the remaining 26 came from acetate.² Thus, the biosynthesis of one molecule of heme requires one atom of iron and eight molecules of glycine. The insufficient delivery of iron to differentiating erythroid cells leads to impaired production of heme. Although defects in glycine transport can cause sideroblastic anemia, thus far there has been no published study directly examining the impact of glycine restriction on the rate of heme synthesis.

Glycine is the simplest amino acid in nature³ and, in animals, it is the main component of extracellular structural proteins such as collagen and elastin.⁴ In mammals, glycine is classified as a non-essential amino acid,⁵ which is synthesized from three distinct substrates: (i) serine, via the enzyme serine hydroxymethyltransferase,⁶ (ii) choline, through sarcosine formation⁷ and (iii) threonine, in a pathway involving the enzyme threonine dehydrogenase.⁸ However, it has been shown, in humans⁹ and pigs,⁵ that the amounts of glycine synthesized *in vivo* are insufficient

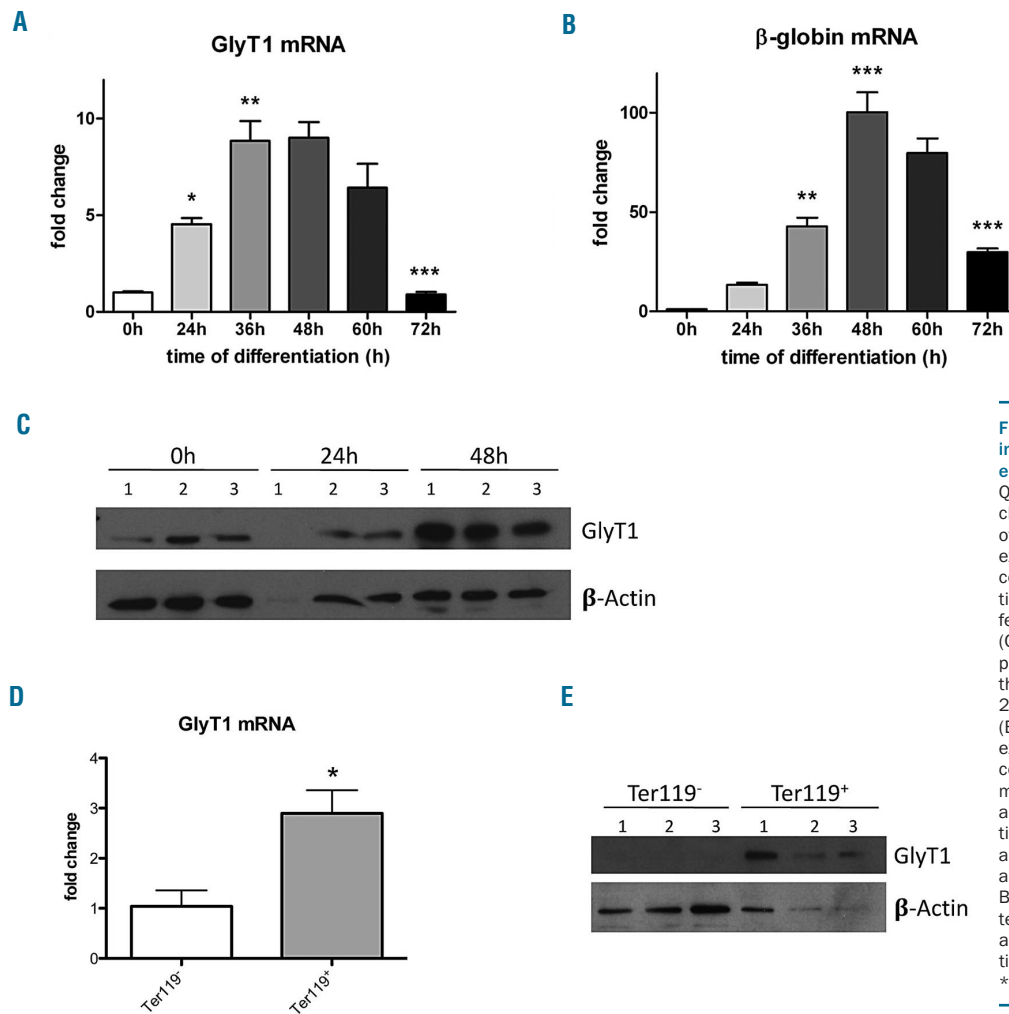


Figure 1. GlyT1 expression increases during erythroid differentiation *in vitro* and *in vivo*. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of (A) GlyT1 and (B) globin mRNA expression in primary erythroid cells kept either in a non-differentiating state (0 h) or induced to differentiate for 24 to 72 h *in vitro*. (C) Western blot analysis of GlyT1 protein expression in primary erythroid cells differentiated for 0 h, 24 h and 48 h. (D) qRT-PCR and (E) western blot analysis of GlyT1 expression in Ter119⁻ and Ter119⁺ cells isolated from mouse bone marrow. qRT-PCR-based results are presented as fold-change relative to the 0 h sample. Statistical analysis was done using one way analysis of variance followed by a Bonferroni multiple comparison test (n=3). The P-value refers always to the value of the previous time point; *P<0.05; **P<0.01; ***P<0.001.

to meet cellular metabolic demands. Therefore, many specialized cells possess different systems to transport glycine actively through their cell membranes: glial cells,¹⁰ enterocytes,¹¹ hepatocytes,¹² placental cells¹³ and erythroid cells.¹⁴ In human erythrocytes, the major glycine uptake pathway, described in the 1980s, is a Na⁺- and Cl⁻-dependent active uptake mechanism dubbed the "System Gly".¹⁴ This system was first described in avian erythrocytes as a high-affinity transporter selective for glycine and sarcosine.¹⁵ Subsequently, it has been shown that System Gly is present in the reticulocytes of all vertebrates analyzed. In some mammalian species its activity was shown to be lost during erythrocyte development to mature red blood cells.¹⁶ With a considerable delay, the two genes (*GLYT1* and *GLYT2*) encoding the glycine carrier system were cloned¹⁷ and shown to possess 12 transmembrane domains with amino and carboxy-terminal ends intracellularly oriented.¹⁸

Although the System Gly was first described in erythroid cells, it has been extensively studied primarily in the central nervous system. Glycine and D-serine are required as co-agonists of NMDA-type glutamate receptors located at excitatory glutamatergic synapses.¹⁹ The levels of glycine within the synapses are controlled by

glycine transporters²⁰ that directly affect glycine homeostasis in the central nervous system and, consequently, control the balance between neuronal inhibition and excitation in several neural circuits.²¹ Inhibition of glycine reuptake by glycine transporters is the rationale for the design of a third generation of anti-schizophrenia drugs.²²

It was initially suggested that glycine transport systems provide glycine mainly for the synthesis of glutathione in immature red blood cells,¹⁴ whereas glycine's crucial importance as one of the substrates for heme synthesis was inconceivably disregarded. Indeed, much earlier Shemin and co-workers had demonstrated that *in vitro* incubation of human reticulocytes with heavy nitrogen [¹⁵N]glycine resulted in the formation of significant amounts of [¹⁵N]heme.²³ This seminal work was the first to demonstrate that reticulocytes internalize glycine and utilize its backbone for heme biosynthesis. Somewhat surprisingly, the role of glycine transporters in supplying glycine for hemoglobin synthesis in developing red blood cells has never been assessed. We hypothesized that the System Gly (more specifically glycine transporter 1, GlyT1), which is responsible for most glycine uptake in red blood cells,¹⁴ actively transports and supplies glycine for heme biosynthesis.

In the present study, we demonstrate that GlyT1 is expressed in fetal liver cells, and its expression increases during erythropoietin-mediated induction of erythroid differentiation. We also show that, compared to wild-type cells, GlyT1 knockout (GlyT1^{-/-}) fetal liver cells internalize less [2-¹⁴C]glycine and incorporate less ⁵⁹Fe from ⁵⁹Fe-transferrin into heme. Moreover, GlyT1^{-/-} fetal liver cells have significantly lower hemoglobin levels as compared to wild-type cells. Finally, newborn mice with a homozygous GlyT1 defect have hypochromic microcytic anemia, whereas adult mice heterozygous for a GlyT1 defect exhibit only mild anemia. Our data show that glycine uptake by erythroblasts is limiting for heme synthesis and thus required for normal erythroid development. These observations are of particular importance in light of the increasing number of clinical trials employing GlyT1 inhibitors for the treatment of schizophrenia.²²

Methods

Study approval

All the animal studies performed in this work were approved by

the Lady Davis Institute animal care committee following the guidelines of the Canadian Council on Animal Care.

Animals

129SvEv mice haplodeficient for GlyT1 (GlyT1^{+/-}), generated in Professor Joseph Coyle's laboratory,²⁴ were maintained and bred in the animal facility at the Lady Davis Institute for Medical Research. All GlyT1 knockout mice die during the first postnatal day;^{24,25} viable neonates were sacrificed within 1-2 h after birth. As indicated, pregnant animals were sacrificed at day E12.5, 14.5 and 16.5 *post coitum* to determine the embryonic phenotype. For studies on erythroid cells *in vitro*, fetal livers were isolated between E12.5 and E13.5. Genotypes were determined by polymerase chain reaction analysis of tail DNA using a previously described protocol.²⁴

Culture of primary mouse erythroblasts

Erythroid cells were isolated and cultured as previously described.²⁶ Briefly, cells were grown from fetal livers obtained from E12.5-13.5. Primary fetal liver cells were kept either in an undifferentiated state (0 h) or induced to terminal differentiation (24, 48 or 72 h). The composition of the medium used to maintain the cells in such states is described in further detail in the *Online Supplementary Information*.

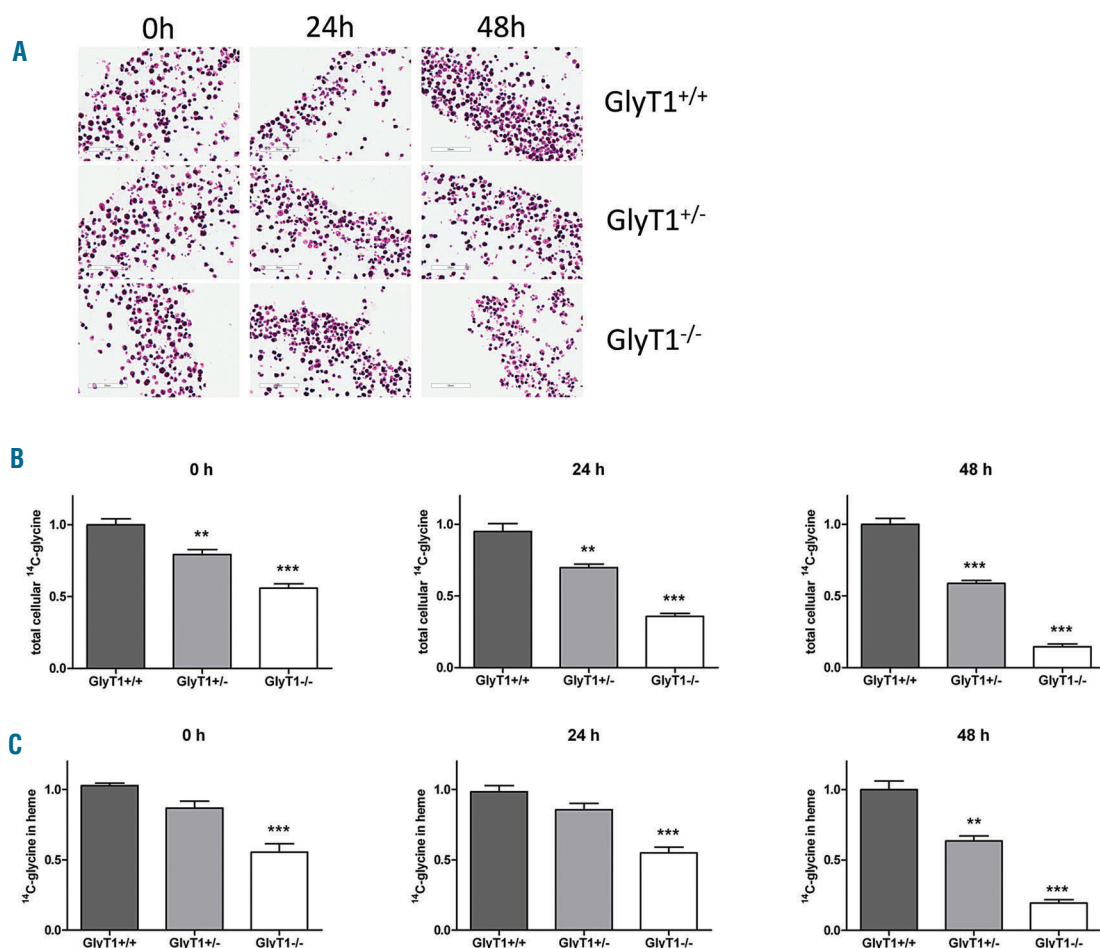


Figure 2. [2-¹⁴C]glycine uptake and incorporation into heme is disrupted in GlyT1-depleted erythroid cells. Erythroid cells isolated from GlyT1^{+/+}, GlyT1^{+/-} and GlyT1^{-/-} embryos were either kept in a non-differentiating state (0 h) or induced to differentiate for 24 and 48 h. (A) Giemsa staining of GlyT1^{+/+}, GlyT1^{+/-} and GlyT1^{-/-} undifferentiated (0 h) and differentiated (24 h and 48 h) fetal liver cells (B) Total uptake of [2-¹⁴C]glycine and (C) [2-¹⁴C]glycine incorporation into heme. [2-¹⁴C]glycine values are presented relative to the wild-type cells at indicated time intervals. Statistical analysis was done using one way analysis of variance followed by a Bonferroni multiple comparison test (n=3). The P value always refers to the value of the previous time point; *P<0.05; **P<0.01; ***P<0.001.

Hemoglobin assay

Hemoglobin measurements were performed as described previously.²⁷ Briefly, 2.5×10^5 cells were transferred in triplicate into a 96-well microtiter plate with conical bottomed wells and washed with 100 μ L phosphate-buffered saline. Cells were lysed in 50 μ L H₂O, following which 125 μ L of dye solution [0.5 mg/mL o-phenylene-diamine-dihydrochloride (Sigma-Aldrich, St. Louis, MO, USA), 50 mM citric acid, 0.1 M Na₂HPO₄ and 1 μ L/mL of 30% H₂O₂] were added. The reaction was stopped within 3–5 min with 25 μ L 8 N H₂SO₄ and the optical density of samples at 492 nm was determined. The results were normalized to total cell volume.

Glycine uptake and its incorporation into heme

Cells were incubated with 4–5 μ Ci of [2-¹⁴C]glycine (100 μ Ci/mL; NEN, Boston, MA, USA) for 1 h, following which cells were washed three times with 1 mL phosphate-buffered saline. Heme was extracted as previously described.²⁸ A detailed protocol is described in the *Online Supplementary Information*.

Iron uptake from iron-transferrin

Radioactive iron-transferrin (⁵⁹Fe₂-Tf) was made from ⁵⁹FeCl₃ (PerkinElmer, Santa Clara, CA, USA; 2 μ Ci) as described previously.²⁸ For measurements of total cellular ⁵⁹Fe uptake, cells were incubated with 2 μ M (final concentration; saturating) ⁵⁹Fe₂-Tf for 3 h, following which samples were washed twice with ice-cold phosphate-buffered saline and collected by centrifugation (200 \times g for 5 min at 4°C). The ⁵⁹Fe in heme and non-heme fractions was measured as described in the *Online Supplementary Methods*.

Statistical analysis

Statistical analysis was done using SPSS v15.0 (IBM Software, Markham, ON, Canada) software. Data were evaluated using one-way analysis of variance (ANOVA) and the Student *t* test. Multiple comparisons were performed using Bonferroni and Dunnett tests. Error bars of graphs represent standard deviations ($n=3$).

Results

GlyT1 expression increases during erythroid differentiation

To analyze the expression of GlyT1 mRNA during erythroid differentiation, we used primary mouse erythroid cells derived from fetal livers.²⁶ We expanded erythroid progenitors isolated from mouse fetal livers and kept them either in a non-differentiated state (0 h) or, after expanding them, stimulated their terminal differentiation using high concentrations of erythropoietin (24–72 h). At the indicated time intervals, mRNA levels of GlyT1 and β -globin were measured using quantitative real-time polymerase

chain reaction analysis. The results shown in Figure 1A demonstrate a strong increase in GlyT1 mRNA within the first 48 h, which matches the maximal rate of hemoglobin synthesis in fetal liver cells at this stage of differentiation.²⁶ In the following 24 h, when hemoglobinization slows down,²⁶ GlyT1 mRNA expression declines to its initial levels. This expression pattern of GlyT1 mRNA closely resembles the expression profile obtained for β -globin mRNA (Figure 1B), suggesting a possible link between GlyT1 and globin synthesis in differentiating fetal liver cells. GlyT1 protein levels were also increased in fetal liver cells after 48 h of differentiation (Figure 1C). We then analyzed GlyT1 expression levels *in vivo* using the erythroid surface marker Ter119²⁹ to separate erythroid cells (Ter119⁺) from non-erythroid cells (Ter119⁻) obtained from mouse bone marrow. Quantitative real-time polymerase chain reaction analysis revealed a 3-fold increase in GlyT1 mRNA expression in the Ter119⁺ fraction compared to the Ter119⁻ one (Figure 1D). In agreement with that, GlyT1 protein levels also increased in Ter119⁺ cells (Figure 1E). Of interest, previous studies determined that the components of System Gly (currently known as GlyT1 and GlyT2) are localized in red blood cell membranes.^{30,31} Our finding of augmented GlyT1 expression, both *in vitro* and *in vivo*, suggests that this transporter is likely required for erythroid differentiation.

Glycine uptake and its incorporation into heme are decreased in GlyT1-depleted erythroid cells

The increase in GlyT1 expression depicted in Figure 1 suggests that an increase in the transporter's levels in erythroid cells is needed to satisfy cellular demands for glycine during erythroid differentiation. To address this issue, we first determined that primary erythroid cells isolated from wild-type (GlyT1^{+/+}), haplodeficient (GlyT1^{+/-}) and knockout (GlyT1^{-/-}) fetal livers showed similar morphology in their undifferentiated (0 h) and differentiated (48 h) state, as demonstrated by Giemsa staining (Figure 2A). The total uptake of [2-¹⁴C]glycine was significantly reduced in undifferentiated (0 h) and differentiated (24 h and 48 h) erythroid cells derived from both haplodeficient and knockout animals when compared to that in wild-type cells (Figure 2B). Non-differentiated GlyT1^{+/-} and GlyT1^{-/-} cells reached only 80 \pm 4.9% and 55 \pm 8.3%, respectively, of the amount of glycine taken up by GlyT1^{+/+} erythroblasts. At 48 h of differentiation, these values further decreased to 60 \pm 1.9% and 15 \pm 1.3%, respectively. Similarly, [2-¹⁴C]glycine incorporation into heme was significantly decreased in both GlyT1^{-/-} and GlyT1^{+/-} fetal liver cells (Figure 2B).

Table 1. GlyT1^{-/-} and GlyT1^{+/-} newborn mice exhibit microcytic hypochromic anemia.

Genotypes	RBC (10 ¹² /L)	Hemoglobin (g/dL)	Hematocrit (%)	MCV (fL)	MCH (pg)
GlyT1 ^{+/+}	3.86 \pm 0.09	14.24 \pm 0.38	42.34 \pm 0.87	109.73 \pm 1.21	36.85 \pm 0.48
GlyT1 ^{+/-}	3.75 \pm 0.06	13.16 \pm 0.19*	39.18 \pm 0.55*	104.86 \pm 1.09*	35.20 \pm 0.39*
GlyT1 ^{-/-}	3.80 \pm 0.12	10.76 \pm 0.28***	32.72 \pm 0.83***	86.72 \pm 0.95***	28.46 \pm 0.27***

Values are mean \pm standard error. Statistical analysis was done using one-way analysis of variance followed by a Dunnett multiple comparison test; * $P<0.05$; *** $P<0.001$. We determined red blood cell indices on GlyT1^{+/-} ($n=20$), GlyT1^{-/-} ($n=36$) and GlyT1^{+/+} ($n=25$) newborn animals by automated analysis. RBC, red blood cell number; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin.

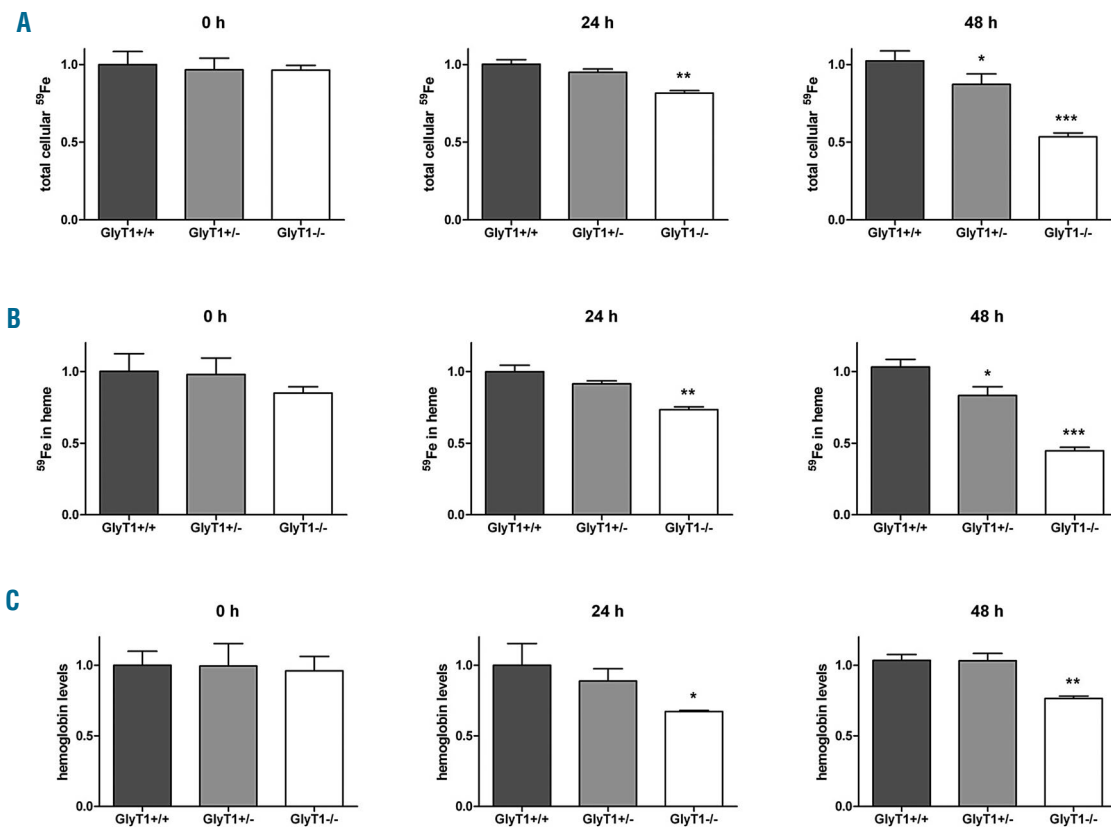


Figure 3. ⁵⁹Fe uptake from ⁵⁹Fe-Tf and ⁵⁹Fe incorporation into heme, as well as hemoglobin levels, are significantly reduced in GlyT1^{-/-} erythroid cells. Erythroid cells isolated from GlyT1^{+/+}, GlyT1^{+/-} and GlyT1^{-/-} embryos were either kept in a non-differentiating state (0 h) or induced to differentiate for 24 and 48 h. (A) Total uptake of ⁵⁹Fe from ⁵⁹Fe-Tf, (B) ⁵⁹Fe incorporation into heme and (C) hemoglobin levels. ⁵⁹Fe and hemoglobin values are presented relative to the wild-type cells at indicated time intervals. Statistical analysis was done using one way analysis of variance followed by a Bonferroni multiple comparison test (n=3); *P<0.05; **P<0.01; ***P<0.001.

Iron uptake and its incorporation into heme is significantly reduced in differentiating GlyT1^{-/-} erythroblasts

Erythroid cells import iron exclusively via the transferrin (Tf)-transferrin receptor (TfR) pathway.¹ Hence, we used ⁵⁹Fe₂-Tf to investigate whether the restriction in glycine supply, shown in Figure 2B, would also affect the uptake of ⁵⁹Fe and its incorporation into heme. As depicted in Figure 3, there were significant reductions in both ⁵⁹Fe uptake (Figure 3A) and its incorporation into heme (Figure 3B) in GlyT1^{-/-} and GlyT1^{+/-} cells when compared to wild-type controls. After 48 h of differentiation, GlyT1^{+/+} fetal liver cells accumulated massive amounts of hemoglobin (a more than 4-fold increase in relation to the 0 h time point), which was expected based on earlier reports.^{26,27,32} Importantly, at 48 h, GlyT1^{-/-} fetal liver cells had significantly lower hemoglobin levels compared to their wild-type counterparts (Figure 3C). In agreement with these results, we observed that cell surface TfR1 levels decreased at 24 h and 48 h in erythropoietin-treated GlyT1^{-/-} and GlyT1^{+/-} cells compared to GlyT1^{+/+} cells (Figure 4).

Of interest, in non-differentiated cells (0 h), the lack or reduction of GlyT1 expression did not significantly affect total ⁵⁹Fe uptake (Figure 3A), ⁵⁹Fe incorporation into heme

(Figure 3B) or hemoglobin levels (Figure 3C), despite the reduction in the total glycine uptake by GlyT1^{-/-} and GlyT1^{+/-} cells at 24 and 48 h (Figure 2). Additionally, in differentiated erythroid cells (48 h), both alleles of GlyT1 were necessary to achieve a rate of heme biosynthesis comparable to that seen in wild-type cells.

GlyT1^{-/-} newborn mice have microcytic hypochromic anemia

We then investigated whether GlyT1 deficiency *in vivo* results in a phenotype similar to that seen in our *in vitro* model. Congruent with earlier reports,^{24,25} mice lacking the gene encoding GlyT1 appear normal at birth, but, 6 to 14 h later, start displaying motor sensory deficits and severe respiratory malfunction, which ultimately leads to death. We sacrificed the newborn pups shortly after birth and observed that GlyT1-deficient mice (GlyT1^{-/-}) were anemic with hemoglobin levels 25% lower than their wild-type (GlyT1^{+/+}) siblings (Table 1). Moreover, the hematocrit was decreased from 42.3 ± 0.9% in GlyT1^{+/+} to 32.7 ± 0.8% in GlyT1^{-/-} animals. This reduction was most likely due to a smaller red cell volume since there was no significant reduction in the total red blood cell counts (Table 1). These differences are further corroborated by the observation that GlyT1 knockout embryos isolated between E12 and

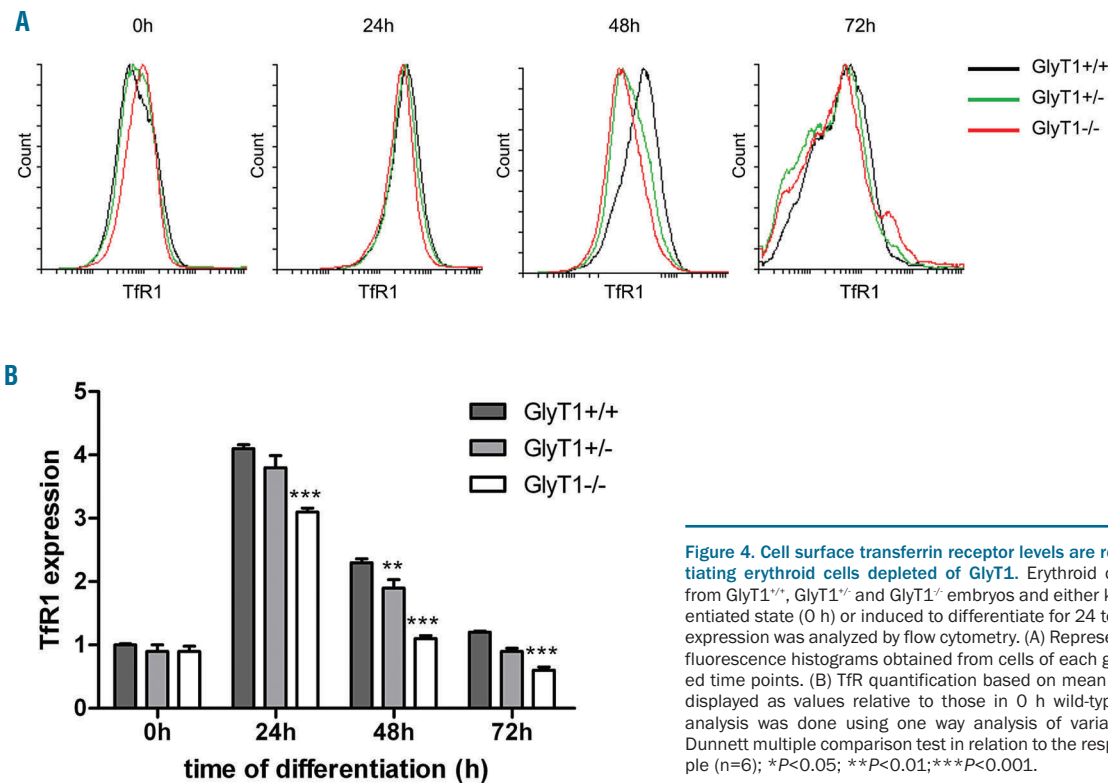


Figure 4. Cell surface transferrin receptor levels are reduced in differentiating erythroid cells depleted of GlyT1. Erythroid cells were isolated from GlyT1^{+/+}, GlyT1^{+/-} and GlyT1^{-/-} embryos and either kept in a non-differentiated state (0 h) or induced to differentiate for 24 to 48 h. Tfr surface expression was analyzed by flow cytometry. (A) Representative Tfr (CD71) fluorescence histograms obtained from cells of each genotype at indicated time points. (B) Tfr quantification based on mean fluorescence intensity displayed as values relative to those in 0 h wild-type cells. Statistical analysis was done using one way analysis of variance followed by a Dunnett multiple comparison test in relation to the respective GlyT1^{+/+} sample (n=6); *P<0.05; **P<0.01; ***P<0.001.

Table 2. Adult GlyT^{-/-} mice exhibit mild microcytic hypochromic anemia.

Genotypes	RBC (x10 ¹² /L)	Hemoglobin (g/dL)	Hematocrit (%)	MCV (fL)	MCH (pg)
GlyT1 ^{+/+}	11.04±0.13	16.59±0.19	54.09±0.74	48.89±0.30	15.04±0.09
GlyT1 ^{-/-}	11.48±0.10*	16.49±0.14	54.31±0.43*	47.42±0.31**	14.38±0.11***

Values are mean±standard error. Statistical analysis was done using one way analysis of variance followed by a Dunnett multiple comparison test; *P<0.05; ***P<0.001. We determined red blood cell indices on GlyT1^{+/+} (n=19) and GlyT1^{-/-} (n=26) adult animals (3- to 4 month old mice) by automated analysis. RBC: red blood cell number; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin.

E16.5 were paler than their wild-type siblings (Figure 5). Moreover, GlyT^{-/-} animals had a significantly increased percentage of reticulocytes in the blood as compared to GlyT1^{+/+} and GlyT1^{+/-} animals (Online Supplementary Figure S1A,B). It is noteworthy that compared to GlyT1^{+/+} animals, haplodeficient (GlyT1^{+/-}) pups also showed a mild anemia, with significantly lower hemoglobin levels and decreased hematocrit, mean corpuscular volume and mean corpuscular hemoglobin (Table 1). Collectively, these data clearly demonstrate that the lack or deficiency of GlyT1 leads to hypochromic microcytic anemia in newborn mice.

Adult mice heterozygous for GlyT1 exhibit mild anemia

Disruption of one GlyT1 allele is sufficient to cause a significant reduction in glycine uptake and heme synthesis during erythroid differentiation *in vitro* (Figures 2 and 3). This is in line with the *in vivo* data showing that haplodeficient GlyT1 newborn pups were anemic (Table 1). In order to evaluate whether adult GlyT1 haplodeficient mice also show any signs of anemia, we analyzed blood from 3- to 4-month old mice. As shown in Table 2, both

mean corpuscular volume and mean corpuscular hemoglobin were slightly reduced in heterozygous animals, whereas red blood cell counts were slightly increased compared to those in wild-type mice. No significant differences in hemoglobin levels or hematocrit were observed between these two genotypes (Table 2).

Discussion

The requirement of the α -carbon of glycine for heme synthesis in vertebrates was established by the groups of Shemin and Neuberger who discovered that one carbon atom and the nitrogen atom of each pyrrole ring of heme is derived from the α -atom and the associated nitrogen atom of glycine³³ and that each of the four methylene bridge carbon atoms of heme is derived from the α -carbon of glycine.³³ However, the first suggestion that extracellular glycine is used in heme synthesis came from Shemin and co-workers who demonstrated that reticulocytes incubated with [¹⁵N]glycine synthesized significant amounts of [¹⁵N]heme.²³

Our first clue that GlyT1 may be required for efficient hemoglobin synthesis was the matching pattern of GlyT1 mRNA expression with that of β -globin during erythroid differentiation (Figure 1A,B). A similar expression profile was obtained in an erythroid global gene expression analysis that showed increased GlyT1 expression at the polychromatic erythroblast stage in mouse primitive, fetal and adult erythroid cells.³⁴ The same analysis did not detect expression of GlyT2, another member of the System Gly, in erythroblasts at any stage of their development. We did not, therefore, examine GlyT2 expression in our study. Our GlyT1 *in vitro* expression data were supported by an *in vivo* experiment in which we also found the increase of GlyT1 mRNA and protein levels in mouse Ter119⁺ bone marrow cells (Figure 1D,E).

Our data show for the first time that the absence of GlyT1 leads to a significant reduction in glycine uptake and its incorporation into heme in erythroid cells (Figure 2B,C), associated with diminished ⁵⁹Fe incorporation into heme (Figure 3B). Decreases in these values were more prominent in differentiated fetal liver cells from Glyt1^{-/-} animals, but a lesser reduction in these parameters was also seen in differentiating fetal liver cells from mice haplodeficient for the *GlyT1* gene, in particular at 48 h of differentiation. Reduced hemoglobin levels, particularly in differentiating fetal liver cells from Glyt1^{-/-} mice (Figure 3C), can be explained by a decrease in heme synthesis in these cells. However, haplodeficient *GlyT1* fetal liver cells have hemoglobin levels similar to those of wild-type controls, which suggest that one functional *GlyT1* allele is sufficient to fully support hemoglobinization. Of interest, sarcosine, a competitive inhibitor of GlyT1,³⁵ also decreases ⁵⁹Fe incorporation into heme from ⁵⁹Fe-Tf (Online Supplementary Figure S2). These observations suggest that, similarly to what happens in other cell types,^{3,9} endogenous glycine sources are insufficient to meet erythroid cell demands for optimal hemoglobinization. However, in undifferentiated erythroid cells, *de novo* glycine synthesis³ and/or glycine transported by systems other than System Gly¹⁴ (band 3, system L, system ASC and simple passive diffusion through the membrane) seem to be supplying sufficient levels of glycine to support housekeeping heme synthesis.

It was demonstrated that System Gly (GlyT1 and GlyT2) accounts for 42% of the total glycine uptake in erythroid cells.¹⁴ Band 3 and system L account for 16% and 15% of glycine uptake, respectively, while system ASC accounts for 11% of uptake.¹⁴ The remaining glycine uptake occurs by passive diffusion.¹⁴ King and Gunn also observed System Gly-independent glycine transport via the band 3 system in erythroid cells.³⁶ The total glycine uptake in erythroid cells is not, therefore, entirely via GlyT1. The existence of alternative glycine transport systems (apart from GlyT1) explains glycine transport to erythroid cells for heme synthesis-unrelated pathways. It seems likely that GlyT1-independent glycine uptake could be responsible for at least some hemoglobin synthesized in Glyt1^{-/-} erythroid cells (Figure 3C) and allows *in utero* survival of Glyt1^{-/-} fetuses (Table 1). Nevertheless, GlyT1 is involved in the transport of glycine, which is specifically committed for heme synthesis and hemoglobin production in erythroid cells.

GlyT1 knockdown caused a decrease in total ⁵⁹Fe uptake by fetal liver cells after 24 and 48 h of differentiation (Figure 3A). At the same time points, we observed a reduc-

tion in cell surface TfR levels (Figure 4) in these cells. As erythroid cells can take up iron only via the transferrin/TfR pathway,¹ it can be expected that the reduction in TfR levels will lead to a decrease in iron uptake and its incorporation into heme. Additionally, heme enhances TfR expression at the transcriptional level in erythroid cells.^{27,37} The reduction in TfR levels observed under glycine restriction is, therefore, most likely due to a decrease in cellular heme levels in fetal liver cells from GlyT1^{-/-} mice.

Our findings that the ablation of *GlyT1* causes a decrease in heme synthesis in erythroblasts and hypochromic microcytic anemia in newborn mice suggest that the transport of glycine to erythroid cells represents a limiting step that controls the rate of heme synthesis. A decrease in heme levels is also well known to cause a reduction in globin synthesis that occurs by well-described mechanisms involving both transcription³⁸ and translation.³⁹ This proposal seems to be in conflict with the idea that in erythroid cells iron acquisition from transferrin is the rate-limiting step in heme synthesis.²⁸ However, all things considered, these two ideas may not be mutually exclusive. The uptake of iron by immature erythroid cells is controlled by a negative feedback mechanism in which non-hemoglobin-bound heme (“uncommitted heme”) inhibits iron acquisition from transferrin.^{1,40} Importantly in this context, heme was also shown to inhibit glycine uptake by erythroid cells.²⁸ The exact molecular mechanisms by which heme affects iron or glycine trafficking in erythroid cells are unknown, but it is tempting to speculate that heme interacts with a yet-to-be-identified entity involved in both iron and glycine conveyance or the regulation of these processes.

Nothing is known about a potential relationship between GlyT1-mediated transport of glycine through the plasma membrane and the import of this amino acid into mitochondria. Nevertheless, some important clues are emerging from the studies of a subtype of hereditary sideroblastic anemias, all of which are characterized by the presence of mitochondrial iron deposits in erythroblasts. Most cases of hereditary X-linked sideroblastic anemia are caused by mutations in the gene encoding erythroid-specific ALAS.⁴¹ However, a certain proportion of patients with hereditary sideroblastic anemia exhibit autosomal recessive inheritance. Guernsey *et al.*⁴² described that at least some such patients have a defect in the gene encoding the erythroid-specific mitochondrial carrier protein, SLC25A38. They demonstrated that this transporter is important for the biosynthesis of heme in eukaryotes and conjectured that this protein may translocate glycine into mitochondria.⁴² Fernández-Murray *et al.* have recently provided convincing evidence that Hem25, the yeast homolog of human SLC25A38, provides glycine for ALAS.⁴³ They also demonstrated that expression of human *SLC25A38* in yeast cells in which the *HEM25* gene was deleted restored heme to a normal level, leading the authors to conclude that Hem25 and SLC25A38 are mitochondrial glycine importers. Taking together these findings and our data, it is tempting to speculate that erythroid cells have a unique pathway to convey glycine from the extracellular milieu to mitochondria, which would involve both GlyT1 and SLC25A38. Such a mechanism would optimize glycine delivery for heme synthesis in developing erythroid cells.

The reduced heme and hemoglobin synthesis occurring in Glyt1^{-/-} fetal liver cells *in vitro* is recapitulated *in vivo*.

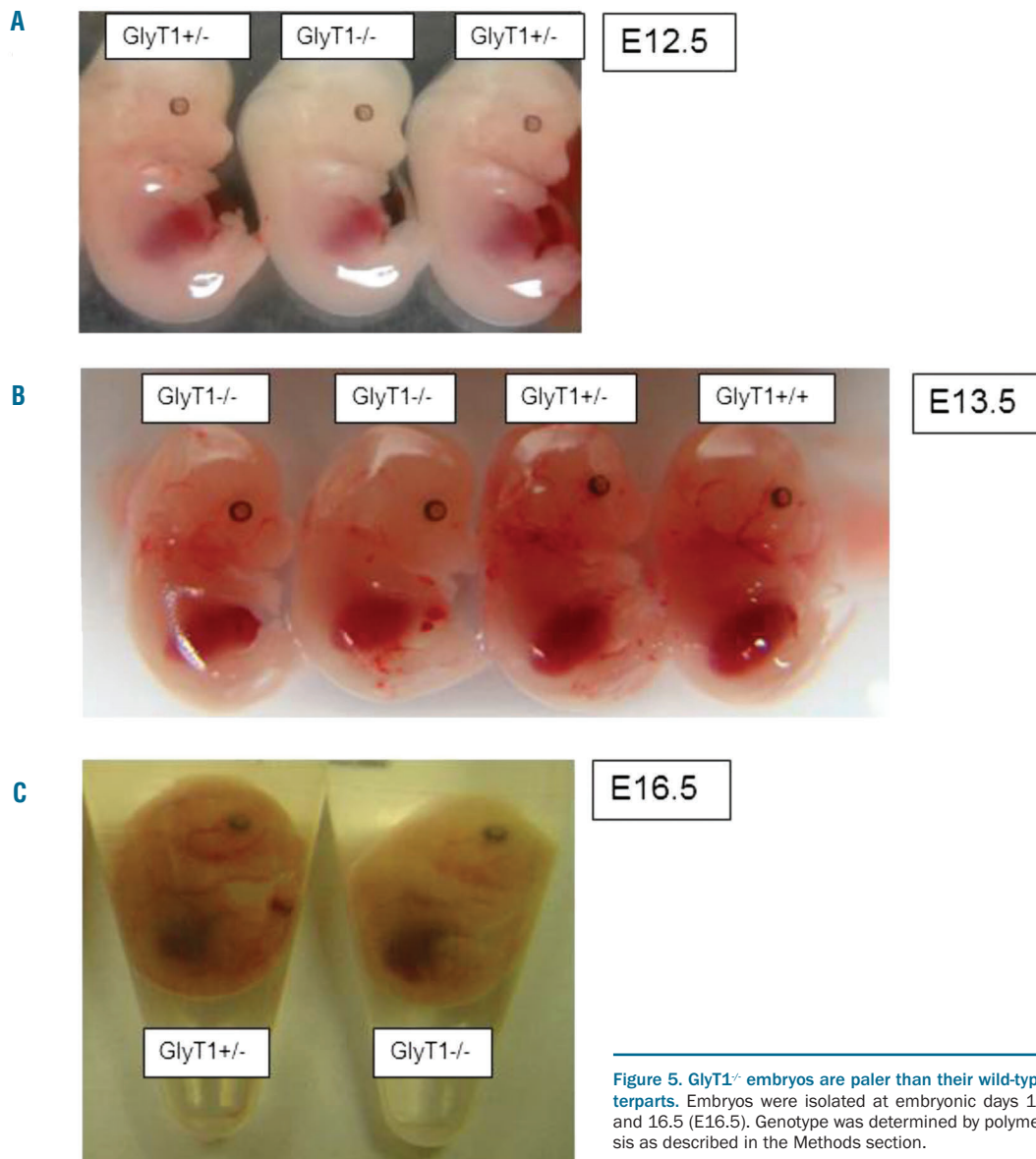


Figure 5. GlyT1^{-/-} embryos are paler than their wild-type and heterozygous counterparts. Embryos were isolated at embryonic days 12.5 (E12.5), 14.5 (E14.5) and 16.5 (E16.5). Genotype was determined by polymerase chain reaction analysis as described in the Methods section.

GlyT1^{-/-} newborn mice display hypochromic microcytic anemia, as documented by significantly lower hemoglobin levels and decreased hematocrit, mean corpuscular volume and mean corpuscular hemoglobin (Table 1). These changes clearly indicate impairment in hemoglobin synthesis. It has already been described that wild-type mice show mean cell volumes between 100 to 110 fL at birth, and these values drop during the first month of life, stabilizing between 48 and 63 fL.⁴⁴ On the other hand, GlyT1^{-/-} adult mice have only a 5% reduction in mean corpuscular volume and mean corpuscular hemoglobin, when compared to their wild-type counterparts (Table 2). Therefore, similarly to what was observed *in vitro* (Figure 3C), one functional *GlyT1* allele is almost sufficient to support normal hemoglobinization *in vivo*.

Glycine is an inhibitory neurotransmitter that acts by directly activating ionotropic glycine receptors, which are predominantly found at inhibitory synapses in the spinal cord, brainstem and retina.⁴⁵ Glycine transporters control

the levels of glycine at the inhibitory and excitatory synapses,²⁰ and GlyT1-mediated glycine reuptake facilitates the activation of NMDA receptors.²¹ Thus, clinical conditions associated with deficient NMDA receptor signaling, such as schizophrenia, may potentially be treated by inhibition of glycine reuptake in the synapses.²¹ The aim of therapeutic GlyT1 inhibitors is to restore glycine homeostasis in specific regions of the central nervous system and alleviate the clinical symptoms of schizophrenia. This new approach represents an alternative to current therapies, which are based primarily on dopamine receptor blockage.⁴⁶

A wide variety of GlyT1 inhibitors have been developed and tested in different pre-clinical models.^{47,48} The GlyT1 inhibitor bitopertin (RG-1678; RO-4917838) is one of these inhibitors. During the preparation of our study for publication, bitopertin was reported to cause anemia in rats.⁴⁹ Similarly to what we observed in GlyT1^{-/-} newborn mice (Table 1), adult rats treated with bitopertin showed

decreases in mean corpuscular volume, mean corpuscular hemoglobin and hemoglobin levels, when compared to those of controls.⁴⁹ Additionally, as expected, bitopertin administration led to an increase in erythropoietin levels in rats.⁴⁹ Our study, for the first time, presents compelling evidence that glycine uptake by erythroblasts represents a limiting step in heme and, consequently, hemoglobin production (Figures 2 and 3).

Concerns regarding the side effects that could be associated with long-term therapy with GlyT1 inhibitors are focused on damage to intestinal epithelial cells⁵⁰ and adverse skin reactions.⁵¹ Importantly, our data show that GlyT1 suppression causes impairment of hemoglobin synthesis *in vitro* and hypochromic microcytic anemia *in vivo* (Tables 1 and 2). Long-term inhibition of glycine uptake by erythroblasts in humans could lead to the development of sideroblastic anemias due to defective heme biosynthesis, which could consequently cause hepatic and systemic iron overload.⁴¹

Umbricht *et al.* performed a randomized, double-blinded, placebo-controlled bitopertin trial involving 323 patients with schizophrenia.⁵² Their study suggested a

modest but clinically relevant effect of bitopertin in improving the negative symptoms associated with schizophrenia. However, at all bitopertin doses tested, a significant number of patients showed a more than 2 g/dL reduction in hemoglobin levels.⁵²

In summary, our data show for the first time that the transport of glycine to erythroid cells is a limiting step in their synthesis of heme. We have also demonstrated that extracellular restriction of glycine due to defective GlyT1 leads to a decrease in hemoglobin levels both *in vivo* and *in vitro*. Collectively, our data reveal a uniqueness of erythroid cells in terms of their high demand for extracellular substrates for heme biosynthesis and also raise the question of how glycine and iron transport are coordinated in these cells.

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