

# Vitamin B6 is essential for serine *de novo* biosynthesis

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**Abstract** Pyridoxal 5'-phosphate (PLP), the metabolically active form of vitamin B6, plays an essential role in brain metabolism as a cofactor in numerous enzyme reactions. PLP deficiency in brain, either genetic or acquired, results in severe drug-resistant seizures that respond to vitamin B6 supplementation. The pathogenesis of vitamin B6 deficiency is largely unknown. To shed more light on the metabolic consequences of vitamin B6 deficiency in brain, we performed untargeted metabolomics in vitamin B6-deprived Neuro-2a cells. Significant alterations were observed in a range of metabolites. The most surprising observation was a decrease of serine and glycine, two amino acids that are known to be elevated in the plasma of vitamin B6 deficient patients. To investigate the cause of the low concentrations of serine and glycine, a metabolic flux analysis on serine biosynthesis was performed. The metabolic flux results showed that the *de novo* synthesis of serine was significantly reduced in vitamin B6-deprived cells. In addition, formation of glycine and 5-methyltetrahydrofolate was decreased. Thus, vitamin B6 is essential for serine *de novo* biosynthesis in neuronal cells,

and serine *de novo* synthesis is critical to maintain intracellular serine and glycine. These findings suggest that serine and glycine concentrations in brain may be deficient in patients with vitamin B6 responsive epilepsy. The low intracellular 5-mTHF concentrations observed *in vitro* may explain the favourable but so far unexplained response of some patients with pyridoxine-dependent epilepsy to folinic acid supplementation.

## Introduction

Pyridoxal 5'-phosphate (PLP), the metabolically active form of vitamin B6, plays a pivotal role in brain metabolism and development (Surtees et al 2006). PLP is an essential cofactor in more than 100 metabolic reactions in humans (Clayton 2006). Most of the PLP-dependent reactions, such as transamination, decarboxylation, deamination, racemization and desulfhydration, are involved in amino acid metabolism (Ebadi 1981; Ueland et al 2015). PLP deficiency, either due to genetic or dietary causes, disrupts the metabolism of neurotransmitters ( $\gamma$ -aminobutyric acid (GABA), dopamine and serotonin), haeme, histamine, amino acids, carbohydrates and nucleotides (da Silva et al 2013; Ueland et al 2015).

Five inborn errors of metabolism are known to affect vitamin B6 concentrations: pyridoxine-dependent epilepsy ( $\alpha$ -amino adipic semialdehyde dehydrogenase (antiquitin) deficiency; OMIM #266100), hyperprolinemia type II (1-pyrroline-5-carboxylate dehydrogenase deficiency; OMIM #239510), pyridox(am)ine 5'-phosphate oxidase deficiency (PNPO deficiency; OMIM #610090), hypophosphatasia (tissue non-specific alkaline phosphatase (TNSALP) deficiency; OMIM #241500) and proline synthetase co-transcribed bacterial homologue deficiency (PROSC deficiency; OMIM #604436). These diseases, except for most cases of

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TNSALP, are characterized by seizures, often beginning in the first days of life, not responsive to anticonvulsants and only controlled by vitamin B6 supplementation (Baxter 1999; Stockler et al 2011). Besides neonatal seizures, many patients suffer from developmental delay or intellectual disability, despite the seizure control (Baxter 2001; van Karnebeek et al 2016; Darin et al 2016; Walker et al 2000; Mills et al 2005, 2014). Little is known about the specific biochemical changes that underlie the clinical symptoms of patients with vitamin B6 deficiency. Low GABA levels were thought to be the main reason for the epilepsy (Gospe et al 1994). GABA is the key inhibitory neurotransmitter in the central nervous system and is synthesized from glutamate (the main excitatory neurotransmitter) through the PLP-dependent enzyme glutamic acid decarboxylase (GAD, EC 4.1.1.15). Animal studies performed in zebrafish larvae showed that upon exposure to ginkgotoxin (4'-O-methylpyridoxine, a pyridoxal 5'-phosphate antimetabolite) a seizure-like behaviour develops. The ginkgotoxin-induced seizures were reversed by the addition of GABA and/or PLP to the fish water, supporting the hypothesis that the seizures are caused by reduced PLP availability, which leads to an imbalance between GABA and glutamate (Lee et al 2012). However, conflicting data on glutamate and GABA levels in the CSF of patients suffering from vitamin B6 deficiency suggest that GABA deficiency may not be the sole cause of symptoms in pyridoxine-dependent epilepsy (Goto et al 2001; Baumeister et al 1994). Several studies have shown that vitamin B6-deficient patients may display biochemical features of aromatic L-amino acid decarboxylase (AADC) deficiency, with low CSF homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA) (Clayton 2006; De Roo et al 2014; Darin et al 2016), raised tyrosine, 3-orthomethylidopa, L-Dopa and 5-hydroxytryptophan (Darin et al 2016). Other studies report increased CSF and plasma concentrations of threonine (Mills et al 2005; Clayton 2006), and/or glycine (Darin et al 2016; Mills et al 2005) and/or branched chain amino acids (Darin et al 2016). Nevertheless, these results are not consistent in all studies (Levtova et al 2015), underlining the complexity of vitamin B6 deficiency. Many additional vitamin B6-dependent reactions may contribute to the clinical phenotype.

To elucidate the pathogenesis of brain vitamin B6 deficiency, we performed untargeted metabolomics on a neuronal cell model deprived of vitamin B6. Our observations indicate that additional factors next to GABA play a role in the pathogenesis of vitamin B6 deficiency.

## Results

To study the consequences of vitamin B6 deficiency in neuronal cells, we developed a model system of vitamin B6-deficient Neuro-2a cells. Neuro-2a cells were cultured in the

presence (100 nM) and absence of pyridoxal (PL). Cells were harvested at different time points.

### Vitamin B6 restriction results in intracellular PLP deficiency

To establish whether the absence of PL in the medium resulted in intracellular vitamin B6 deficiency, we quantified the intracellular concentrations of PL, pyridoxamine (PM), pyridoxine (PN), the 5'-phosphorylated forms (PLP, PMP and PNP, respectively) and the breakdown product pyridoxic acid (PA) (Fig. S1). PLP concentrations were significantly decreased in cells that were cultured in the absence of vitamin B6 ( $P < 0.01$  for all time points, with a 41–75% reduction), compared to 100 nM PL. PL concentrations were relatively conserved, being significantly decreased at  $t = 14$  days only ( $P < 0.05$ ). Intracellular PMP concentrations were also decreased ( $P < 0.01$  at  $t = 7$  and 18 days, with a 48% and 53% reduction, respectively;  $P < 0.05$  at  $t = 4$  and 14 days, with a 27% and 49% reduction, respectively). PN, PNP, PM and PA were below the limit of quantification (LOQ), for both vitamin B6-deficient and -proficient conditions, at all time points. Thus, absence of PL in the medium leads to decreased concentrations of the active cofactor of vitamin B6, making this model suitable for the study of the consequences of vitamin B6 deficiency on the intracellular metabolome.

### Vitamin B6 deficiency alters the metabolome of Neuro-2a cells

We compared the metabolomes of Neuro-2a cells cultured in 0 and 100 nM PL at all time points. By direct-infusion high-resolution mass spectrometry (DI-HRMS) of the extracts we detected 15,765 features (a feature is defined as a mass over charge,  $m/z$ ) of which 62 features in negative scan mode and 83 in positive scan mode were significantly different ( $P < 0.05$  after Bonferroni correction) and identified using the Human Metabolome Database (HMDB, [www.hmdb.ca](http://www.hmdb.ca)). The most significantly altered intracellular metabolites, in positive and negative scan modes, are shown in Tables 1 and 2, respectively. Among others, we observed decreased signals of the masses corresponding to the amino acids serine, glycine and cystathionine, GABA, the Krebs cycle intermediates malate, citrate/isocitrate, fumarate, and pyruvate. Phosphoserine, the direct precursor of serine, was also decreased in B6-deficient cells. The masses of the features that were increased corresponded to those of glutamine and glutamine adducts (Tables 1 and 2). This increase was confirmed by targeted LC-MS/MS (Suppl. Fig. S2a).

The most striking observations were the decreased signals of serine and glycine, two amino acids that are increased in plasma of vitamin B6-deficient patients and animal models. Thus, we confirmed the amino acid findings by quantitative

**Table 1** The 15 most significantly altered intracellular compounds (positive scan mode)

#	<i>m/z</i>	Identification	<i>P</i> value	Change	Fold change <sup>a</sup>
1	144.006	serine [M + K] <sup>+</sup>	5.82E <sup>-15</sup>	decrease	2.7
2	106.050	serine [M] <sup>+</sup>	3.95E <sup>-13</sup>	decrease	2.2
3	223.075	L-cystathionine [M] <sup>+</sup> ; cysteinyl-threonine [M] <sup>+</sup> ; threoninyl-cysteine [M] <sup>+</sup>	4.57E <sup>-13</sup>	decrease	4.0
4	128.032	serine [M + Na] <sup>+</sup>	2.87E <sup>-12</sup>	decrease	1.8
5	104.070	(α/β/γ)-aminobutyric acid [M] <sup>+</sup> ; dimethylglycine [M] <sup>+</sup>	5.14E <sup>-12</sup>	decrease	1.5
6	165.988	serine [M + NaK-H] <sup>+</sup>	8.90E <sup>-11</sup>	decrease	3.0
7	150.014	serine [M + 2Na-H] <sup>+</sup>	4.85E <sup>-10</sup>	decrease	1.9
8	207.014	glutamine [M + NaK-H] <sup>+</sup>	8.50E <sup>-09</sup>	increase	1.2
9	157.011	malic acid [M + Na] <sup>+</sup>	2.82E <sup>-08</sup>	decrease	1.3
10	178.993	malic acid [M + 2Na-H] <sup>+</sup>	3.75E <sup>-08</sup>	decrease	1.3
11	215.016	citric acid [M + Na] <sup>+</sup> , isocitric acid [M + Na] <sup>+</sup>	4.21E <sup>-08</sup>	decrease	1.2
12	76.039	glycine [M] <sup>+</sup>	5.51E <sup>-08</sup>	decrease	1.4
13	135.977	glycine [M + NaK-H] <sup>+</sup>	9.44E <sup>-08</sup>	decrease	1.7
14	191.040	glutamine [M + 2Na-H] <sup>+</sup>	3.42E <sup>-07</sup>	increase	1.4
15	113.995	glycine [M + K] <sup>+</sup>	6.88E <sup>-07</sup>	decrease	1.4

Direct infusion high resolution mass spectrometry (DI-HRMS) in positive scan mode was used to detect the overall intracellular metabolic consequence of vitamin B6 deprivation. A t-test was performed on the area under the curve (AUC, in a plot of intensities against time) for every compound. Compounds are sorted on the *P* value between PL absence and presence (100 nM)

<sup>a</sup> Fold change was calculated from the arithmetic mean values of the AUC of each group

**Table 2** The 15 most significantly altered intracellular compounds (negative scan mode)

#	<i>m/z</i>	Identification	<i>P</i> value	Change	Fold change <sup>a</sup>
1	181.051	homovanilic acid [M] <sup>-</sup> ; 4-hydroxyphenyllactic acid [M] <sup>-</sup>	2.39E <sup>-13</sup>	decrease	1.6
2	104.035	serine [M] <sup>-</sup>	6.67E <sup>-12</sup>	decrease	2.6
3	221.060	L-cystathionine [M] <sup>-</sup> ; cysteinyl-threonine [M] <sup>-</sup> ; threoninyl-cysteine [M] <sup>-</sup>	4.48E <sup>-11</sup>	decrease	3.8
4	129.056	3-methyl-2-ketovaleric acid [M] <sup>-</sup> ; 4-methyl-2-ketovaleric acid [M] <sup>-</sup>	5.91E <sup>-11</sup>	decrease	2.1
5	215.033	hexose [M + Cl] <sup>-</sup>	6.98E <sup>-11</sup>	decrease	1.4
6	87.009	pyruvic acid [M] <sup>-</sup>	2.31E <sup>-10</sup>	decrease	1.5
7	181.039	glutamine [M + Cl] <sup>-</sup>	6.09E <sup>-10</sup>	increase	1.3
8	140.012	O-phosphoethanolamine, serine [M + Cl] <sup>-</sup> , α/β/γ-aminobutyric acid [M + K-H] <sup>-</sup> , dimethylglycine [M + K-H] <sup>-</sup>	2.24E <sup>-09</sup>	decrease	2.9
9	184.002	phosphoserine [M] <sup>-</sup>	1.08E <sup>-08</sup>	decrease	3.5
10	145.062	glutamine [M] <sup>-</sup> , ureidoisobutyric acid [M] <sup>-</sup> ; alanyl-glycine [M] <sup>-</sup>	2.53E <sup>-08</sup>	increase	1.3
11	195.051	gluconic acid [M] <sup>-</sup> , galactonic acid [M] <sup>-</sup>	3.20E <sup>-08</sup>	decrease	1.2
12	218.033	3-methyladipic acid [M] <sup>-</sup> , pimelic acid [M + NaCl] <sup>-</sup>	1.06E <sup>-07</sup>	decrease	1.4
13	190.973	glyceraldehyde-3-P [M + Na-H] <sup>-</sup>	1.13E <sup>-07</sup>	decrease	1.3
14	243.038	glutamine [M + H <sub>2</sub> PO <sub>4</sub> ] <sup>-</sup>	2.37E <sup>-07</sup>	increase	1.3
15	150.980	maleic acid [M] <sup>-</sup> , fumaric acid [M] <sup>-</sup>	7.49E <sup>-07</sup>	decrease	1.4

Direct infusion high resolution mass spectrometry (DI-HRMS) in negative scan mode was used to detect the overall intracellular metabolic consequence of vitamin B6 deprivation. A t-test was performed on the area under the curve (AUC, in a plot of intensities against time) for every compound. Compounds are sorted on the *P* value between PL absence and presence (100 nM)

<sup>a</sup> Fold change was calculated from the arithmetic mean values of the AUC of each group

LC-MS/MS (Fig. 1 and S2). The intracellular amino acid fluctuations observed along the course of the study were due to the media refreshment schedule. Media was refreshed every 24 h before sampling. As a consequence, cells were exposed to the same medium for 2 or 3 days, depending on the collection time point. Nevertheless, intracellular serine concentrations were significantly lower ( $P < 0.01$ ) at all time points, while intracellular glycine concentrations were lower at  $t = 4, 11, 14$  and  $18$  days ( $P < 0.01, P < 0.01, P < 0.01$  and  $P < 0.05$ , respectively). Reasoning that the decrease of serine could be either due to reduced biosynthesis or reduced import from the culture medium, we analysed serine and glycine concentrations in the medium that was sampled from the cells during the experiment (Fig. 1). In the presence of  $100$  nM PL, cells were found to have net export of serine into the medium at later time points. In contrast, in vitamin B6-deficient cells import of serine from the medium exceeds export. Glycine is exported, both in PL presence and absence but vitamin B6-deficient cells exported 17–21% less glycine than cells cultured in  $100$  nM PL. This suggests that biosynthesis of serine is hampered when cells are deficient in vitamin B6, making them more dependent on extracellular serine. This proposition is strengthened by the decrease of phosphoserine, the immediate precursor of serine, observed in the untargeted metabolomics analysis (Table 2).

Cystathionine was strongly decreased in B6-deficient conditions. Cystathionine is an intermediary metabolite in the homocysteine transsulfuration pathway. Cystathionine  $\beta$ -synthase (CBS, EC 4.2.1.22), the first enzyme in the homocysteine transsulfuration pathway, catalyzes cystathionine synthesis from homocysteine and serine, using pyridoxal 5'-phosphate as cofactor. Our results suggest that the additive effects of vitamin B6 and serine deficiencies led to a strong decrease of cystathionine production.

### Serine biosynthesis is decreased when vitamin B6 is deficient

To investigate serine *de novo* biosynthesis we performed a metabolic flux analysis. Vitamin B6-deficient and -proficient cells were incubated with  $^{13}\text{C}_6$ -glucose. In the vitamin B6 deficiency condition, serine synthesis was significantly decreased ( $P < 0.05$ ) with a 50% reduction at  $t = 12$  h. Intracellular  $^{13}\text{C}_2$ -glycine concentrations were significantly decreased at  $t = 0.5$  ( $P < 0.01$ ) and  $12$  h ( $P < 0.05$ ), being 38% less in the B6-deficient cells at  $t = 12$  h (Fig. 2).

We analysed the intracellular concentrations of 5-mTHF, the other product of SHMT activity, in addition to glycine, and established they were significantly decreased at  $t = 1$  and  $3$  days (\*\* $P < 0.01$ , and \* $P < 0.05$ , respectively) in vitamin B6-deficient cells (Fig. 2).

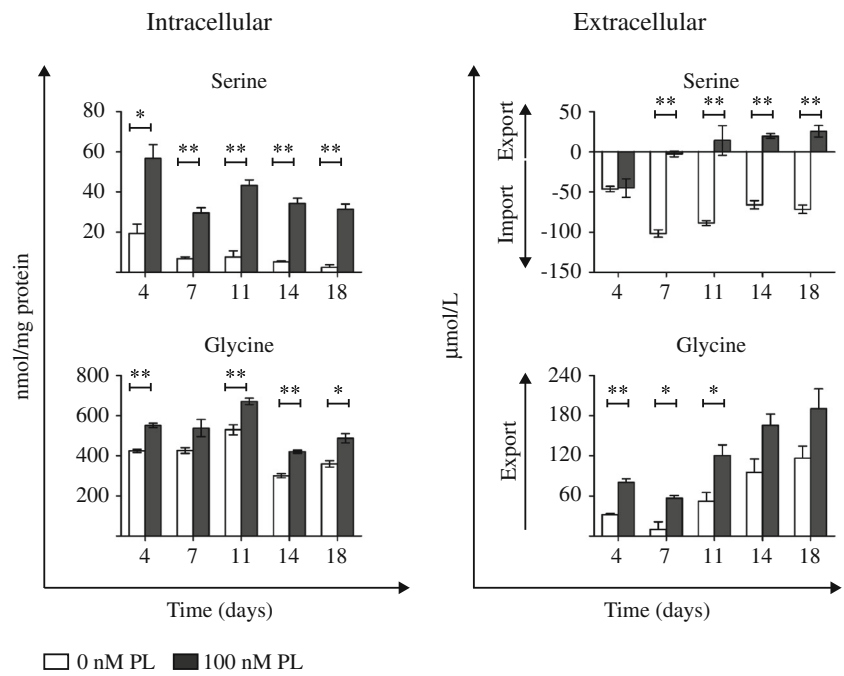
## Discussion

Vitamin B6 has an important role in development and functioning of the brain by catalyzing essential reactions in neurotransmitter and amino acid metabolism (Surtees et al 2006). To investigate the metabolic consequences of vitamin B6 deficiency in the brain, we employed a model system of Neuro-2a cells that were cultured in vitamin B6-deficient medium. Neuro-2a cells have a neuronal origin and are easily cultured in high amounts. In previous (yet unpublished) work we have investigated the presence of the enzymes involved in vitamin B6 metabolism and found that all are present in these cells, confirming that they provide a suitable model. This model mimics vitamin B6 deficiency as it results in strongly decreased intracellular concentrations of PLP (63% reduction at the latest time point) and PMP (50% reduction at the latest time point). The low PLP is a direct consequence of PL absence in the medium, whereas the decrease of PMP may reflect less transaminating activity secondary to the low PLP. It is unknown how the brain intracellular concentrations of vitamin B6 relate between B6-deficient and B6-proficient humans. However, some *in vivo* animal studies have documented vitamin B6 levels in the brain of B6-deficient animals. In Dakshinamurti et al, adult rats were fed PN-supplemented and PN-deficient diets and the PLP concentrations in the brains were documented. In the whole brain of B6-deficient rats, PLP concentrations were 28% reduced, while in their hypothalamus the reduction was 57% compared to PN-supplemented (control) rats (Dakshinamurti et al 1985). These results are in close accordance with the ones presented in this study. Thus, we successfully created a vitamin B6 deficiency model system.

Investigation of metabolism by untargeted metabolomics yielded a range of altered metabolites: amino acids, Krebs cycle intermediates, GABA and homovanillic acid. Among the most significantly changed metabolites were serine and glycine, which were both decreased as validated by targeted LC-MS/MS analysis. Flux studies clearly illustrated reduced serine biosynthesis in vitamin B6-deficient Neuro-2a cells and thus made evident that serine biosynthesis depends on vitamin B6. The PLP-dependent enzyme phosphoserine aminotransferase (PSAT, EC 2.6.1.52) cannot fully function in vitamin B6 deficient conditions, which is expected to result in less synthesis of phosphoserine and serine, as observed.

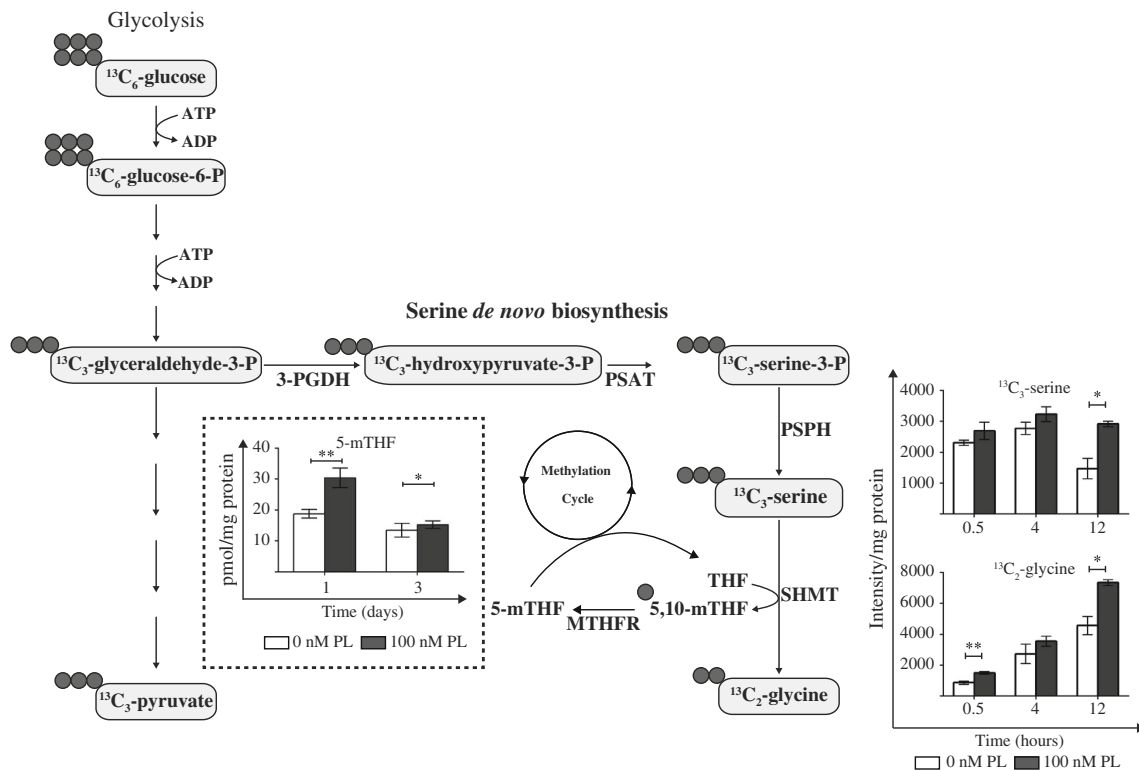
The low medium and intracellular serine concentrations are in contrast with reports on the effect of vitamin B6 deprivation on human plasma, in which the concentrations of glycine showed an increase of 28% after 2 weeks of vitamin B6 depletion and serine showed an increase of 47% after 1 week of depletion (Park and Linkswiler 1971). This behaviour was also observed in a 28-day vitamin B6 restriction diet study, where the plasma levels of healthy men and women for glycine and serine showed an increase of 15% and 12%,

**Fig. 1** Serine and glycine levels are decreased in vitamin B6-deficient cells. Neuro-2a cells were grown in medium in the presence (100 nM) and absence of PL for 18 days. Extracellular serine and glycine concentrations are presented as the difference to the media basal amino acid levels. Intracellular concentrations are normalized to total protein content. All results are represented as the mean of triplicates  $\pm$  SD; \*  $P < 0.05$ , \*\*  $P < 0.01$



respectively (da Silva et al 2013). Furthermore, cerebrospinal fluid (CSF) studies of PNPO-deficient patients have reported

elevated levels of glycine prior to B6 supplementation (Mills et al 2005). For the one individual in this study in whom CSF



**Fig. 2** Vitamin B6 deficiency hampers serine *de novo* biosynthesis and decreases 5-methyltetrahydrofolate levels. Neuro-2a cells were grown in the presence (100 nM) and absence of PL for 3 days. On day 3 cells were incubated with <sup>13</sup>C<sub>6</sub>-glucose, and the formation of the labelled <sup>13</sup>C<sub>3</sub>-serine and <sup>13</sup>C<sub>2</sub>-glycine was analysed at  $t = 0.5, 4$  and  $12$  h after exposure. Results are represented as the mean of triplicates  $\pm$  SD. The

insert reflects the steady state levels of 5-methyltetrahydrofolate. For 5-methyltetrahydrofolate study, Neuro-2a cells were grown in medium in the presence (100 nM) and absence of PL for 3 days. The results are normalized to total protein content and represented as the mean of  $n = 6$  ( $t = 1$  day) and  $n = 12$  ( $t = 3$  days)  $\pm$  SD; \*  $P < 0.05$ , \*\*  $P < 0.01$

analysis was repeated after supplementation this normalized. Elevation of CSF glycine has been reported to occur secondary to a deficiency of the activity of the glycine cleavage system, which is PLP-dependent. It should be noted however that this does not occur in all PNPO-deficient patients, with some only showing a transient increase of glycine in CSF (Hoffmann et al 2007). Glycine levels have also been reported to be slightly raised in the CSF of patients with mutations in PROSC prior to B6-supplementation (Darin et al 2016). In patients with mutations in ALDH7A1, however, CSF glycine levels have been reported to be normal (Hoffmann et al 2007) or just slightly elevated (Mills et al 2010). Interestingly few studies report on serine levels leading us to assume that serine is kept within normal values in the CSF of vitamin B6 deficient patients. Additionally, in a B6-deprivation study in HepG2 cells, vitamin B6 deficiency yielded large increases in glycine concentrations and no effect on serine (da Silva et al 2014). However, our findings in the Neuro-2a model are in accordance with *in vivo* studies performed by Tews, in which mice fed on a PN-deficient diet for 4 weeks presented a progressive decrease in brain serine concentrations. Brain glycine concentrations significantly decreased during the first 2 weeks of PN-deprivation and increased after 4 weeks when compared to normal-PN fed mice. Upon reinstating a complete-PN diet, serine and glycine concentrations returned to control levels (Tews 1969). This suggests that a decrease in serine and concomitant decrease in glycine concentrations is tissue or cell type dependent.

Serine in brain originates from two sources: uptake and biosynthesis (de Koning et al 2003). Serine *de novo* biosynthesis is a side-pathway of glycolysis. The first and rate-limiting step is the oxidation of 3-phosphoglycerate (3-PG) to 3-phosphohydroxypyruvate, by 3-phosphoglycerate dehydrogenase (PHGDH, EC 1.1.1.95). The conversion of 3-phosphohydroxypyruvate to 3-phosphoserine is catalyzed by phosphoserine aminotransferase, a PLP-dependent enzyme. Serine biosynthesis seems to be particularly important in the brain, as illustrated by the severe clinical symptoms in patients affected with a defect in serine synthesis, including congenital microcephaly, severe epilepsy and very little development (Jaeken et al 1996; Furuya 2008). In CSF of these patients, serine (both L- and D-serine) and glycine are decreased (van der Crabben et al 2013).

Glycine production depends on serine availability and on the PLP-dependent enzyme SHMT. SHMT catalyzes the transfer of the methyl group of serine to tetrahydrofolate (THF), allowing the formation of 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) and glycine (de Koning et al 2003). 5,10-methyleneTHF is converted by the enzyme methylenetetrahydrofolate reductase (MTHFR, EC 1.5.1.20) to 5-mTHF, the main circulating form of folate which can serve as a methyl donor in the generation of S-adenosylmethionine (SAM). Indeed, the combination of less serine and PLP may

lead to a lower activity of SHMT, explaining the decrease in the intracellular concentration of 5-mTHF in vitamin B6-deficient cells.

Our findings are important in considering pathogenesis and treatment of patients with vitamin B6-dependent epilepsy. Generally, it was thought that a reduction of GABA concentrations in the brain of these patients was the main cause of the epilepsy, due to suboptimal activity of the PLP-dependent enzyme glutamic acid decarboxylase. We demonstrate that low serine, glycine and 5-methyltetrahydrofolate may also contribute to pathogenesis. Probably, these amino acids are low in brain cells *in vivo*, as suggested by the B6-deficient mouse studies (Tews 1969). Although theoretically uptake of serine and glycine from blood to brain may compensate a lower serine biosynthetic capacity, two observations suggest clinical relevance of our findings. Patients with a defect in serine synthesis need high doses of serine to normalize serine in CSF (de Koning et al 2003; van der Crabben et al 2013). Furthermore, some patients with vitamin B6-dependent epilepsy clinically respond to supplementation of folinic acid, a 5-mTHF precursor (Nicolai et al 2006; Gallagher et al 2009; Stockler et al 2011; Dill et al 2011; van Karnebeek et al 2016). Our work provides an explanation for this hitherto puzzling observation.

## Material and methods

### Cell culture

Neuro-2a cells were purchased from ATCC Cell Biology Collection. Dulbecco's modified eagle medium (DMEM) GlutaMAX™ (31966), B6 vitamer-free DMEM GlutaMAX™ (custom made 31966-like) medium, foetal bovine serum (FBS; 10270), penicillin-streptomycin (P/S; 15140) and trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA, 0.5%) were purchased from Gibco (Invitrogen Life Technologies). Pyridoxal hydrochloride (PL-HCl) was purchased from Sigma-Aldrich (Steinheim, Germany). Cells were grown in 75 cm<sup>2</sup> flasks and maintained in DMEM GlutaMAX™ supplemented with 10% heat-inactivated FBS and 1% P/S, in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C. When cells reached optimal confluence (>70%) they were washed twice with PBS and passed into 6-well plates (1.5 × 10<sup>5</sup> cells per well) by trypsinization with 0.05% trypsin-EDTA. Confluent cells (>70%) were exposed to the experimental medium conditions: 1:1, PBS:B6 vitamer-free DMEM GlutaMAX™ (with 10% FBS and 1% P/S), with 100 nM of PL-HCl (content of vitamin B6: PL 97.4 ± 5.6 nM; PN 2.6 ± 2.6 nM; PM 1.4 ± 1.3 nM; PLP 1.9 ± 1.5; PNP and PMP are below the LOQ) or without vitamin B6 (residual content of vitamin B6: PL

4.7 ± 3.8 nM; PN 2.3 ± 0.4 nM; PM 1.1 ± 1.1 nM; PLP, PNP and PMP are below the LOQ).

### Direct-infusion high-resolution mass spectrometry (DI-HRMS)

Direct-infusion was performed using chip-based infusion (400 nozzles, nominal internal Ø 5 µm) on the TriVersa NanoMate (Advion, Ithaca, NY, USA). High-resolution mass spectrometry (140,000) was performed using a Q-ExactivePlus (Thermo Scientific GmbH, Bremen, Germany) using a scan range of  $m/z$  70 to 600 in positive and negative modes. Besides mass calibration of the instrument, internal lock masses were used for high mass accuracy. Cells were harvested in biological triplicates.

### Untargeted metabolomics pipeline (DI-HRMS)

RAW data files were converted to mzXML format using MSConvert (Chambers et al 2012). The data were processed using an in-house developed untargeted metabolomics pipeline written in the R programming language (<http://www.r-project.org>). First, the mzXML files were converted to readable format by the XCMS package (Smith et al 2006). For every sample, peak finding was done and peaks with the same  $m/z$  (within 0.5\**fwhm*) were grouped over different samples. Peak groups that were not present in three out of three technical replicates in at least one biological sample were discarded. The intensities of the technical replicates were averaged. Peak groups were identified using all entries in the HMDB, including their most likely adducts (Na<sup>+</sup>, K<sup>+</sup>, NH<sub>4</sub><sup>+</sup> in positive mode and Cl<sup>-</sup> and formate in negative mode) and isotopes, using an accuracy of 3 ppm or better. The statistical analysis was a t-test on the area under the curve (AUC) in a plot of intensities against time for every metabolite. Raw metabolomics data can be supplied upon request.

### Ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS)

#### Amino acids

Amino acid concentrations were determined using the UPLC-MS/MS method described by Prinsen et al (2016). Apart from adapting the range of the calibrators and quality control (QC) samples to resemble the concentrations in the samples, no further adaptations were needed for sample preparation or analysis of the amino acids.

#### Vitamin B6 vitamers

Vitamin B6 vitamers were quantified according to the method of van der Ham et al (2012).

#### 5-methyltetrahydrofolate (5-mTHF)

5-mTHF was purchased from Sigma-Aldrich (Steinheim, Germany). The internal standard <sup>13</sup>C<sub>5</sub>-5-mTHF was purchased from Merck KGaA (Darmstadt, Germany). 5-mTHF analyses were performed on a Waters Micromass Quattro Ultima triple quadrupole mass spectrometer (Manchester, U.K.), using an Acquity UPLC<sup>®</sup> BEH C18 (130 Å, 17 µm 2.1 × 50 mm column) (Waters, Manchester, UK), which was kept at 30 °C, while the autosampler temperature was kept at 15 °C. The dwell time was set at 0.3 s. The capillary voltage was 3.00 kV and the cone voltage was 35 V. The source and desolvation temperatures were 120 and 450 °C respectively. The cone gas flow rate was 158 L/h. Quantitative analysis was achieved using a negative ion multiple reaction monitoring (MRM) mode with the  $m/z$  transitions of 460.2 > 313.2 and 465.2 > 313.2 with a collision energy of 17 and 18 V, for 5-mTHF and <sup>13</sup>C<sub>5</sub>-5-mTHF respectively. The specific MRM transitions were determined by direct infusion of both standards and internal standards.

#### Metabolic flux analysis: serine de novo biosynthesis

Neuro-2a cells were grown in 6-well plates and maintained in DMEM GlutaMAX<sup>™</sup>. When cells reached a confluence of >70%, they were washed twice with room temperature PBS and incubated with B6 vitamer-free DMEM GlutaMAX-I (supplemented with 10% FBS, 1% P/S, and with either 100 nM PL-HCl or without PL-HCl): PBS, 1:1. Cells were grown for 72 h in these media before collection. At 72 h the medium was refreshed with the exposition medium: B6 vitamer-free DMEM GlutaMAX-I (supplemented with 10% FBS, 1% P/S, and without or with 100 nM PL-HCl): PBS (<sup>13</sup>C<sub>6</sub>-glucose, 25 mM), 1:1. Cells were harvested at  $T = 0.5, 4$  and 12 h. Uniformly labelled <sup>13</sup>C<sub>6</sub>-glucose (99%) was purchased from Cambridge Isotope Laboratories, Inc. (MA, USA). To quantify the intracellular <sup>13</sup>C<sub>3</sub>-serine and <sup>13</sup>C<sub>2</sub>-glycine, we adapted the LC-MS/MS method described by Prinsen et al (2016).

#### Protein analysis

Protein concentrations were quantified using the 96-well microplate protocol of the colorimetric bicinchoninic acid (BCA) Pierce<sup>™</sup> BCA Protein Assay Kit (Thermo Fisher Scientific Incorporated), in accordance with the manufacturer's protocol, with BSA as standard.

#### Statistical analysis

Statistical significance was determined with unpaired two-tailed t-test, using GraphPad Prism 6 (version 6.0.2, GraphPad Software Inc.) software.

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#### Compliance with ethical standards

**Conflict of interest** R. Ramos, M. Pras-Raves, J. Gerrits, M. van der Ham, M. Willemsen, H. Prinsen, B. Burgering, J. Jans, and N. Verhoeven-Duif declare that they have no conflict of interest.

**Informed consent** This article does not contain any studies with human or animal subjects performed by the any of the authors.

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