Impaired folate one-carbon metabolism causes formate-preventable hydrocephalus in glycine decarboxylase-deficient mice

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Ventriculomegaly and hydrocephalus are associated with loss of function of glycine decarboxylase (Gldc) in mice and in humans suffering from Non-Ketotic Hyperglycinemia (NKH), a neurometabolic disorder characterised by accumulation of excess glycine. Here, we showed that ventriculomegaly in Gldc-deficient mice is preceded by stenosis of the Sylvian aqueduct and malformation or absence of the sub-commissural organ and pineal gland. Gldc functions in the glycine cleavage system, a mitochondrial component of folate metabolism, whose malfunction results in accumulation of glycine and diminished supply of glycine-derived one-carbon units to the folate cycle. We showed that inadequate one-carbon supply, as opposed to excess glycine is the cause of hydrocephalus associated with loss of function of the glycine cleavage system. Maternal supplementation with formate prevented both ventriculomegaly, as assessed at pre-natal stages, and post-natal development of hydrocephalus in Gldc-deficient mice. Furthermore, ventriculomegaly was rescued by genetic ablation of 5,10-methylene tetrahydrofolate reductase (Mthfr), which results in retention of one-carbon groups in the folate cycle at the expense of transfer to the methylation cycle. In conclusion, a defect in folate metabolism can lead to pre-natal aqueduct stenosis and resultant hydrocephalus. These defects are preventable by maternal supplementation with formate, which acts as a one-carbon donor.
Impaired folate one-carbon metabolism causes formate-preventable hydrocephalus in glycine decarboxylase-deficient mice

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Abstract

Ventriculomegaly and hydrocephalus are associated with loss of function of glycine decarboxylase (Gldc) in mice and in humans suffering from Non-Ketotic Hyperglycinemia (NKH), a neurometabolic disorder characterised by accumulation of excess glycine. Here, we showed that ventriculomegaly in Gldc-deficient mice is preceded by stenosis of the Sylvian aqueduct and malformation or absence of the sub-commissural organ and pineal gland. Gldc functions in the glycine cleavage system, a mitochondrial component of folate metabolism, whose malfunction results in accumulation of glycine and diminished supply of glycine-derived one-carbon units to the folate cycle. We showed that inadequate one-carbon supply, as opposed to excess glycine is the cause of hydrocephalus associated with loss of function of the glycine cleavage system. Maternal supplementation with formate prevented both ventriculomegaly, as assessed at pre-natal stages, and post-natal development of hydrocephalus in Gldc-deficient mice. Furthermore, ventriculomegaly was rescued by genetic ablation of 5,10-methylene tetrahydrofolate reductase (Mthfr), which results in retention of one-carbon groups in the folate cycle at the expense of transfer to the methylation cycle. In conclusion, a defect in folate metabolism can lead to pre-natal aqueduct stenosis and resultant hydrocephalus. These defects are preventable by maternal supplementation with formate, which acts as a one-carbon donor.
Introduction

Hydrocephalus results from abnormal cerebrospinal fluid (CSF) hydrodynamics (over-production, diminished drainage or impaired flow) leading to progressive enlargement of the cerebral ventricular system and subsequent pathology (1). Hydrocephalus can be acquired (following injury, infection, tumour formation or trauma) or arise as a congenital condition, in isolation or associated with structural abnormalities such as Dandy-Walker or Chiari II malformations (2;3). Although common (affecting 0.5-1 per 1,000 live births), the molecular pathophysiology of isolated congenital hydrocephalus is known in only a relatively small proportion of cases owing to heterogeneity and multifactorial etiology (4-6).

A potential causal effect of impaired function of the glycine cleavage system (GCS) is highlighted by occurrence of hydrocephalus in association with Non-Ketotic Hyperglycinemia (NKH), a life-limiting autosomal recessive neuro-metabolic disorder characterised by accumulation of glycine in body fluids and tissues (7;8). NKH results from mutation of GCS-encoding genes, with the majority of patients carrying mutations in GLDC (glycine decarboxylase) (9;10). Hydrocephalus arises in around 8% of NKH patients and enlarged ventricles are commonly found on imaging (15 of 41 patients in one clinical survey) (8;11).

A direct link between GLDC loss of function and hydrocephalus was confirmed by analysis of mice carrying hypomorphic (GldcGT1) or null (GldcGT2) alleles of the murine homologue, Gldc. A proportion of Gldc mutants die peri-natally owing to neural tube defects (NTDs), resulting from failed neural tube closure (12;13). However, among Gldc-deficient mice that survive post-natally, hydrocephalus becomes evident by 5-7 weeks of age in 20-25% of homozygotes (GldcGT1/GT1), with a characteristic domed head, distorted cranium and severely enlarged lateral ventricles (12). These mice also show signs of NKH, including elevated glycine concentration in body fluids and tissues and premature lethality (12).

A pre-natal origin of hydrocephalus was demonstrated by histological analysis of litters at E18.5 which revealed enlarged lateral ventricles in Gldc-deficient (GldcGT1/GT1 or GldcGT2/GT2) mice (12;14). This is consistent with the association of ventriculomegaly, enlargement of the cerebral ventricles, with post-natal hydrocephalus in humans (15). Serial imaging of individual GldcGT1/GT2 fetuses at successive gestational time points by in utero high-frequency ultrasound showed onset of ventriculomegaly between E16.5 and E18.5 (14).
The physiological (communicating vs non-communicating) and metabolic mechanisms underlying GLDC-related hydrocephalus have not been determined. The GCS is a mitochondrial enzyme complex which mediates decarboxylation of glycine, with concomitant transfer of a one-carbon group to tetrahydrofolate (THF), generating 5,10-methylene THF in folate one-carbon metabolism (FOCM)(9). Hence, GCS loss-of-function not only causes accumulation of glycine, but also prevents transfer of glycine-derived one-carbon units to FOCM (13). Excess glycine is thought to lead to neurological features of NKH, such as epilepsy (8;9), whereas NTDs result from impaired FOCM (13). Potential mechanisms underlying the development of ventriculomegaly and hydrocephalus could therefore include consequences of FOCM suppression or glycine accumulation in CSF or neural tissues. For example, it is proposed that altered osmolality of CSF (a potential effect of excess glycine) could result in net movement of water into the ventricles, leading to abnormal CSF hydrodynamics (16). Alternatively, FOCM is required for provision of one-carbon groups for key cellular processes including nucleotide biosynthesis and methylation reactions, whose disturbance could plausibly lead to ventriculomegaly. Here, we investigated the cause of hydrocephalus and the requirement for FOCM in ventricular development in Gldc-deficient mice.

**Results and Discussion**

In our previous ultrasound analysis of litters at E18.5, Gldc-deficient (GldcGT1/GT2) fetuses that exhibited enlarged lateral ventricles did not differ from wild-types in volume of the fourth ventricle or cerebellum (14). Similarly, we found that among GldcGT1/GT1 fetuses with obvious dilatation of the lateral and third ventricles (7 of 13 GldcGT1/GT1), more posterior structures including the fourth ventricle appeared comparable to wild-types (Fig. 1).

Specific expansion of the ventricular system rostral to the aqueduct of Silvius indicated the possibility of mechanical obstruction. Consistent with this hypothesis, after injection into the lateral ventricles, aqueous dye circulated to the posterior aqueduct and fourth ventricles in wild-type and GldcGT/+ neonatal mice but not in some GldcGT1/GT1 littermates (Fig. 1P-U, Fig. S1). Among wild-type and unaffected GldcGT1/GT1 fetuses at E18.5, the aqueduct lumen was continuous and could be followed in serial sections from the third to fourth ventricles (Fig. 2). In contrast, the aqueduct was very narrow or completely occluded in all GldcGT1/GT1 fetuses which displayed ventriculomegaly (Fig. 2, Fig. S2).

At E18.5, the subcommissural organ (SCO) and pineal gland are visible in the roof of the aqueduct in wild-type and unaffected Gldc-deficient fetuses (Fig. 2A-L). However, in all the affected Gldc-
deficient fetuses the SCO was absent and the pineal gland was absent or malformed (Fig. 1M, Fig. 2M-O). SCO agenesis is associated with development of both communicating and non-communicating forms of hydrocephalus in mice, as seen in genetic mutants for Msx1, Pax6 and Rfx3 or over-expression of Sox3 (17;18). A hypoplastic pineal gland is also observed with post-natal hydrocephalus in Lhx9 null mice (19).

*Gldc* is expressed throughout the neuroepithelium at E9.5 (12). At developmental stages when ventriculomegaly arose, we found widespread expression of *Gldc* in the brain at E16.5, including the pineal gland, SCO and pituitary gland (Fig 2Q, T, R). Immunostaining confirmed the presence of Gldc protein in these structures at E18.5 (Fig. 2S, U, V).

Hydrocephalus has been associated with denudation of the ependymal cell layer lining the ventricles and subsequent occlusion of the aqueduct (17;20). Lack or abnormal function of ependymal motile cilia may also result in hydrocephalus without aqueduct stenosis, possibly owing to impaired CSF flow (17;20). We found that the ependymal cell layer was intact in the ventricular system of wild-type (n=5) and unaffected *Gldc*<sup>GT/GT</sup> (n=4) fetuses at 18.5 (Fig. 2W, Fig. S2). Abnormalities were not observed in the ependymal cell layer of the lateral ventricles in *Gldc*<sup>GT/GT</sup> fetuses with ventriculomegaly (Fig. S2G-L), but this layer appeared disrupted in the dorsal region of the third ventricle in most (4 out of 5; Fig. 2Y) but not all (Fig. S3) fetuses. The fetal onset of ventriculomegaly, prior to maturation of multiciliated ependymal (in the first post-natal week mice), and largely intact ependyma suggest that disruption of this cell layer is unlikely to be causal. Together, the presence of enlarged lateral and third ventricles, unaffected fourth ventricle, impaired dye distribution and late-fetal onset of ventriculomegaly, implicate aqueduct stenosis as the structural abnormality causing a non-communicating form of hydrocephalus in Gldc-deficient fetuses.

We next investigated the metabolic basis of hydrocephalus in *Gldc*-deficient mice. Loss of function of GCS causes accumulation of glycine, with significantly elevated levels in *Gldc*-deficient embryos by E11.5 (12). Metabolic labelling shows that the contribution of glycine-derived one-carbon units to FOCM is also ablated in *Gldc*-deficient embryos, with consequent alterations in the relative abundance of folates (12;13).

We found that exogenous supply of one-carbon units by maternal formate-supplementation normalised the folate profile in *Gldc*-deficient embryos and prevented NTDs, despite tissue glycine concentration remaining elevated (12;13). Based on this strategy, pregnant dams were
supplemented with formate prior to collection of litters for evaluation of ventriculomegaly (Fig. 3A). Histological examination divided Gldc<sup>GT1/GT1</sup> fetuses into two main categories: a group which exhibited severely dilated lateral ventricles with absent SCO and pineal gland (as Fig. 1M) and another with normal appearance, indistinguishable from wild-type (as Fig. 1H; Fig. S4). A few fetuses displayed an intermediate phenotype in which the SCO or pineal gland were detectable but appeared abnormal, accompanied by mild ventricle dilation (Fig. S4). Formate-supplementation led to significant normalisation of development (Fig. 3B), with none of the treated Gldc<sup>GT1/GT1</sup> fetuses displaying severe ventriculomegaly or absence of the SCO and pineal gland. Similarly, the ependymal cell layer was intact in all formate-treated fetuses examined (n = 6 unaffected and 1 intermediate; Fig. 3D-I).

Having found that formate supplementation could prevent fetal ventriculomegaly, we asked whether this pre-natal treatment rescued post-natal onset of hydrocephalus. Gldc<sup>GT1/+</sup> dams were supplemented with formate for the first 15 days of pregnancy and offspring were monitored for development of abnormalities until 6-7 weeks of age. Notably, none of the Gldc<sup>GT2/GT2</sup> offspring (n = 35) of formate-supplemented mice developed hydrocephalus, unlike non-supplemented Gldc<sup>GT1/GT1</sup> in which hydrocephalus arose in 30% of mice (Fig. 3C), similar to previous studies (12;14).

Prevention of ventriculomegaly/hydrocephalus by pre-natal formate-supplementation implicates insufficient supply of one-carbon groups to FOCM as the underlying causative mechanism. The folate cycle intermediates, 5,10-methylene tetrahydrofolate (THF) and 10-formyl THF, act as one-carbon donors in biosynthesis of thymidylate and purines. Alternatively, one-carbon groups can be transferred to the methionine cycle via donation of a methyl group from 5-methyl THF to homocysteine (Fig. 4A). To ask whether ventriculomegaly derives from suppression of the folate cycle or inadequate supply of one-carbon groups to the methionine cycle we bred Gldc-deficient fetuses that lack 5,10-methylene tetrahydrofolate reductase (Mthfr). Compound null Gldc<sup>GT2/GT2</sup>; Mthfr<sup>-/-</sup> embryos cannot generate 5-methyl THF and one-carbon groups are therefore retained in the folate cycle (13).

Analysis of litters produced by inter-cross of compound heterozygous, Gldc<sup>GT2/+</sup>; Mthfr<sup>-/-</sup> mice revealed ventriculomegaly in Gldc<sup>GT2/GT2</sup> fetuses that were wild-type or heterozygous at the Mthfr locus (Fig. 4). In contrast, all Gldc/Mthfr double-knockout fetuses lacked ventriculomegaly, demonstrating a significant protective effect of the Mthfr null mutation. This preventive effect highlights the folate cycle, rather than methionine cycle, as the subset of FOCM reactions that are
critical in prevention of aqueduct stenosis. Given the key role of the folate cycle in providing precursors for thymidylate and purine synthesis we speculate that defects result from impaired nucleotide biosynthesis in the neuroepithelium. The next step in this research will be to further refine the developmental stage and tissue site(s) in which folate cycle disruption subsequently leads to malformations of the SCO, pineal gland and aqueduct.

In animal models, a potential link between folate status and hydrocephalus was noted in dietary studies examining nutritional effects of folate and vitamin B₁₂ (21;22). Some neonatal offspring of rats fed synthetic diets lacking folic acid and vitamin B₁₂ exhibited enlarged ventricles attributed to possible blockage of the cerebral aqueduct (21). However, speculation remained over the relative importance of folic acid and vitamin B₁₂ and whether FOCM was impaired in these models (22).

Abnormal folate transport to the brain has been proposed to contribute to aqueduct obstruction in the hydrocephalic Texas (H-Tx) rat (23;24). Interestingly however, hydrocephalus is not commonly described among neurological signs associated with the very low levels of folate in CSF that occur in hereditary folate malabsorption, caused by mutations in SLC46A1 (proton-coupled folate transporter; PCFT) or cerebral folate deficiency, caused by mutations in FOLR1 (folate receptor; FRα)(25-27). On the other hand, hydrocephalus can arise in patients with severe MTHFR deficiency or remethylation disorders (including cblC disease) (28). MTHFR-related hydrocephalus may be of the communicating form (29), and it is not clear to what extent aqueduct stenosis also contributes. Nevertheless, impaired methionine synthase activity (as in cblC patients) could lead to accumulation of 5-methyl THF and thereby deplete one-carbon units from the folate cycle (methyl trap). This suggests the potential for a shared biochemical mechanism underlying hydrocephalus caused by methionine synthase or Gldc deficiency.

Here, we found that impaired enzymatic activity within mitochondrial FOCM, as opposed to diminished exogenous supply or a methyl trap, can be a direct cause of aqueduct stenosis, the most common known cause of congenital hydrocephalus in humans. The presence of hydrocephalus and/or enlarged ventricles in NKH patients and in Gldc-deficient mice suggests that the requirements for GCS activity are shared. Mutations in GLDC may also predispose to failed neurulation in humans as in mice, missense variants having been identified in some patients with NTDs (30-32), including functional mutations found in both NTDs and NKH (30;31).
Further evidence for a molecular link between congenital hydrocephalus and NTDs has come from identification of putative causal mutations in TRIM71, SMARCC1 and PTCH1 in patients with communicating and obstructive forms of hydrocephalus (6). Loss of function of each of these genes in mice causes cranial NTDs (33-35). Although the association of hydrocephalus with spina bifida is usually considered a secondary manifestation, one could speculate that they may be independent malformations with a shared genetic origin in some individuals. Hence, although NTDs and hydrocephalus arise at different developmental stages, the potential for prevention of both defects by formate supplementation suggests that there may be a related cellular mechanism, serving as a starting point for further research towards understanding the causation, pathogenesis and primary prevention of these related conditions.

**Methods** (further detail in Supplementary Material)

*Gldc*-deficient mice carried gene-trap alleles denoted *Gldc*<sup>GT1</sup> (12) or *Gldc*<sup>GT2</sup> (13). *Mthfr* null mice were previously described (36). Litters were generated by overnight matings with the following day designated embryonic day 0.5 (E0.5). Injection of the lateral ventricles was performed at P1. Mice were genotyped by PCR of genomic DNA (12;13). Sodium formate (30 mg/ml) was added to drinking water (12;13) from E0.5 to E15.5 and dams were then returned to normal drinking water.

Bouin’s fixed, paraffin embedded samples were sectioned (8 µm) and stained with haematoxylin and eosin. *In situ* hybridisation was performed on sections using a digoxigenin-labelled anti-sense probe to *Gldc* (12). Immunostaining was performed using anti-*Gldc* (1:300; Atlas Antibodies, HPA002318) with anti-rabbit AlexaFluor secondary (Invitrogen Thermo Fisher, A11034) antibodies.

**Study approval:** Studies were carried out under regulations of the Animals (Scientific Procedures) Act 1986 of the UK Government and approved by UCL Animal Welfare and Ethical Review Body, London, UK.

**Statistical analysis** was performed by Fisher-Exact test with p<0.05 considered significant.

**Author contributions:** Study design, NDEG, AJC, KL; Investigation, CS, YJP, MRM, KYL, DS, SW; Writing, NDEG; Editing, NDEG, AJC. The authors have declared that no conflict of interest exists.

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References


Figure 1. *Gldc*-deficiency results in ventriculomegaly. Unlike wild-type (A-E) and ‘unaffected’ *Gldc*<sup>GT1/GT1</sup> (F-J) fetuses, a subset (7 of 13) of *Gldc*<sup>GT1/GT1</sup> fetuses (K-O) were ‘affected’ by enlargement of the lateral (Lv) and third (III) ventricles at E18.5. The fourth ventricle (IV) does not differ in size between genotypes (compare D-E, I-J, N-O), nor does the aqueduct (aq) at this posterior axial level. Choroid plexus is detected in lateral, third and fourth ventricles (arrows in B) of all genotypes. However, the pineal gland (pg) and sub-commissural organ (arrowheads in C, H) are absent in *Gldc*<sup>GT1/GT1</sup> fetuses displaying ventriculomegaly (M). (P-U) Following bilateral injection into the lateral ventricles of neonatal mice (P-Q, S-T), dye distributed throughout the ventricular system including the fourth ventricle (IV) of *Gldc*<sup>+/+</sup> (R) but not *Gldc*<sup>GT1/GT1</sup> (U) mice. Scale bars: 1 mm (C, H, M), 5 mm (P-U), 0.5 mm (other panels).
Figure 2. Ventriculomegaly is associated with aqueduct stenosis in Gldc-deficient fetuses. Coronal sections in a rostral-caudal sequence (at levels shown in A-D in wild-type brain) show continuity of the aqueduct of Sylvius in Gldc\textsuperscript{+/+} (arrows in E-H) and unaffected Gldc\textsuperscript{GT1/GT1} (I-L) fetuses at E18.5. In contrast, the aqueduct narrows and exhibits discontinuities in Gldc\textsuperscript{GT1/GT1} mutants with ventriculomegaly (‘affected’) (M-P). Boxed areas (A-D) show enlarged regions in E-P. At E16.5, Gldc mRNA is widely expressed in the brain (Q-R, T), with abundant expression in the pineal gland (pg), sub-commissural organ (sco) and pituitary (pi). Immunohistochemistry confirms localisation of Gldc protein at these sites at E18.5 (S, U, V). The ependymal cell lining of the third ventricle (boxed in W-Y, enlarged in Z-EE) appears disrupted in Gldc\textsuperscript{GT1/GT1} fetuses at E18.5 (Y). Scale bars: 0.1 mm (R-V, Z-EE), 0.5 mm (A-P), 1 mm (Q, W-Y).
Figure 3. Maternal formate supplementation prevents ventriculomegaly and hydrocephalus. (A) *Gldc*\textsuperscript{GT1/+} mice were mated and pregnant females were supplemented with formate from E0.5 to E15.5. (B) At E18.5, there was a significantly greater proportion of *Gldc*\textsuperscript{GT1/GT1} fetuses that were ‘unaffected’ (n = 8/10) in formate-treated litters than among *Gldc*\textsuperscript{GT1/GT1} fetuses (n = 6/13) in control litters (\(*p < 0.05\); Fisher Exact test). (C) Among offspring of formate-supplemented mice that were monitored at post-natal stages none of the *Gldc*\textsuperscript{GT1/GT1} mice (n = 0/35) developed hydrocephalus, whereas 30% (6/20) of non-supplemented *Gldc*\textsuperscript{GT1/GT1} offspring developed hydrocephalus by 6-7 weeks (\(**p<0.002\) Fisher Exact test). Number of litters: n = 19 supplemented and 35 non-supplemented. (D-I) The ependymal lining of the third ventricle appeared intact in formate-treated fetuses at E18.5. Scale bars; 1 mm (D, G) or 100 µm (other panels).
Figure 4. Mthfr deletion in Gldc-deficient mice normalises development of the aqueduct, subcommisural organ and pineal gland. (A) Outline of folate and methionine cycles with key outputs in green text and relevant enzymes in blue text. Lack of Mthfr activity prevents transfer of one-carbon units from 5,10-methylene THF to the methionine cycle via 5-methyl THF. (B) Among offspring of Gldc<sup>G{T2}/+</sup>; Mthfr<sup>+/−</sup> intercrosses, ventriculomegaly was not detected at E18.5 in Gldc<sup>G{T2}/G{T2}; Mthfr<sup>−/−</sup></sup> fetuses (n = 5), but occurred at high frequency among Gldc<sup>G{T2}/G{T2}</sup> fetuses that were heterozygous (n = 7) or wildtype (n = 2) for Mthfr (p<0.025; Fisher Exact test). (C-E) Sections of E15.5 wild-type (C), Gldc<sup>G{T2}/G{T2}</sup> (D) and Gldc<sup>G{T2}/G{T2}; Mthfr<sup>−/−</sup></sup> (E) brains showing that absence of Mthfr restores the wild-type appearance of the pineal, SCO and aqueduct, and prevents ventriculomegaly, in Gldc null fetuses. Scale bars indicate 1mm.