

# Mutations in genes encoding the glycine cleavage system predispose to neural tube defects in mice and humans

Ayumi Narisawa<sup>1,2</sup>, Shoko Komatsuzaki<sup>1</sup>, Atsuo Kikuchi<sup>3</sup>, Tetsuya Niihori<sup>1</sup>, Yoko Aoki<sup>1</sup>, Kazuko Fujiwara<sup>4</sup>, Mitsuyo Tanemura<sup>5</sup>, Akira Hata<sup>6</sup>, Yoichi Suzuki<sup>6</sup>, Caroline L. Relton<sup>7</sup>, James Grinham<sup>8</sup>, Kit-Yi Leung<sup>8</sup>, Darren Partridge<sup>8</sup>, Alexis Robinson<sup>8</sup>, Victoria Stone<sup>8</sup>, Peter Gustavsson<sup>9</sup>, Philip Stanier<sup>8</sup>, Andrew J. Copp<sup>8</sup>, Nicholas D.E. Greene<sup>8,\*</sup>, Teiji Tominaga<sup>2</sup>, Yoichi Matsubara<sup>1</sup> and Shigeo Kure<sup>1,3,\*</sup>

<sup>1</sup>Department of Medical Genetics, <sup>2</sup>Department of Neurosurgery and <sup>3</sup>Department of Pediatrics, Tohoku University School of Medicine, Sendai, Japan, <sup>4</sup>Institute for Enzyme Research, University of Tokushima, Tokushima, Japan, <sup>5</sup>Tanemura Women's Clinic, Nagoya, Japan, <sup>6</sup>Department of Public Health, Chiba University School of Medicine, Chiba, Japan, <sup>7</sup>Human Nutrition Research Centre, Institute for Ageing and Health, Newcastle University, Newcastle upon Tyne, UK, <sup>8</sup>Institute of Child Health, University College London, London, UK and <sup>9</sup>Department of Molecular Medicine and Surgery, Karolinska Institute, Stockholm, Sweden

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Neural tube defects (NTDs), including spina bifida and anencephaly, are common birth defects of the central nervous system. The complex multigenic causation of human NTDs, together with the large number of possible candidate genes, has hampered efforts to delineate their molecular basis. Function of folate one-carbon metabolism (FOCM) has been implicated as a key determinant of susceptibility to NTDs. The glycine cleavage system (GCS) is a multi-enzyme component of mitochondrial folate metabolism, and GCS-encoding genes therefore represent candidates for involvement in NTDs. To investigate this possibility, we sequenced the coding regions of the GCS genes: *AMT*, *GCSH* and *GLDC* in NTD patients and controls. Two unique non-synonymous changes were identified in the *AMT* gene that were absent from controls. We also identified a splice acceptor site mutation and five different non-synonymous variants in *GLDC*, which were found to significantly impair enzymatic activity and represent putative causative mutations. In order to functionally test the requirement for GCS activity in neural tube closure, we generated mice that lack GCS activity, through mutation of *AMT*. Homozygous *Amt*<sup>-/-</sup> mice developed NTDs at high frequency. Although these NTDs were not preventable by supplemental folic acid, there was a partial rescue by methionine. Overall, our findings suggest that loss-of-function mutations in GCS genes predispose to NTDs in mice and humans. These data highlight the importance of adequate function of mitochondrial folate metabolism in neural tube closure.

## INTRODUCTION

Neural tube defects (NTDs), such as spina bifida and anencephaly, are severe birth defects that result from failure of

closure of the neural folds during embryonic development (1). Although NTDs are among the commonest birth defects in humans, the causes are still not well understood. This is most likely due to their complex, multifactorial causation

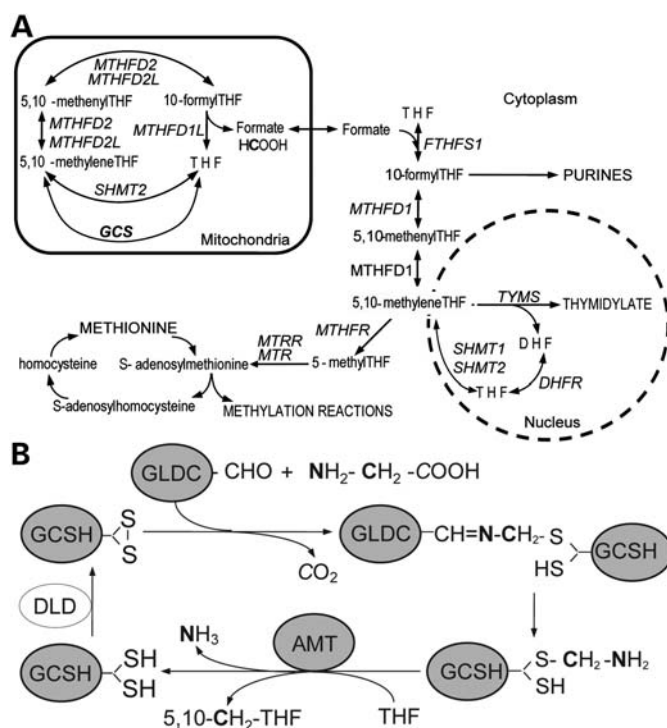
\*To whom correspondence should be addressed at: Neural Development Unit, UCL Institute of Child Health, Guilford Street, London, WC1N 1EH, UK. Email: n.greene@ucl.ac.uk (N.D.E.G.); Department of Pediatrics, Tohoku University School of Medicine, 1-1 Seiryomachi, Aobaku, Sendai 980-8574, Japan. Email: kure@med.tohoku.ac.jp (S.Ku.)

which is thought to involve contributions from both genetic and environmental factors (2–4). The potential complexity of NTD genetics is illustrated by the fact that more than 200 different genes give rise to NTDs when mutated in mice (5,6). Moreover, inheritance patterns in humans suggest a multigenic model in which an affected individual may carry two or more risk alleles, which by themselves may be insufficient to cause NTDs (2).

Folate one-carbon metabolism (FOCM) is strongly implicated as a determinant of susceptibility to NTDs since sub-optimal maternal folate status and/or elevated homocysteine are established risk factors, whereas periconceptional maternal folic acid supplementation can reduce the occurrence and recurrence of NTDs (7,8). Nevertheless, the precise mechanism by which folate status influences NTD risk remains elusive (7,9). FOCM comprises a network of enzymatic reactions required for synthesis of purines and thymidylate for DNA synthesis, and methionine, which is required for methylation of biomolecules (Fig. 1A) (9). In addition to the cytosol, FOCM also operates in mitochondria, supplying extra one-carbon units to the cytosolic FOCM as formate (Fig. 1A) (10).

Genes that are functionally related to folate metabolism have been subjected to intensive genetic analysis in relation to NTD causation, principally through association studies (reviewed in 3,4,11). In the most extensively studied gene, *MTHFR*, the c.677C>T SNP is associated with NTDs in some, but not all, populations. However, other FOCM-related genes have largely shown non-significant or only mild associations. Given the apparently complex inheritance of the majority of human NTDs, many association studies have been hampered by limitations on sample size. Moreover, although positive associations have been noted for other genes including *DHFR*, *MTHFD1*, *MTRR* and *TYMS* (12,13), these have not been replicated in all populations, and additional studies are required. The hypothesis that genetically determined abnormalities of folate metabolism may contribute to NTD susceptibility is supported by the observation of defects of thymidylate biosynthesis in a proportion of primary cell lines derived from NTDs (14). However, these defects do not correspond with known polymorphisms in FOCM-related genes. Overall, it appears likely that genetic influences on folate metabolism remain to be identified in many NTDs.

A potential link between mitochondrial FOCM and NTDs was suggested by the finding of an association of increased NTD risk with an intronic polymorphism in *MTHFD1L* (15). Another component of mitochondrial FOCM, the glycine cleavage system (GCS), acts to break down glycine to donate one-carbon units to tetrahydrofolate (THF), generating 5,10-methylenetetrahydrofolate (methylene-THF; Fig. 1B) (16,17). The GCS consists of four enzyme components, each of which is required for the glycine cleavage reaction (18,19). The components—glycine dehydrogenase (decarboxylating) (GLDC; P-protein), aminomethyltransferase (AMT; T-protein), glycine cleavage system protein H (GCSH; H-protein) and dihydrolipoamide dehydrogenase (DLD; L-protein)—are encoded by distinct genes: *GLDC*, *AMT*, *GCSH* and *DLD*, respectively. The functions of *GLDC*, *AMT* and *GCSH* are specific to the GCS, whereas *DLD* encodes a housekeeping enzyme. GCS components



**Figure 1.** Schematic diagrams summarizing the key reactions of folate-mediated one-carbon metabolism and the GCS. (A) Foliates donate and accept one-carbon units in the synthesis of purines, thymidylate and methionine. Mitochondrial FOCM supplies one-carbon units to the cytoplasm via formate. The GCS is a key component of mitochondrial FOCM that breaks down glycine and generates 5,10-methylene-THF from THF. Genes encoding enzymes for each reaction are indicated in italics. DHF, dihydrofolate; THF, tetrahydrofolate. (B) Summary of the GCS. The glycine cleavage reaction is catalysed by the sequential action of four individual enzymes: GLDC, GCSH, AMT and DLD. The first three of these (shaded grey) are specific to the GCS. Glycine is broken down into CO<sub>2</sub> and NH<sub>3</sub>, and donates a one-carbon unit (indicated in bold) to THF, generating 5,10-methylene-THF. The other carbon in glycine (indicated in italics) enters CO<sub>2</sub>.

have been found to be abundantly expressed in the neuroepithelium during embryogenesis in the rat (20).

We hypothesized that modulation of GCS activity has the potential to influence efficacy of cellular FOCM during the period of neural tube closure and, hence, susceptibility to NTDs. Therefore, in the current study, we screened genes encoding GCS components for possible mutations in NTD patients and controls. We tested variant proteins for loss of function by enzymatic assay and mice lacking GCS function were generated, to test the effect on embryonic development.

## RESULTS

The hypothesis that genes of the GCS represent candidates for involvement in NTDs prompted us to screen for potential mutations in patient samples. Coding exons of *AMT* (9 exons), *GCSH* (5 exons) and *GLDC* (25 exons) were sequenced in a total of 258 NTD patients comprising cohorts from Japan, the UK and Sweden. Each of the major categories of NTDs was represented among study samples, including anencephaly ( $n = 38$ ), spina bifida ( $n = 198$ ) and craniorachischisis ( $n = 22$ ).

**Table 1.** Nucleotide changes in NTD patients and controls identified by exon sequencing of *AMT*, *GLDC* and *GCSH*

Location	Nucleotide change	Effect	Number of mutation carriers in UK cohorts		Number of mutation carriers in the Japanese cohort		Number of mutation carriers in the Swedish cohort		Variant <i>GLDC</i> enzyme activity <sup>a</sup>
			NTD group (type <sup>b</sup> ) (n = 166) <sup>c</sup>	Control group (n = 189) <sup>c</sup>	NTD group (type <sup>b</sup> ) (n = 14) <sup>c</sup>	Control group (n = 36) <sup>c</sup>	NTD group (type <sup>b</sup> ) (n = 76) <sup>c</sup>	Control group (n = 145) <sup>c</sup>	
<i>AMT</i>									
Exon 2	c.103A>C	p.R35R	0	1	0	0	0	—	
	c.214A>G	p.T72A	0	0	0	1	0	—	
Exon 6	c.623C>A	p.A208D	0	2	0	0	0	—	
	c.631G>A	p.E211K <sup>d</sup>	2 (SBA)	0	0	0	1	—	
Exon 7	c.589G>C	p.D197H	0	0	1 (An)	0	0	—	
	c.825T>A	p.N275K	0	1	0	0	0	—	
	c.850G>C	p.V284L	1 (SBA)	0	0	0	0	—	
<i>GLDC</i>									
Exon 1	c.52G>T	p.G18C	2 (SBO/SBA)	2	0	0	2 (SBA)	2	84%
Exon 5	c.668C>G	p.P223R	0	0	0	1	0	—	92%
Exon 12	c.1508A>C	p.E503A	1 (SBA)	0	0	0	0	0	—
	c.1512G>C	p.E504D	1 (SBA)	0	0	0	0	0	99%
	c.1519G>C	p.G507R	1 (An)	0	0	0	0	0	17%
	c.1525C>G	p.P509A <sup>e</sup>	1 (An)	0	0	0	0	0	41%
	c.1550G>C	p.S517T	0	0	0	0	1 (SBA)	0	—
Exon 14	c.1570G>C	p.V524L	1 (SBA)	0	0	0	0	0	34%
	c.1705G>A	p.A569T <sup>f</sup>	3 (SBA/SBO/SBO)	1	0	0	1 (SBA)	0	40%
Exon 17	c.1953T>C	p.H651H	0	1	0	0	0	—	—
Exon 19	c.2203G>T	p.V735L	0	2	0	0	0	—	81%
Intron 19	c.2316-1G>A	splice	1 (SBA)	0	0	0	0	—	—
Exon 20	c.2380G>A	p.A794T	2 (SBASBA)	0	0	0	2 (SBA)	2	88%
	c.2406G>A	p.A802A	1 (An)	0	0	0	0	0	—
Exon 21	c.2474G>A	p.G825D	0	0	1 (An)	0	0	—	24%
	c.2487C>T	p.A829A	0	1	0	0	0	—	—
	c.2565A>C	p.A855A	1 (An)	0	0	0	0	—	—
Exon 23	c.2746C>T	p.L916L	1 (Crm)	0	0	0	0	—	—
Exon 25	c.2964G>A	p.R988R	0	0	0	0	1 (SBA)	0	—
	c.2965A>G	p.I989V	0	1	0	0	0	0	130%
<i>GCSH</i>									
Exon 1	c.53C>T	p.A18V	1 (An)	1	0	0	—	—	—

All nucleotide changes were found in heterozygous form. One individual carried c.52G>T and c.1705G>A in *GLDC*, whereas no other individuals carried more than one of the nucleotide changes listed here. Eight silent polymorphisms and four missense variants present in dbSNP (<http://www.ncbi.nlm.nih.gov/projects/SNP/>) are not listed in this table and include: *AMT*: c.954G>A (p.R318R, rs11715915); *GLDC*: c.249G>A (p.G83G, rs12341698), c.438G>A (p.T146T, rs13289273), c.501G>A (p.E167E, rs13289273), c.660C>T (p.L220L, rs2228095), c.666T>C (p.D222D, rs12004164), c.671G>A (p.R224H, rs28617412) and c.1384C>G (p.L462V, rs73400312); and for *GCSH*: c.62T>C (p.S21L, rs8052579), c.90C>G (p.P30P, rs8177847), c.159C>T (p.F53F, rs177876), c.218A>G (N73S, rs8177876), c.252T>C (Y84Y, rs8177907) and c.261C>G (L87L, rs8177908). Grey shading indicates loss-of-function mutations, based on enzymatic activity in the *in vitro* expression study or splicing defect.

<sup>a</sup>Residual enzymatic activity of *GLDC* mutant protein is expressed as %activity of the wild-type enzyme (Fig. 2).

<sup>b</sup>SBA, spina bifida aperta; SBO, spina bifida occulta; An, anencephaly; Crm, craniorachischisis.

<sup>c</sup>Total number of UK, Japanese or Swedish NTD patients.

<sup>d</sup>This variant was previously established as likely to be a non-functional polymorphism by segregation in an NKH family (21).

<sup>e</sup>A biochemical test of folate metabolism, the dU suppression test, was previously performed on primary fibroblasts derived from this patient and showed a defect of thymidylate biosynthesis to be present (14).

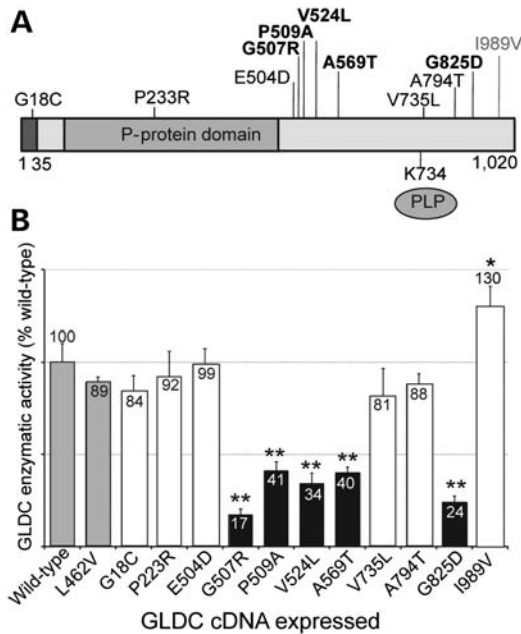
<sup>f</sup>p.A569T has previously been reported as a pathogenic mutation in a patient with typical NKH (21).

In *AMT*, we identified two novel sequence variants predicted to result in non-synonymous missense changes, c.589G>C (D197H) and c.850G>C (V284L), in anencephaly and spina bifida patients, respectively, from the UK cohort (Table 1). Neither variant was present in 526 UK or 36 Japanese control subjects or in the SNP databases dbSNP and 1000 Genomes. An additional missense variant, E211K, was also identified in three spina bifida patients, two from the UK and one from Sweden. Causative mutations in *AMT* have been found previously in an autosomal recessive inborn error of metabolism, non-ketotic hyperglycaemia (NKH) (17). The E211K variant had previously been identified in

an NKH family but was established as likely to be a non-functional polymorphism by segregation (21). Therefore, this variant is considered unlikely to be causally related to NTDs.

Exon sequencing of *GCSH* revealed eight single-base substitutions, one of which (c.53C>T, p.A18V) was a novel change found in both an NTD and a single control (Table 1). The others all corresponded to known SNPs, which did not suggest a role for *GCSH* in NTDs.

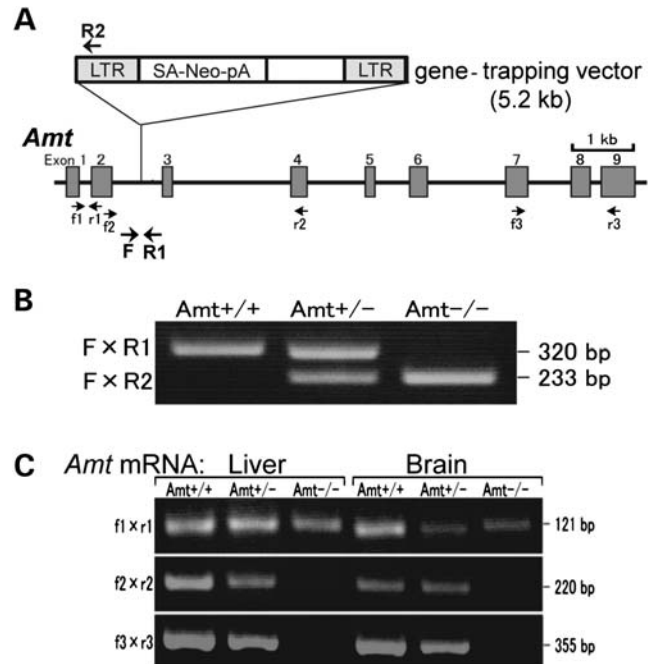
Next we turned our attention to *GLDC*, in which we identified 27 single-base substitutions (Table 1), including 11 silent nucleotide changes, 15 non-synonymous changes and a splicing acceptor variant of intron 19 (c.2316-1G>A). The



**Figure 2.** Characterization of *GLDC* missense mutations identified through DNA sequence analysis. (A) The schematic represents the 1020 amino acid residue *GLDC* polypeptide with the positions of the identified missense variants indicated. Mutations conferring significantly reduced activity (B) are indicated in bold. The leader peptide for mitochondrial import (shaded black) and the lysine 754-binding site for the co-factor pyridoxal phosphate (PLP) are indicated (49). (B) Enzymatic activity of *GLDC* missense variants. Expression vectors with wild-type and mutant *GLDC* cDNAs were transfected into COS7 cells for the evaluation of *GLDC* activity, which is expressed as relative activity (%) of cells expressing wild-type cDNA (shaded grey). The L462V *GLDC* enzyme (shaded grey) was tested as an example of a normally occurring variant (rs73400312). Variant proteins whose activities were significantly diminished compared with wild-type are indicated by black shading. The I989V variant, identified in a control parent, showed significantly elevated activity. Values are given as mean  $\pm$  SD of triplicate experiments (\* $P < 0.05$ ; \*\* $P < 0.01$ , compared with wild-type).

latter is deduced to abolish normal splicing of the *GLDC* mRNA, with predicted skipping of exon 19 resulting in loss of the reading frame. Among the 15 missense variants identified in *GLDC*, 5 were unique to the NTD group, being absent from all 562 control individuals as well as from the SNP databases. A further three novel variants were found only in controls, whereas the remainder were found in both NTDs and controls, and included previously reported SNPs.

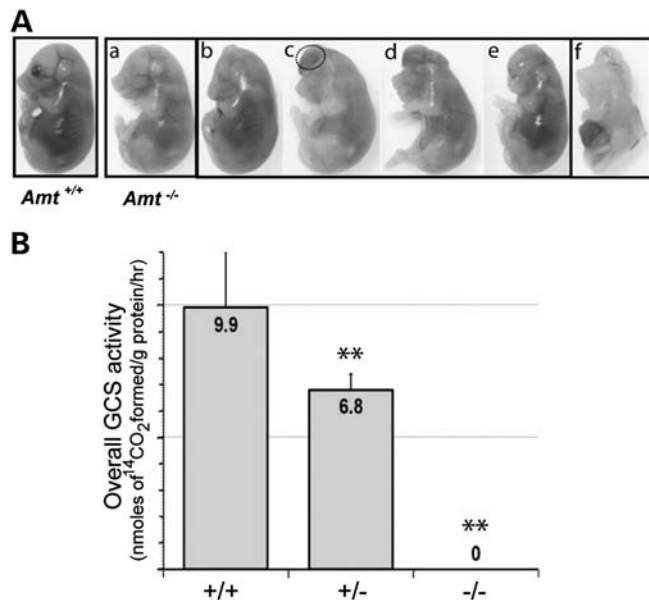
We investigated the possible functional effects of *GLDC* missense variants by expressing wild-type and mutant cDNA constructs in COS7 cells, followed by enzymatic assay of *GLDC* activity involving a decarboxylation reaction using [ $^{14}$ C]glycine (22). Twelve *GLDC* variants were tested, including those that were unique to NTD patients and, therefore, hypothesized to be potentially pathogenic (Fig. 2). The L462V variant, which corresponds to a known SNP (rs73400312), was included as an example of a known normally occurring form. Five of the missense changes, G507R, P509A, V524L, A569T and G825D, resulted in a significant reduction in *GLDC* activity compared with the wild-type protein ( $P < 0.001$ ). Notably, all five of these deleterious variants were present solely in NTD cases, whereas none of the variants that were unique to controls (P223R, V735L and I989V) showed loss of



**Figure 3.** Generation of *Amt* knockout mouse by gene trapping. (A) The location of the gene-trap vector in *Amt* intron 2 in the ES cell line OST181110 was determined by inverse PCR. Mice carrying this mutation were generated using standard methods of blastocyst microinjection with OST181110 ES cells to generate chimeras, and germ-line transmission. LTR, long terminal repeats; SA, splicing acceptor site; Neo, neomycin phosphotransferase gene; pA, polyadenylation sequence. (B) For genotyping, mouse genomic DNA was subjected to allele-specific amplification with F, R1 and R2 primers (Supplementary Material, Table S1). A genomic fragment of 320 bp was amplified from the wild-type allele, whereas a 233 bp fragment was amplified from the *Amt*-mutant allele. (C) RT-PCR analysis of *Amt* mRNA expressed in the brain and liver of *Amt*-mutant mice. Primers in exon 1–2 generated a 121 bp band irrespective of mouse genotypes. RT-PCR in which either one (f2-r2) or both (f3-r3) primers were located in exons 3' to the insertion site produced 220 and 355 bp cDNA fragments, respectively, in *Amt*<sup>+/+</sup> and *Amt*<sup>+/-</sup> mice, but not in *Amt*<sup>-/-</sup>. The *Amt* mRNA in mice carrying the trap vector was, therefore, aberrantly spliced at the end of exon 2, resulting in truncation of *Amt* mRNA in *Amt*<sup>-/-</sup> mice.

enzymatic function. In the case of G18C and A794T, which occurred in both NTDs and controls, there was no significant loss of enzymatic activity, suggesting that these are unlikely to be causative mutations.

Having identified putative mutations in *AMT* and *GLDC* in NTD patients, we hypothesized that loss of GCS function could predispose to development of NTDs. In order to directly test the functional requirement for GCS activity in neural tube closure, we generated mice that lacked GCS activity, using a gene trap (OmniBank, OST181110) of the *Amt* gene. The vector was located in intron 2, resulting in a truncated transcript that lacked exons 3–9 (Fig. 3). The efficacy of the gene-trap vector in trapping expression of *Amt* (*Amt*<sup>-</sup>) was confirmed by RT-PCR analysis (Fig. 3). Heterozygous *Amt*<sup>+/-</sup> mice were viable and fertile and exhibited no obvious malformations. Homozygous *Amt*<sup>-/-</sup> mice were not observed among post-natal litters from heterozygote intercrosses, and so fetuses were examined at embryonic day (E) 17.5. Strikingly, 87% of *Amt*<sup>-/-</sup> fetuses (34 out of 39) exhibited NTDs, whereas no malformations were observed in *Amt*<sup>+/+</sup> ( $n = 33$ ) or *Amt*<sup>+/-</sup>



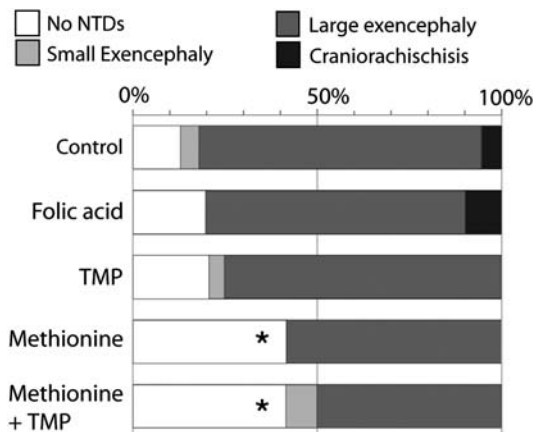
**Figure 4.** Mice lacking GCS activity exhibit NTDs. (A) Phenotypes of *Amt* mutant mice. NTDs were evident in the majority (88%) of *Amt*<sup>-/-</sup> fetuses (examples shown are at E17.5). Various types of NTDs were observed in *Amt*<sup>-/-</sup> fetuses, which principally affected the cranial region; a, no NTDs; b, small exencephaly (dotted circle); c–e, large exencephaly; f, craniorachischisis. (B) Enzymatic activity of the GCS in *Amt* knockout mice. *Amt*<sup>+/-</sup> and *Amt*<sup>-/-</sup> fetuses had significantly lower GCS activity in the liver than *Amt*<sup>+/+</sup> fetuses, with activity in *Amt*<sup>-/-</sup> samples below the level of detection (\*\**P* < 0.01 compared with *Amt*<sup>+/+</sup>).

(*n* = 66) fetuses. Defects mainly comprised exencephaly (82%), in which the cranial neural folds persistently failed to close (Fig. 4). There was also a low frequency of the more severe condition, craniorachischisis (5%), in which the neural tube remains open from the mid- and hindbrain, and throughout the spinal region (Fig. 4). Fetal liver samples were subjected to enzyme assay to determine overall activity of the GCS. In *Amt*<sup>-/-</sup> mice, overall GCS activity was effectively ablated being below the detection level of the assay (0.01 nmoles of <sup>14</sup>C<sub>2</sub>O<sub>2</sub> formed/gram protein/h), consistent with the *Amt*<sup>-</sup> allele being a functional null (22) (Fig. 4). These findings confirm that *AMT* function is essential for GCS activity, and that the latter is necessary for successful neural tube closure.

Given that GCS is a component of FOCM (Fig. 1), we evaluated the possible prevention of NTDs by folate-related metabolites. Maternal supplementation was performed with folic acid, thymidine monophosphate (TMP), methionine or methionine plus TMP (23). Neither folic acid nor TMP significantly affected the frequency of NTDs among the homozygous *Amt*<sup>-/-</sup> offspring. However, we observed a significant protective effect of maternal supplementation with methionine or methionine plus TMP, compared with the non-treated group (*P* < 0.05; Fig. 5).

## DISCUSSION

NTDs remain among the commonest human birth defects and understanding their genetic basis presents a considerable



**Figure 5.** Maternal supplementation of *Amt* mutant embryos with folic acid, TMP or methionine. Maternal treatment with folic acid (*n* = 10 homozygous mutant fetuses) or TMP (*n* = 12) had no significant effect on NTD frequency, whereas the frequency of unaffected embryos was significantly increased following treatment with methionine (*n* = 12) or methionine plus TMP group (*n* = 12). The asterisk indicates significant difference compared with non-treated group (*P* < 0.05).

challenge owing to their multigenic inheritance and the potential influence of environmental factors, either predisposing or ameliorating. Several lines of evidence indicate a requirement for FOCM in neural tube closure and, therefore, GCS-encoding genes provide excellent candidates for possible involvement in NTD susceptibility. We identified putative mutations in *AMT* and *GLDC* which include a splice acceptor mutation and a number of non-synonymous variants that were absent from a large group of population-matched controls, as well as from public SNP databases. In the case of *GLDC*, enzymatic assay confirmed that several mutations resulted in significant loss of enzyme activity. Finally, *in vivo* functional evidence of a requirement for GCS function in neural tube closure was provided by the occurrence of NTDs in *Amt*<sup>-/-</sup> mice lacking GCS activity. Together these findings indicate that mutations in *GLDC* and *AMT* predispose to NTDs in both mice and humans.

Where parental samples were available (6 of the 11 NTD cases that involved putative mutations in *GLDC*), we demonstrated parent-to-child transmission (Supplementary Material, Table S2). Six were instances of maternal transmission and one involved paternal transmission. We hypothesize that absence of an overt NTD phenotype in parents who carry a deficient *GLDC* allele may result from incomplete penetrance, or lack of additional genetic or environmental factors which are predicted to be necessary for NTDs owing to their multifactorial aetiology. We also note that partial penetrance is a feature of numerous mouse models of NTDs (5,8).

Inherited GCS deficiency, owing to mutation of *AMT* and/or *GLDC*, has been shown to cause NKH in humans (17). NKH is a rare, autosomal recessive, inborn error of metabolism, characterized by accumulation of glycine and encephalopathy-like neurological signs, including coma and convulsive seizures in neonates. GCS activity is greatly diminished in NKH patients and they would, therefore, be predicted to be at increased risk of NTDs. It is possible that NTDs may occur in combination with NKH but as anencephaly is a lethal condition, co-existing

NKH would go undetected. Lack of NTDs in NKH patients may also reflect the multigenic nature of NTDs, which require the presence of additional risk alleles in non-GCS genes. NKH is a relatively rare condition, with a prevalence of 1/63 000 births in British Columbia (24) and 1/250 000 in the USA (25). It is therefore possible that an increased risk of NTDs among carriers of GCS mutations in NKH families may not have been noted and this possibility is worthy of investigation. Based on estimated carrier frequency and the incidence of mutations among NTD patients, we predict that NTDs might be expected among 1/150 of the siblings of NKH patients (see Supplementary Material, Table S3 for estimate calculation). One case report of an NKH patient with a *GLDC* mutation describes the additional presence of spinal cord hydromyelia (19). This condition is often associated with low spinal defects (involving secondary neurulation), but it is also possible that the expanded spinal canal was also present at a higher level and might indicate a limited defect in primary neurulation.

The mutations described in the current study were all present in heterozygous form and, therefore, are hypothesized to be insufficient to cause NKH while predisposing to NTDs. For example, in the current study we found four NTD patients and one control individual to be heterozygous for the A569T mutation, which is shown to result in reduced enzyme activity. This mutation was previously identified in a Caucasian patient with typical NKH, in combination with a second mutation, P765S (26), confirming that it is deleterious *in vivo*. Hence, we predict that, depending on the co-existing genetic milieu, the A569T variant may cause NKH, predispose to NTDs or be compatible with normal development.

The high incidence of NTDs in *AMT* mutant mice is particularly notable as NTDs have not previously been found to be a common feature of mouse models deficient for folate-metabolizing enzymes. This includes null mutants that have been reported for eight other genes that encode enzymes in FOCM (Fig. 1A) (27). Four have normal morphology at birth (*Cbs*, *Mthfd1*, *Mthfr* and *Shmt1*) (28–31), *Mthfd2* null embryos die by E15.5 but neural tube closure is complete (32) and null mutants for *Mtr*, *Mtrr* and *Mthfs* die before E9.5, prior to neural tube closure (33–35). Although analysis of mouse mutants has not supported a role for single-gene mutations in FOCM as major causes of NTDs, a requirement for cellular uptake of folate for neural tube closure has been demonstrated in *Folr1* null embryos, in which NTDs occur when rescued from early lethality by folic acid supplementation (36). There is also considerable evidence for possible involvement of gene–environment and/or gene–gene interactions in NTDs. For example, in *Pax3* mutant (*splotch*) embryos, which exhibit a defect of thymidylate biosynthesis, dietary folate-deficiency increases the frequency of cranial NTDs (23,37). Similarly, a diet deficient in folate and choline causes NTDs in *Shmt1* mutant embryos, whereas *Shmt1* and *Pax3* mutations exhibit genetic interaction (38).

Regarding the mechanisms by which GCS mutations affect neural tube closure, a key question is whether NTDs are caused by impairment of FOCM or by another cause such as glycine accumulation. Modelling of hepatic FOCM, based on biochemical properties of folate-metabolizing enzymes (39), predicts that loss of the mitochondrial GCS reaction

would reduce the efflux rate of formate to the cytosol by ~50%. This results in reduced synthesis of purines and thymidylate, which are essential for the rapid cell division in the closing neural folds. Interestingly, a UK patient with anencephaly who was found to carry the *GLDC* loss-of-function mutation P509A in the current study (Table 1) was previously found to have impaired thymidylate biosynthesis, assayed in cultured fibroblasts (14). These findings support the hypothetical link between diminished *GLDC* function, reduced thymidylate biosynthesis and development of NTDs. Reduced thymidylate biosynthesis and diminished cellular proliferation are proposed to underlie folate-related cranial NTDs in *splotch* (*Pax3*) mouse mutants (37,38).

As well as impairment of nucleotide biosynthesis, the predicted effect of diminished GCS activity in reducing production of methionine (39) may also be of relevance as methionine is the precursor for the methyl donor *S*-adenosylmethionine. Indeed, metabolic tracing experiments suggest that ~80% of 1C units in the methylation cycle are generated within mitochondrial FOCM (40). Impairment of the methylation cycle and/or DNA methylation is known to cause NTDs in mice (41) and is proposed as a possible cause of human NTDs (7,42). It was therefore notable that we found a preventive effect of methionine supplementation in *Amt*<sup>-/-</sup> mice. Together, these findings suggest that FOCM, required for both thymidylate biosynthesis and methylation reactions that are essential for neural tube closure, may be functionally deficient in individuals who have mutations in *GLDC* or *AMT*.

## MATERIALS AND METHODS

### Patient cohorts and sequencing

Mutation analysis by DNA sequencing was performed on all exons of *AMT*, *GCSH* and *GLDC* as described (26). Cases comprised Japanese patients with anencephaly ( $n = 14$ ) and two separate cohorts of UK patients with a diagnosis of anencephaly (combined  $n = 24$ ), spina bifida ( $n = 122$ ) or craniorachischisis ( $n = 22$ ). In addition, the exons of *AMT*, *GCSH* and *GLDC* were sequenced in 76 Swedish patients with spina bifida. Unaffected controls, completely sequenced for these genes, comprised 36 Japanese and 189 unrelated UK subjects. Exons found to contain missense mutations were also sequenced in a further cohort of 192 well-characterized UK controls (43) and in 145 Swedish controls. This study was approved by the Ethical Committees of Tohoku University School of Medicine, UCL Institute of Child Health, Newcastle University and the Karolinska Institute.

### Enzymatic assay of GCS activity and *GLDC* activity

GCS activity was measured in mouse liver samples by a decarboxylation reaction using [1-<sup>14</sup>C]glycine as described (22). For analysis of *GLDC* activity, wild-type and mutant *GLDC* cDNAs were cloned into pCAG expression vector, kindly provided by Professor Jun-ichi Miyazaki (Osaka University, Japan) (44). Constructs were transfected into COS7 cells, which were harvested as described previously and cell pellets stored at -80°C prior to analysis (45). *GLDC*

enzymatic activity was determined, in triplicate, by exchange reaction between carbon dioxide and glycine using  $\text{NaH}^{14}\text{CO}_3$  in the presence of excess recombinant bovine GCSH protein as described (22). An expression system of lipoylated bovine GCSH protein in *Escherichia coli* was kindly provided by Dr Kazuko Fujiwara (Tokushima University, Japan) (46). Statistical analysis was performed using SPSS software version 11.0 (SPSS, Inc., Chicago, IL, USA).

### Knockout of Amt by insertion of a gene-trap vector

Mice carrying a gene-trap allele of *Amt* (here denoted *Amt*<sup>-</sup>) were generated at Lexicon Genetics, Inc. (Houston, TX, USA) using the OST181110 ES cell line. The genomic insertion site of the gene-trap vector was determined by inverse PCR and localized to intron 2 (Supplementary Material, Fig. S1). Total RNA was prepared from the mouse liver and brain at E18 for RT-PCR analysis (Supplementary Material, Fig. S1 and Table S1). *Amt*<sup>+/-</sup> mice were backcrossed with wild-type C57BL/6 mice for nine generations to generate a congenic line of mice on the C57BL/6 background, for use in biochemical and histological analyses. This study was approved by the Animal Experiment Committee of Tohoku University.

### Maternal supplementation with folic acid and related metabolites

Dams were treated with folic acid (25 mg/kg), thymidine-1-phosphate (TMP; 30 mg/kg) or L-methionine (70 mg/kg) by intra-peritoneal injection, 2 h prior to mating and daily from E7.5–10.5. Doses were based on previous studies (23,47,48).

### SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

*Conflict of Interest statement.* None declared.

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