A requirement for \textit{Gch1} and tetrahydrobiopterin in embryonic development

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**A B S T R A C T**

\textbf{Introduction:} GTP cyclohydrolase I (GTPCH) catalyses the first and rate-limiting reaction in the synthesis of the enzymatic cofactor, tetrahydrobiopterin (BH4). Loss of function mutations in the \textit{GCH} gene lead to congenital neurological diseases such as DOPA-responsive dystonia and hyperphenylalaninemia. However, little is known about how GTPCH and BH4 affects embryonic development \textit{in utero}, and in particular whether metabolic replacement or supplementation in pregnancy is sufficient to rescue genetic GTPCH deficiency in the developing embryo.

\textbf{Methods and results:} \textit{Gch1} deficient mice were generated by the insertion of loxP sites flanking exons 2–3 of the \textit{Gch1} gene. \textit{Gch1}\textsuperscript{fl/fl} mice were bred with Sox2\textsuperscript{cre} mice to generate mice with global \textit{Gch1} deficiency. Genetic ablation of \textit{Gch1} caused embryonic lethality by E13.5. Despite loss of \textit{Gch1} mRNA and GTPCH enzymatic activity, whole embryo BH4 levels were maintained until E11.5, indicating sufficient maternal transfer of BH4 to reach this stage of development. After E11.5, \textit{Gch1}\textsuperscript{+/fl} embryos were deficient in BH4, but an unbiased metabolomic screen indicated that the lethality was not due to a gross disturbance in metabolic profile. Embryonic lethality in \textit{Gch1}\textsuperscript{−/−} embryos was not caused by structural abnormalities, but was associated with significant bradycardia at E11.5. Embryonic lethality was not rescued by maternal supplementation of BH4, but was partially rescued, up to E15.5, by maternal supplementation of BH4 and L-DOPA.

\textbf{Conclusion:} These findings demonstrate a requirement for \textit{Gch1} in embryonic development and have important implications for the understanding of pathogenesis and treatment of genetic BH4 deficiencies, as well as the identification of new potential roles for BH4.

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\textbf{Introduction}

GTP cyclohydrolase I (GTPCH) catalyses the first and rate-limiting reaction in the synthesis of the enzymatic cofactor, tetrahydrobiopterin (BH4). BH4 is essential for several enzymes with critical physiologic and metabolic functions, including the three nitric oxide synthases (NOS1–3; EC 1.14.13.39), the aromatic amino acid hydroxylases (phenylalanine (EC 1.14.16.1), tyrosine (EC 1.14.16.2) and tryptophan (EC 1.14.16.4) hydroxylases) and alkylglycerol mono-oxygenase (AGMO; EC 1.14.16.5) (Werner et al., 2011). Through these enzymes, BH4 is required for nitric oxide (NO) production, phenylalanine catabolism, synthesis of the neurotransmitters dopamine, norepinephrine, epinephrine and serotonin, and for the metabolism of ether lipids.

Genetic deficiency in GTPCH, due to loss of function mutations in \textit{GCH1}, cause dopa-responsive dystonias and hyperphenylalaninemia, resulting from deficiency in BH4. Clinically, GTPCH deficiency is characterised by low levels of BH4, particularly in the cerebrospinal fluid (Furukawa et al., 1998), leading to dopamine and serotonin depletion in the CNS, with or without hyperphenylalaninemia due to insufficiency in hepatic phenylalanine metabolism (Opladen et al., 2012). GTPCH deficiency can have a mild to severe phenotype and is associated with a wide range of symptoms including muscle hypotonia/hypertonia, dystonia, movement disorders without dystonia, convulsions and autonomic symptoms (Opladen et al., 2012). Recommended treatment strategies for patients with GTPCH deficiency include...
supplementation of neurotransmitters, with L-DOPA and 5-HT, and BH4 replacement therapy. In addition to the critical role of BH4 in these severe genetic diseases, BH4 also plays a role in the pathogenesis of common, acquired diseases. These include vascular disease states such as high blood pressure, atherosclerosis and pulmonary hypertension, diabetes, neuropathic pain, depression and sickle cell disease (Bellantoni et al., 2013; Costigan et al., 2012; Cunnington and Channon, 2010; Katusic et al., 2009).

Although GTPCH deficiencies are treatable conditions, delayed diagnosis and treatment can result in significant neurocognitive impairment (Opladen et al., 2012), and early treatment does not result in normal neurocognitive development in all patients (Ponzoni et al., 1990). A recent study suggested that prenatal treatment with L-DOPA in normal neurocognitive development in all patients (Ponzone et al., 2005; Mitchell et al., 2003), or the hph–1 hyperphenylalaninemic mouse, generated by ENU mutagenesis, that has moderate systemic BH4 deficiency due to reduced gene expression from the GCH1 locus (Cosentino et al., 2001; Khoo et al., 2004). However, in the hph–1 mouse, variable BH4 deficiency in different cell and tissue types, and the moderate reduction in GTPCH activity, results in no gross abnormality in foetal development or survival, and limit the interpretation of the role in GTPCH in development (McDonald and Bode, 1988). Indeed, no genetic mouse model has tested the requirement for GTPCH and genetic deletion of Gch1, and assessed embryonic development and the effectiveness of metabolic supplementation during pregnancy to treat embryonic BH4 deficiency.

Materials and methods

Generation of the GCH1fl/fl mouse and GCH1 global knock out mouse

A plasmid containing a FRT-PGK-promoter and a neomycin-FRT-LoxP positive selection cassette was inserted in the intron 3 sequence of Gch1. An additional LoxP site was inserted into intron 1 to flox exon 2–3. The Gch1 floxed mouse was generated using homologous recombination in ES cells. Two chimeric male offspring with 90–100% chimerism were selected for breeding, with one chimera generating a novel mouse model with genetic deletion of Gch1, and assessed embryonic development and the effectiveness of metabolic supplementation during pregnancy to treat embryonic BH4 deficiency.

Gch1fl/fl mice were mated to generate Gch1−/−, Gch1fl/fl and Gch11+/− littermate embryos. Dams were treated with sepiapterin, (twice daily subcutaneous dosing, 5 mg/kg in PBS) and/or L-DOPA (drinking water, 1 mg/ml with 0.25% ascorbic acid) from E7.5 until either E13.5 or E15.5. Embryos were harvested from sacrificed dams at either E13.5 or E15.5. At harvest embryos were examined for the presence of a heart beat and gross abnormalities.

Embryonic heart rate analysis

At E10.5 and E11.5; dams were anesthetised with isoflurane (1–1.5%) in 100% oxygen and placed on a homeothermic mat, the abdomen was shaved and embryonic heart rates were quantified in utero using a Visualsonics Vevo 2100 with a 22–55 MHz transducer. Maternal body temperature was maintained between 36 and 37.5 °C and maternal heart rate was maintained between 400 and 500 bpm. An initial scan was made and a uterine map drawn of the location of each embryo. Embryonic heart rates were measured by counting waveforms in pulse wave Doppler mode over a 30 s period. Embryos were harvested from the dam under general anaesthetic and each embryo was assigned an identification number corresponding to their location on the scan to allow for genetic identification of the scanned embryos.

Histology and immunohistochemistry

Embryo morphology was assessed in paraffin-embedded embryo sections stained with haematoxylin and eosin (Merck, Germany). Whole embryos were sectioned, stained and all section assessed for abnormalities. Whole mount PECAM staining was performed on embryos as described before (Smart et al., 2010), using rat anti-PECAM. Embryos were then subjected to DAB substrate (Vector Labs) and cleared (50% glycerol). Histological quantification was carried out on digitised microscopic images using Image-Pro Plus.

RNA

Total RNA was obtained from homogenised embryos or adult tissue by Trizol extraction. Reverse transcription was carried out using the QuantiTect reverse transcription kit (Qiagen, UK) on 1 µg total RNA. Quantitative real-time RT–PCR was performed with an iCycler IQ real-time detection system (BioRad Laboratories, USA) using primers and probes from the TaqMan Gene Expression Assay system (Applied Biosystems, UK).

LC-MS-based Metabolomic screening

E11.5 embryos were exposed to three subsequent rounds of homogenisation in cold (−80 °C) methanol (80%) to rapidly quench metabolism and extract small molecule metabolites. The supernatant from each round was pooled and dried in a speed-vac. Protein assays were carried out on the tissue pellet to allow for normalisation. Metabolite extracts were resuspended in 70% acetonitrile+0.2% ammonium hydroxide to their appropriate protein concentrations. Resuspended metabolite samples were spun to remove residual debris, transferred to 250 µL conical polypropylene vials, capped, and loaded into the LC autosampler for subsequent LC-MS analysis. 3 µL of each sample was injected into the LC-MS platform. Further details regarding the LC-MS platform, data analysis and the identification of sample metabolites are described in detail within the Supplementary Material.

BH4, Phenylalanine and catecholamine analysis

BH4 levels in embryos and adult tissue was determined by HPLC followed by electrochemical and fluorescent detection, as previously
described (Crabtree et al., 2009). Briefly, embryos were homogenised in phosphate-buffered saline (50 mM), pH 7.4, containing dithioerythritol (1 mM) and EDTA (100 μM), centrifuged (15 min at 13,000 rpm and 4 °C), and the supernatant removed and the protein precipitated with ice cold phosphoric acid (1 M), TCA (2 M), and dithioerythritol (1 mM) and centrifuged. Samples were injected onto an isotropic HPLC system and quantified using sequential electrochemical (Dionex Coulochem III; Thermoscientific, Buckinghamshire, UK) and fluorescence (Jasco, Essex, UK) detection. HPLC separation was performed using a 250-mm, ACE C-18 column (Hichrom, Berkshire, UK) and a mobile phase comprising of sodium acetate (50 mM), citric acid (5 mM), EDTA (48 μM), and dithioerythritol (160 μM) (pH 5.2) at a flow rate of 1.3 ml/min. Background currents of +500 and −50 μA were used for the detection of BH4 on electrochemical cells E1 and E2, respectively. Quantification of BH4 was done by comparison with authentic external standards, processed identically.

For i-DOPA analysis, embryos were homogenised in PBS containing 0.1 M perchloric acid solution (PCA), homogenised and centrifuged (13,000 g for 15 min at 4 °C). i-DOPA levels were quantified as previously described (Ryan et al., 2014). Briefly, samples were injected onto an isotropic HPLC system and quantified using a Decade SDC detector and a carbon working electrode held at +0.7 V vs Ag/AgCl reference electrode (Antec). HPLC separation was performed using a 250-mm Microsorb C18 reverse-phase column (Agilent) and a mobile phase comprising methanol (13% v/v), NaH2PO4 (120 mM), EDTA (0.8 mM), and sodium octane sulphonate (3.2 mM), pH 3.27, and the flow rate was fixed at 1 ml/min. i-DOPA content was determined relative to authentic, freshly prepared standards and normalised to sample protein content.

For phenylalanine and tyrosine analysis embryos were homogenised in PBS, deproteinated by the addition of PCA (0.6 mol/L) in a ratio of 1:1 and centrifuged (13,000 rpm for 15 min at 4 °C). The supernatant was injected onto a Jasco Plus HPLC system and quantified using UV spectrophotometric detection (wavelength 210 nm). HPLC separation was performed using a Aeras PEPTIDE C18 column (3.6 μm, 150 × 4.6 mm, Phenomenex) and a mobile phase comprising acetonitrile (2.5%), octylamine (10 μL/L) and perchloric acid (0.8 mL/L), pH 2, at a flow rate of 0.75 ml/min. Quantification of phenylalanine and tyrosine was done by comparison with external standards and normalised to sample protein content.

Measurement of GTPCH activity

GTPCH activity was assessed by the quantification of the conversion of GTP to 78-dihydroxopterin. Embryos and adult tissue was homogenised in 300 μl Tris–HCl (100 mM), KCl (300 mM), EDTA (2.5 mM), phenylmethylsulfonyl fluoride (100 μM), and glycerol (10% v/v), pH 7.8. After centrifugation (5 min at 13,000 rpm and 4 °C) the supernatant was incubated with 10 mM GTP (final volume 300 μl) for 1 h at 37 °C. Samples, or neopterin standards, were subsequently incubated with 10 μl HCl (1 M) and 10 μl Lj/Kl solution (1% w/v l in 2% KI) and incubated for 1 h. Samples were centrifuged (2 min at 13,000 rpm) and 10 μl ascorbate (100 mM), 10 μl NaOH (1 M), and calf intestinal alkaline phosphatase (0.32U) were added to the supernatant (final volume 340 μl). Samples were centrifuged and then assessed using the biotyper electrochemical/fluorescence HPLC system detailed above.

Statistical Analysis

Data are presented as mean ± SEM. Groups were compared using the Mann-Whitney U test for non-parametric data or the Student’s two-sided t-test for parametric data. When comparing multiple groups data were analysed by analysis of variance (ANOVA) with Newman–Keuls post-test for parametric data or Kruskal–Wallis test with Dunns post-test for non-parametric data. Chi Square was used to test the differences between categorical variables. A value of P < 0.05 was considered statistically significant. Statistical analysis was carried out using GraphPad Prism version 6 (GraphPad Software, USA).

Results

Generation of a Gch1 knock out mouse

For conditional deletion of the Gch1 gene, exons 2–3 were flanked with loxP sites, as these exons encode amino acids involve in the GTPCH active site (Fig. 1A and B). We first confirmed that insertion of loxP sites in the Gch1 gene at these positions did not result in a hypomorphic allele. There was no difference in BH4 levels between Gch1+/+, Gch1+/− and Gch1−/− mice in heart, liver or lung tissues, indicating that the floxed Gch1 allele leads to normal levels of BH4 synthesis in gene-targeted mice (Supplementary Fig. 1).

In order to generate mice with global deletion of Gch1, we first crossed Gch1fl/fl mice with Sox2Cre mice. Gch1+/− mice from Gch1+/− x Gch1+/− matings were born in the expected ratio with no obvious defects. Adult Gch1+/− mice had a significant reduction in Gch1 mRNA expression in the heart compared to Gch1+/− littermates (Fig. 1C). As expected, due to the decrease in mRNA expression there was a concomitant reduction in GTPCH activity and BH4 levels in the hearts of adult Gch1+/− mice compared with Gch1+/− littermates (Fig. 1D and E). A significant reduction in BH4 levels was also observed in the lung and spleen of adult Gch1+/− mice compared with Gch1+/− littermate controls. However, no difference was observed in liver or plasma BH4 levels in these mice (Supplementary Fig. 2).

In order to generate homozygous Gch1 knockout mice (Gch1−/−) we mated Gch1+/− mice. However, this mating did not yield any Gch1−/− pups from more than 20 litters. To determine the onset of embryonic lethality, embryos from Gch1+/− x Gch1+/− matings were harvested after timed matings, for genotyping and analysis. Up to E10.5, Gch1−/− embryos were present at the expected Mendelian ratio, however, from E11.5 onwards alive Gch1−/− embryos were significantly underrepresented. At E11.5 and E12.5 only 55% and 39% of Gch1−/− embryos were alive. By E13.5, all Gch1−/− embryos were either absent or dead and by E15.5 dead Gch1−/− embryos were under represented in the litter with only 4 Gch1−/− embryos present compared with 18 Gch1+/− and 23 Gch1+/− embryos indicating significant reabsorption (Fig. 1F).

At E11.5, surviving Gch1−/− embryos appeared normal with no obvious oedema or blood spots present (Fig. 3A). There were no differences in either crown to rump length or placenta weights of surviving Gch1−/− embryos compared with Gch1+/− or Gch1+/− littermates (Fig. 3B and C).

Embryonic Gch1 deletion abolishes GTPCH activity and reduces both BH4 and i-DOPA, but without global metabolic derangement

Gch1 mRNA expression increased significantly with the age of the embryos from E9.5 to E13.5 (Fig. 2A). Gch1−/− embryos had no detectable Gch1 mRNA expression, whereas Gch1 mRNA was maintained in Gch1+/− embryos. A significant reduction in GTPCH activity was observed in Gch1−/− compared to Gch1+/− embryos and this was further reduced in Gch1+/− embryos to almost non-detectable levels (Fig. 2B). Surprisingly, given the lack of GTPCH activity in Gch1−/− embryos a significant reduction in BH4 levels was not detected until E11.5 (Fig. 2C-E). The maintenance of normal BH4 levels in Gch1−/− embryos at E9.5–10.5 might be indicative of maternal transfer of BH4. However, at E11.5 where the embryo is larger, maternal transfer appears insufficient to maintain Gch1+/− levels of BH4 in the Gch1−/− embryos.

The reduction in BH4 levels at E11.5 resulted in a concurrent decrease in i-DOPA levels in Gch1−/− embryos (Fig. 2F). There was no
difference in phenylalanine levels between Gch1+/+ and Gch1−/− embryos (Fig. 2G), which could be due to the low expression levels of PAH at this time point (Supplementary Fig. 2). No difference was observed in tyrosine levels between Gch1+/+ and Gch1−/− embryos (Fig. 2H). There was also no compensatory up regulation of mRNA of other BH4 dependent enzymes, or critical enzymes in BH4 biosynthesis downstream of GTPCH (Supplementary Fig. 3).

Since BH4 is a critical co-factor for a number of important metabolic enzymes, we next sought to determine whether global BH4 deficiency altered the metabolic profile of the embryos. Untargeted metabolomics analysis was used to compare metabolic profiles between surviving E11.5 Gch1+/+ and Gch1−/− embryos. We quantified the relative abundance of 590 features (metabolites) that were detected in at least 80% of samples from either Gch1+/+ and Gch1−/− embryos. However, no significant differences were observed in metabolite levels between the two groups (P > 0.05). Furthermore, unsupervised principal component analysis (data not shown) and hierarchical clustering, based on these 590 compounds, did not result in separation or clustering of the embryos by genotype (Fig. 2I).

We further analysed potential metabolic differences between Gch1+/+ and Gch1−/− embryos, using a curated metabolite library of 150 compounds, generated with pure chemical standards, known to be implicated in BH4 biology and related metabolic processes. Thirty-eight of these compounds (listed in supplementary Table 1) were observed in at least 80% of samples from at least one experimental group. This targeted approach yielded similar results to the untargeted metabolomics screen, with no significant difference in any of the observed metabolites between the two genotypes (P > 0.05) and no distinct separation by genotype with unsupervised hierarchical analysis (Fig. 2J). These experiments indicate that Gch1−/− embryo lethality observed at E11.5 is not due to gross disruption in metabolic pathways.

**Embryonic Gch1 deletion does not cause structural developmental defects, but results in foetal bradycardia**

Since embryonic deficiency in Gch1 did not lead to any difference in metabolic profile at the whole embryo level, we reasoned that Gch1 deficiency could result in cell specific defects in tissues that require de novo BH4 synthesis and may not be capable of BH4 uptake. As the embryonic cardiovascular system is critical for foetal survival beyond E9.5, we determined whether Gch1 deficiency leads to defects in the cardiovascular system. At E11.5 no excessive pericardial fluid was
Fig. 2. A, Whole embryo mRNA expression of Gch1 in Gch1+/+, Gch1+/- and Gch1-/- embryos. Gch1 expression increased with development (*P < 0.05 of 4-6 embryos) in Gch1+/+ and Gch1+/- embryos whereas in Gch1-/- embryos Gch1 was not detected at any time point. B, Whole embryo Gch1 activity in E11.5 embryos, activity was significantly reduced in Gch1+/- and further reduced in Gch1-/- embryos (*P < 0.05, n=8-6 per group). C, BH4 levels in E9.5 embryos; D, E10.5 embryos and E, E11.5 embryos, no difference in embryonic BH4 levels between genotypes at either E9.5 or E10.5. At E11.5 there was a significant reduction in BH4 levels in Gch1-/- embryos (P<0.05, n=7-8 per group). F, L-DOPA levels in E11.5 embryos were significantly reduced in Gch1-/- embryos compared with Gch1+/+ and Gch1+/- embryos (*P < 0.05, n=8-6 per group). G. Douglas et al. / Developmental Biology 399 (2015) 129–138
Fig. 3. A, Representative photographs of E11.5 Gch1+++, Gch1+/- and Gch1-/- embryos. B, Embryos crow to rump length at E11.5, no differences between groups was observed. C, Placenta weights of Gch1+++, Gch1+/- and Gch1-/- embryos at E10.5 and E11.5, no significant difference was observed between groups. D, Representative images of the heart, outflow tracks and dorsal aorta from E11.5 Gch1+++ and Gch1-/- embryos. E, Representative images of whole mount PECAM stained E9.5 and E10.5 Gch1+++ and Gch1-/- embryos.
observed in surviving Gch1<sup>−/−</sup> embryos. Histological analysis confirmed no cardiovascular development abnormalities in the heart or outflow tract and well organised trabeculas in the hearts of Gch1<sup>−/−</sup> embryos, with no difference observed in trabeculated area between the two groups (0.056 ± 0.05 vs. 0.064 ± 0.007 Gch1<sup>+/+</sup> vs. Gch1<sup>−/−</sup>, p < 0.05; n = 5 per group). No blood pooling was observed in any tissues, the dorsal aortas appeared normal and blood was contained within the artery (Fig. 3A and D). Whole mount PECAM staining at E9.5 and E10.5 did not reveal any abnormality in the embryonic vasculature. Blood vessels of the intersomitic regions were normal and an intact vascular network was observed in the head region of both Gch1<sup>+/+</sup> and Gch1<sup>−/−</sup> embryos suggesting that lack of embryonic Gch1 did not affect vasculogenesis (Fig. 3E).

We next examined if the embryo lethality could in part be due to an altered heart rate, as deletion of tyrosine hydroxylase which reduces catecholamine synthesis results in embryonic bradycardia. Conversely a deficiency in Gch1 may lead to an indirect failing of the heart. We assessed heart rate in utero using ultrasound imaging and demonstrated no difference in embryonic heart rate between Gch1<sup>+/+</sup> and Gch1<sup>−/−</sup> embryos at E10.5 (Fig. 4A). At E11.5, 7 out of 12 Gch1<sup>−/−</sup> embryos were already dead and the remaining alive Gch1<sup>−/−</sup> had a significantly decreased in heart rate compared to their Gch1<sup>+/+</sup> and Gch1<sup>+/−</sup> littermates (83 ± 4 vs. 70 ± 4 in Gch1<sup>+/−</sup> vs. Gch1<sup>−/−</sup>; Fig. 4B).

Embryonic Gch1 deficiency is not rescued by maternal supplementation with BH4

Post-natal lethality observed in other genetic models of BH4 deficiencies can be rescued by post-partum supplementation with neurotransmitters and/or BH4. Accordingly, we next sought to establish whether embryonic lethality due to Gch1 deletion could be rescued by maternal treatment with BH4 and catecholamine. l-DOPA (10 mg/kg), administered to pregnant dams in the drinking water, resulted in a significant increase in embryonic l-DOPA levels in E11.5 Gch1<sup>+/+</sup> embryos (Fig. 5A). Maternal treatment with a BH4 precursor sepiapterin (5 mg/kg twice daily by subcutaneous injection) significantly increased embryonic BH4 levels, which peaked at 6 h and declined back to baseline 14 h after administration (Fig. 5B). Based on these observations, and to ensure continuous embryonic supplementation with BH4, dams were dosed twice daily with sepiapterin until E11.5. Quantification of BH4 in embryos, harvested 14 h hours after maternal BH4 injection (i.e. at trough level) confirmed that BH4 levels in Gch1<sup>+/+</sup> embryos were significantly elevated compared with embryos from untreated dams, and achieved levels of embryonic BH4 similar to Gch1<sup>+/+</sup> embryos. Indeed, in embryos from dams treated with sepiapterin there was no difference in whole embryo BH4 between Gch1<sup>−/−</sup>, Gch1<sup>+/−</sup> or Gch1<sup>−/−</sup> embryos (Fig. 5C), indicating effective supplementation of maternal BH4 that is able to restore the biochemical deficiency in BH4 in Gch1<sup>−/−</sup> embryos.

To determine whether biochemical rescue of embryonic BH4 deficiency would prevent embryonic lethality due to Gch1 deletion, we treated Gch1<sup>−/−</sup> dams, mated with Gch1<sup>−/−</sup> males, with either l-DOPA + sepiapterin, or sepiapterin alone. Treatment with sepiapterin alone did not result in a significant increase in Gch1<sup>−/−</sup> embryo survival at E13.5, with only one Gch1<sup>−/−</sup> embryo alive compared with 13 dead. However, supplementation with both l-DOPA and sepiapterin resulted in a significant increase in embryonic survival, with 8 out of 18 Gch1<sup>−/−</sup> embryos alive at E13.5, compared with no surviving Gch1<sup>−/−</sup> embryos out of 16 in control dams treated with saline (Fig. 5D). We next tested whether the partial rescue in embryo survival at E13.5 with sepiapterin and l-DOPA treatment would be sustained. At E15.5 we observed some surviving Gch1<sup>−/−</sup> embryos (4 alive out of a total of 9), but in both the control and treated group Gch1<sup>−/−</sup> embryos were significantly under represented compared with the expected Mendelian ratio, with only 4 embryos alive representing only 8% of the litter (P < 0.02), indicating a significant loss of Gch1<sup>−/−</sup> embryos prior to E15.5, despite continued maternal supplementation with sepiapterin and l-DOPA (Fig. 5E).

Discussion

In this study we report the first gene targeted mouse model of Gch1 deficiency. We show that deletion of Gch1 causes loss of embryonic BH4 synthesis and embryonic lethality by E13.5. Furthermore, lethality cannot be rescued with BH4 supplementation, indicating a requirement for Gch1 in embryonic development. Embryonic lethality in Gch1 deletion is not associated with any gross perturbation in the metabolic pathways or with any gross organogenesis defects, but causes embryonic bradycardia.

Our study provides novel insights into the role of Gch1 in development. Clinically, loss of function mutations in the Gch1 gene lead to rare congenital neurological diseases such as dystonia, DOPA-responsive dystonia and hyperphenylalaninemia. The majority of mutations are heterozygous autosomal dominant with a smaller number of autosomal recessive mutations (Thöny and Blau, 2006). These disorders are characterised by low levels of BH4 and decreased GTPCH activity (Furukawa et al., 1998) (Opladen et al., 2012, 2011). Infants with GTPCH deficiency are born normally but develop muscle hypotonia and movement disorders usually within the first year of life, with more severe BH4 deficiency leading to symptoms at an earlier age (Opladen et al., 2012). In our study global deficiency of Gch1 resulted in embryo lethality at mid-gestation. Clinically, non-functional mutations resulting in complete loss of BH4 synthesis have not been described, suggesting that complete loss of Gch1 in humans is also likely to be embryonically lethal. Unlike the eNOS KO mouse, which shows significant growth restriction at E17.5 (Hefler et al., 2001; van der Heijden et al., 2005),
there was no difference in embryonic size or placenta weights between Gch1+/− and Gch1+/+ littersmates at E11.5, indicating that up to this time point embryo growth was not retarded. As there were no apparent histological differences in heart or vascular structure between the Gch1−/− and Gch1+/+ littersmates, it is unlikely the lethality was due to structural abnormalities. Both catecholamine-deficient embryos and eNOS−/− embryos show signs of an altered development of the vasculature and the myocardium, with blood congestion and thinning of the myocardium (Thomas et al., 1995; Zhou et al., 1995) (Liu et al., 2012). However, these defects predominantly present at E12.5, by which time significantly lethality in Gch1−/− has already occurred.

GTPCH catalyses the first and rate limiting step in the synthesis of BH4, a critical co-factor for a number of important metabolic enzymes. At E11.5 Gch1−/− embryos were significantly deficient in both BH4 and L-DOPA, we hypothesise that as the embryos appeared structurally normal that the lethality was in part due to a gross perturbation of metabolic pathways. We used a targeted and untargeted metabolomics screen approach to look for candidate pathways which may have been altered due to Gch1 deficiency. Surprisingly, given the role of BH4 in multiple pathways there was no difference in the metabolic profile between Gch1−/− and Gch1+/+ littersmates, suggesting that any metabolic disturbance can be effectively buffered by maternal metabolism, and indicating that the embryonic lethality in Gch1 deficiency is not due to gross alteration in metabolic pathways. Nevertheless, the metabolic analysis carried out at the whole embryo level does not exclude the possibility that cell or tissue specific differences in the metabolic profile may in part account for embryonic lethality.

Other mouse models with genetic deletion of the downstream enzymes involved in the synthesis of BH4 have been previously investigated. PTPS knockout mice are born alive with the expected Mendelian ratio but die within 48 h of birth (Elzaouk et al., 2003; Sumi-Ichinose et al., 2001). It had previously been thought that there was no alternative pathway for the synthesis of BH4 in the absence of PTPS. However, if this was true then it would be expected that PTPS knock out mice would have the same phenotype as Gch1−/− mice. The finding of a more severe phenotype in Gch1−/− mice suggests that either an alternative pathway for the synthesis of BH4 exists, or that GTPCH has a role in embryonic development that is independent of BH4 biosynthesis. Studies in rats have indicated that GTPCH could act as a chaperone, and/or interact with other proteins (Du et al., 2012), such that loss of these functions could be a contributing mechanism to the embryonic lethality of Gch1 deletion.

In Gch1+/− embryos Gch1 mRNA and BH4 levels were detectable from E9.5 and increased markedly at E11.5 onwards, indicating a growing role for BH4 as the embryo develops beyond this gestational stage. Despite having no detectable Gch1 mRNA and minimal GTPCH enzymatic activity, Gch1+/− mice did not become deficient in BH4 until
E11.5, and even by E11.5 the embryonic deficiency in BH4 was proportionately much less than the observed loss of both Gch1 expression and GTPCH enzymatic activity. These findings support previous indications that BH4 can cross the placenta (Thomas et al., 1995; Vásquez-Vivar et al., 2009), and indicate that up to E10.5 maternal transfer of BH4 across the placenta is sufficient to maintain normal BH4 levels in the embryo, and beyond E10.5 remains sufficient to substantially mitigate the effects of Gch1 deletion on BH4 levels. It is possible that BH4 has a physiological role in embryo development before E10.5 which is masked in possible that BH4 has a physiological role in embryo development. Interestingly, the finding that sepiapterin supplement Gch1+/- embryos had whole embryo BH4 levels comparable to WT littermates at trough when BH4 levels in supplemented Gch1+/- embryos were no different from control Gch1+/- embryos raises the possibility that BH4 in the Gch1+/- embryos is being actively maintained.

Since some BH4 can cross the placenta, we hypothesised that Gch1+/- embryos could be rescued by maternal supplementation with BH4. Indeed, administration of sepiapterin significantly increased embryonic BH4 such that, even at trough levels, Gch1+/- embryos had comparable BH4 levels to those observed in WT littermates. However, despite this biochemical restoration of BH4 levels at the level of the whole embryo, embryonic lethality could not be rescued. It is possible that despite reaching super-physiological levels of BH4 for at least 6 h post-injection and maintain WT levels of BH4 in Gch1+/- embryos at trough that key BH4-requiring cells in the embryo did not reach WT levels. Additional experiments with alternative dosing regimens may help to answer this question.

In common with previous observations in the PTPS knock out mouse, we found no gross organ defects but a significant bradycardia (Sumi-Ichinose et al., 2001; indicating that the cause of lethality in Gch1+/- embryos is physiological rather than anatomical. The observed bradycardia in Gch1+/- embryos is most likely due to a deficiency in catecholamines, as tyrosine hydroxylase (TH) knockout mice (leading to deficient l-DOPA, dopamine, noradrenaline and adrenaline) also show significant bradycardia at E12.5 (Zhou et al., 1995). In common with the PTPS knock out mice and Gch1+/- embryos, both TH and dopamine β-hydroxylase KO embryos show no gross organ abnormalities but display embryo lethality between E16.5-E18.5 (Kobayashi et al., 1995; Sumi-Ichinose et al., 2001; Thomas et al., 1995; Zhou et al., 1995). Catecholamines are released by the embryo in response to in utero stress such as hypoxia which acts to increase heart rate via the β-adrenergic receptor (Portbury et al., 2003). Post-partum lethality in PTPS pups can be rescued with BH4 and neurotransmitter replacement therapy (Elzaouk et al., 2003; Sumi-Ichinose et al., 2001). Both TH and dopamine β-hydroxylase KO embryos can be rescued to term by maternal supplementation with l-DOPA (Zhou et al., 1995) or DOPS (Thomas et al., 1995). In Gch1+/- embryos l-DOPA levels were significantly decreased compared with Gch1+/- littermates. The maternal supplementation of embryos observed in this study indicates that BH4 can cross cell membrane. However, the mechanism of this transport is unclear and it has yet to be established if all cells are capable of taking up BH4 to the same extent and thus some BH4 requiring cells may still be significantly deficient. If catecholamine producing cells in the embryo were unable to uptake BH4 this would account for the failure of BH4 supplementation alone to rescue Gch1+/- embryos. At E13.5 supplementation with BH4 and l-DOPA appeared to part rescue the Gch1+/- embryos with 8/18 Gch1+/- embryos alive at harvest, indicating that the lethality observed in Gch1+/- embryos was in part due to a catecholamine deficiency. As dams were treated with both sepiapterin and l-DOPA it is likely that the combination of these two agents was required for the partial rescue, reflected in the slight delay in embryonic lethality. However, unlike mice with genetic deletion of either TH (Zhou et al., 1995) or dopamine β-hydroxylase (Thomas et al., 1995), which were rescued to term with catecholamine supplementation, Gch1+/- embryos were significantly underrepresented in the litter (8% of the litter at E15.5) indicating that an additional factor other than catecholamine deficiency was responsible for the lethality. Indeed, the bradycardia observed in untreated E11.5 Gch1+/- embryos could also be an indirect consequence of Gch1 deficiency resulting from a failing heart due to an alternative consequence of Gch1 deficiency.

Recently, AGMO has been identified as a BH4 dependent enzyme. AGMO is the only enzyme known to cleave the ether bond of xylglycerol and lyso-alkylglycerol phospholipids, including lyso-platelet activation factor (Watschinger et al., 2010: Watschinger and Werner, 2013). In our study only minimal AGMO expression was observed in Gch1+/- embryos up to E11.5 with a marked increase in expression observed from E12.5, hence it is unlikely that loss of AGMO function due to BH4 deficiency is the cause of embryo lethality observed between E10.5 to E12.5 in this study. However, further research into potential roles for AGMO in embryo development will help to answer this question.

In conclusion, we have demonstrated a requirement for Gch1 in embryonic development. lethality in Gch1+/- embryos is not associated with major metabolic disturbance, nor embryonic structural abnormalities, but causes embryonic bradycardia. Gch1 deletion is not rescued by BH4 supplementation (sepiapterin 5 mg/kg twice daily), and is only minimally impacted by catecholamine supplementation. Thus, cellular requirements for Gch1 in embryonic development have important implications for understanding the pathogenesis and treatment of genetic BH4 deficiencies, and for identifying new potential roles for BH4.

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Appendix A. Supporting information

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References

Bendall, J.K., Douglas, G., McNeill, E., Channon, K., Crabtree, M.J., 2013. Tetrahydrobiopterin in cardiovascular health and disease. Antioxid. Redox Signal. Bruggemann, N., Spiegler, J., Hellenbroich, Y., et al., 2012. BEneficial prenatal levodopa therapy in autosomal recessive guanosine triphosphate cyclohydrolase 1 deficiency. Arch. Neurol. 69, 1071–1075. Cosentino, F., Barker, J.E., Brand, M.P., Heales, S.J., Werner, E.R., Tippins, J.R., West, N., Channon, K.M., Volpe, M., Luscher, T.F., 2001. Reactive oxygen species mediate endothelium-dependent relaxations in tetrahydrobiopterin-deficient mice. Arterioscler. Thromb. Vasc. Biol. 21, 496–502. Costigan, M., Latremoliere, A., Woolf, C.J., 2012. Analgesia by inhibiting tetrahydrobiopterin synthesis. Curr. Opin. Pharmacol. 12, 92–99. Cotton, R.G.H., 1986. A model for hyperphenylalaninaemia due to tetrahydrobiopterin deficiency. J. Inherit. Metab. Dis. 9, 4–14. Crabtree, M.J., Tatham, A.L., Al-Wakeel, Y., Warrick, N., Hale, A.B., Cai, S., Channon, K.M., Alp, N.J., 2009. Quantitative regulation of intracellular endothelial nitric oxide synthase (eNOS) coupling by both tetrahydrobiopterin-eNOS stoichiometry and biotinoprenexostatus: insights from cells with tet-regulated GTP cyclohydrolase I expression. J. Biol. Chem. 284, 1336–1344.
