Note

Homocysteine Hypothesis on the Impaired Peripheral but Not Central Nervous System Oxytocin Responses in Cystathionine γ -Lyase-Deficient Dam Mice

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An elevated plasma homocysteine level is an independent risk factor for cardiovascular diseases, neurological disorders, and pregnancy complications. We recently demonstrated partial lactation failure in cystathionine γ -lyase-deficient (*Cth*^{−/−}) dam mice and their defective oxytocin responses in peripheral tissues: uterine (*ex vivo*) and mammary gland (*in vivo*). We reasoned that elevated levels of circulatory homocysteine in *Cth*^{−/−} dam mice counteract with oxytocin-dependent milk ejection from the mammary gland. Based on our observation that those mice displayed normal maternal behaviors against their pups and adult *Cth*^{−/−} male mice exhibited normal social behaviors against adult wild-type female mice, both of which are regulated by oxytocin in the central nervous system (CNS), we conducted the present study to investigate the amino acid profiles, including total homocysteine, in both blood and cerebrospinal fluid (CSF) of wild-type and *Cth*^{−/−} female mice before pregnancy and at day 1 of lactation (L1). Serum levels of total homocysteine in wild-type and *Cth*^{−/−} L1 dam mice were 9.44 and 188 μ mol/L, respectively, whereas their CSF levels were below 0.21 (limit of quantification) and 3.62 μ mol/L, respectively. Their CSF/serum level ratio was the lowest (1/51.9) among all 20 proteinogenic amino acids, sulfur-containing amino acids, and citrulline/ornithine in *Cth*^{−/−} mice. Therefore, we hypothesize that the blood–brain barrier protects the CNS from high levels of circulatory homocysteine in *Cth*^{−/−} dam mice, thereby conferring normal oxytocin-dependent maternal behaviors.

Key words blood–brain barrier; cerebrospinal fluid; cystathionine γ -lyase; homocysteine; maternal behavior; oxytocin

INTRODUCTION

Elevated levels of plasma homocysteine (Hcy: HS-(CH₂)₂-CH(NH₂)-COOH), an amino acid intermediate in methionine metabolism, are widely known as an independent risk factor for cardiovascular diseases such as myocardial infarction, stroke, and venous thromboembolism.¹⁾ They have also been implicated in the onset or progression of various neurological disorders, including autism spectrum disorder, attention deficit hyperactivity disorder, amyotrophic lateral sclerosis (ALS), Parkinson's disease, and Alzheimer-type dementia in adults^{2–4)} as well as neural tube defects in neonates.⁵⁾ Moreover, increased plasma Hcy levels are attributed to pregnancy complications, including preeclampsia, spontaneous (recurrent) abortion, and premature/low-body-weight infants.⁶⁾ However, the molecular mechanisms through which Hcy exerts its pleiotropic effects *in vivo* yet remain unexplored.

In the present study, we have addressed this issue using the following two lines of homocysteinemic mutant mice: cystathionine β -synthase (Cbs)-deficient mice (*Cbs*^{−/−}, a model of homocystinuria [OMIM 236200])⁷⁾ and cystathionine γ -lyase (*Cth*)-deficient mice (*Cth*^{−/−}, a model of cystathioninuria [OMIM 219500]).⁸⁾ The majority of *Cbs*^{−/−} mice died at 2–4 weeks of age perhaps due to hepatic dysfunction,⁹⁾ whereas *Cth*^{−/−} mice appeared normal,⁸⁾ just as observed in patients with cystathioninuria.¹⁾ However, we found that *Cth*^{−/−} mice exhibited increased susceptibility to oxidative injury⁸⁾ and exposures to acetaminophen or various environmental electrophiles,^{10,11)} as well as partial lactation failure.¹²⁾

The lactation failure observed in *Cth*^{−/−} dam mice was attributed to reduced oxytocin responses in the mammary gland (*i.e.*, milk ejection) perhaps due to their homocysteinemia.¹²⁾ Oxytocin is a hormone/neuropeptide that consists of nine amino acids with an intramolecular disulfide bond between two cysteine (Cys) and is normally produced in the hypothalamus and released by the posterior pituitary into the circulation to mainly regulate uterine contraction and milk ejection. It acts on Gq-coupled oxytocin receptor (Oxtr) to activate phospholipase C and intracellular calcium signaling. Previous studies conducted using oxytocin-deficient (*Oxt*^{−/−}) mice and Oxtr-deficient (*Oxtr*^{−/−}) mice have shown that either *Oxt*^{−/−} or *Oxtr*^{−/−} female mice displayed normal fertility, pregnancy, and parturition, but failed to eject milk; therefore, all pups born to these mice died within 24 h after birth.^{13,14)} Moreover, adult *Oxtr*^{−/−} male mice exhibited deficits in social discrimination and elevated aggressive behaviors.¹⁴⁾

Furthermore, *Cth*^{−/−} female mice displayed normal fertility, pregnancy, parturition, and maternal behaviors but insufficient milk ejection, and *Cth*^{−/−} male mice exhibited normal social behaviors¹²⁾; only their central nervous system (CNS) phenotypes were contrasted with those of *Oxt*^{−/−} and *Oxtr*^{−/−} mice. Therefore, in the present study, we investigated the amino acid profiles in the blood and cerebrospinal fluid (CSF) of wild-type and *Cth*^{−/−} female mice before pregnancy and at day 1 of lactation (L1). We observed very low levels of Hcy in the CSF of homocysteinemic *Cth*^{−/−} mice compared to their circulation levels.

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MATERIALS AND METHODS

Cth-heterozygous ($Cth^{+/-}$) mice were generated and backcrossed for 10 generations to C57BL/6J inbred strain (CLEA Japan, Tokyo, Japan), and thereafter, $Cth^{+/-}$ males and females were bred to obtain $Cth^{-/-}$ mice.⁸⁾ Blood and CSF samples were collected from both wild-type and $Cth^{-/-}$ mice (at 8–12 weeks of age) before pregnancy (virgin) and at L1 after anesthesia with isoflurane inhalation. Blood was collected by cardiocentesis (approx. 200 μ L/mouse) and used for serum preparation. CSF was collected by ventricular puncture with a glass capillary tube (5–10 μ L/mouse). Serum and CSF levels of amino acids were measured as described previously.¹⁵⁾ Briefly, thiol-containing amino acids (Hcy, Cys and glutathione (GSH)) were reductively cleaved at their intramolecular disulfide bonds and then derivatized with the thiol-labeling reagent SBD-F (Dojindo, Kumamoto, Japan). All other amino acids (except tryptophan (Trp)) were derivatized with the amino acid-labeling reagent NBD-F (Dojindo). Trp was detected using its own fluorescence without labeling. The levels of all amino acids were determined using HPLC.¹⁵⁾ Because a minimum volume of 10 μ L was required for all these assays, equal volumes of CSF samples from 2–3 mice were often pooled. All animal procedures were conducted according to the Guide for the Care and Use of Laboratory Animals, 8th Edition published by the US National Research Council, and were approved by the Animal Care Committees of Showa Pharmaceutical University (No. P-2018-07).

RESULTS AND DISCUSSION

Serum levels of total Hcy in virgin and L1 $Cth^{-/-}$ mice were 153 and 188 μ mol/L, and those of cystathionine were 98.9 and 137 μ mol/L, respectively; all these levels were much higher than those of the respective wild-type mice (Table 1), as we had previously observed in adult $Cth^{-/-}$ male mice.¹⁶⁾ Except for lower total Cys/total GSH and higher citrulline levels in $Cth^{-/-}$ mice, the amino acid levels were generally comparable between wild-type and $Cth^{-/-}$ mice or between virgin and L1 mice (Table 1). Meanwhile, the CSF samples displayed completely altered amino acid profiles, and their amino acid levels were generally much lower than their serum levels (except taurine). In particular, the CSF levels of total Hcy in virgin and L1 $Cth^{-/-}$ mice were only 3.69 and 3.62 μ mol/L, respectively, and both those levels in wild-type mice were below the limit of quantification (LOQ: <0.21 μ mol/L). The CSF/serum level ratio in $Cth^{-/-}$ L1 mice was the lowest (1/51.9) among all the measured amino acids (Table 1), and the ratios in wild-type mice were also estimated to be less than 1/35.8 (virgin) or 1/45.0 (L1) (Table 1). These results suggest that the blood–brain barrier protects the CNS from the circulatory homocysteine, especially in $Cth^{-/-}$ dam mice.

In our previous study, we observed an impaired peripheral oxytocin response (milk ejection) in the epithelial ducts of the mammary gland of $Cth^{-/-}$ mice.¹²⁾ Furthermore, preadministration of Hcy was found to interfere with oxytocin-induced uterine contraction in *ex vivo* experiments.¹²⁾ We hypothesized that the high level of circulatory Hcy somehow counteracts

Table 1. Serum and CSF Amino Acid Levels of Virgin and Lactating Wild-Type and $Cth^{-/-}$ Mice

(μM)	Serum				CSF				CSF/serum ratio in <i>Cth</i> ^{-/-} L1
	WT		<i>Cth</i> ^{-/-}		WT		<i>Cth</i> ^{-/-}		
	Virgin	L1	Virgin	L1	Virgin	L1	Virgin	L1	
t-Hcy	7.52 ± 1.79 (9)	9.44 ± 1.85 (8)	153 ± 26 (10)	188 ± 55 (12)	N.D. (< 0.21) (6)	N.D. (< 0.21) (6)	3.69 ± 1.27 (6)	3.62 ± 0.59 (5)	1/51.9
t-Cys	322 ± 24 (9)	355 ± 48 (8)	182 ± 17 (10)	224 ± 39 (12)	9.71 ± 3.68 (6)	8.96 ± 2.74 (5)	9.13 ± 3.57 (6)	11.6 ± 4.4 (5)	1/19.3
t-GSH	66.5 ± 15.9 (9)	64.8 ± 12.0 (8)	45.8 ± 12.8 (10)	53.5 ± 15.2 (12)	27.0 ± 6.0 (6)	27.6 ± 14 (5)	26.1 ± 6.5 (6)	24.1 ± 11.3 (5)	1/2.22
Ala	500 ± 131 (9)	476 ± 173 (8)	409 ± 101 (9)	397 ± 83 (12)	145 ± 24 (6)	151 ± 39 (5)	148 ± 34 (5)	146 ± 56 (5)	1/2.71
Arg	161 ± 27 (9)	217 ± 89 (8)	177 ± 35 (9)	234 ± 81 (12)	46.3 ± 8.7 (6)	60.4 ± 10.9 (5)	72.5 ± 2.7 (5)	77.0 ± 18.4 (5)	1/3.03
Asp/Asn	9.68 ± 3.96 (9)	9.66 ± 3.66 (8)	12.5 ± 1.9 (9)	10.2 ± 4.8 (12)	7.9 ± 4.29 (6)	12.3 ± 6.2 (5)	9.81 ± 4.04 (5)	6.29 ± 1.95 (5)	1/1.62
Gln	839 ± 136 (9)	969 ± 125 (8)	852 ± 62 (9)	916 ± 119 (12)	851 ± 93 (6)	968 ± 126 (5)	1,039 ± 411 (5)	907 ± 219 (5)	1/1.01
Glu	28.3 ± 7.6 (9)	34.4 ± 10.2 (8)	30.2 ± 8.7 (9)	30.7 ± 13.3 (12)	31.5 ± 18.0 (6)	31.0 ± 8.8 (5)	28.7 ± 3.2 (5)	25.2 ± 9.6 (5)	1/1.22
Gly	397 ± 77 (9)	347 ± 67 (8)	468 ± 85 (9)	336 ± 68 (12)	116 ± 28 (6)	99.1 ± 21.1 (5)	134 ± 56 (5)	87.3 ± 44.5 (5)	1/3.85
His	51.5 ± 25.3 (9)	55.7 ± 27.6 (8)	72.7 ± 24.9 (9)	74.0 ± 31.0 (12)	13.2 ± 10.3 (6)	13.5 ± 12.1 (5)	22.2 ± 6.6 (5)	22.1 ± 7.3 (5)	1/3.34
Ile	124 ± 37 (9)	138 ± 37 (8)	92.0 ± 15.5 (9)	129 ± 41 (12)	10.4 ± 1.8 (6)	11.1 ± 1.6 (5)	9.87 ± 0.92 (5)	12.4 ± 4.4 (5)	1/10.5
Leu	198 ± 64 (9)	202 ± 59 (8)	147 ± 26 (9)	201 ± 79 (12)	15.8 ± 2.8 (6)	16.3 ± 2.9 (5)	16.0 ± 2.9 (5)	18.6 ± 8.2 (5)	1/10.9
Lys	539 ± 158 (9)	1,092 ± 649 (8)	355 ± 136 (9)	786 ± 381 (12)	116 ± 14 (6)	166 ± 56 (5)	104 ± 9 (5)	178 ± 81 (5)	1/4.42
Met	98.7 ± 36.0 (9)	85.1 ± 24.3 (8)	104 ± 33 (9)	104 ± 32 (12)	17.0 ± 6.9 (6)	16.6 ± 2.4 (5)	23.9 ± 6.2 (5)	18.0 ± 7.4 (5)	1/5.77
Phe	105 ± 21 (9)	101 ± 47 (8)	88.9 ± 31.8 (9)	87.2 ± 23.6 (12)	10.1 ± 1.4 (6)	8.89 ± 1.02 (5)	11.3 ± 3.8 (5)	8.68 ± 2.75 (5)	1/10.0
Pro	159 ± 69 (9)	175 ± 94 (8)	158 ± 88 (9)	172 ± 72 (12)	11.9 ± 4.3 (6)	11.5 ± 1.3 (5)	14.0 ± 2.7 (5)	13.5 ± 5.9 (5)	1/12.8
Ser	195 ± 43 (9)	222 ± 63 (8)	175 ± 40 (9)	185 ± 52 (12)	127 ± 26 (6)	135 ± 16 (5)	125 ± 13 (5)	139 ± 49 (5)	1/1.33
Thr	233 ± 58 (9)	405 ± 147 (8)	225 ± 57 (9)	351 ± 124 (12)	57.5 ± 8.6 (6)	92.9 ± 29.9 (5)	64.3 ± 8.6 (5)	112 ± 58 (5)	1/3.12
Trp	101 ± 16 (9)	107 ± 26 (8)	108 ± 23 (10)	114 ± 35 (12)	4.53 ± 0.71 (6)	4.02 ± 1.34 (5)	3.97 ± 0.78 (5)	4.72 ± 1.37 (5)	1/24.2
Tyr	197 ± 46 (9)	145 ± 41 (8)	172 ± 71 (9)	133 ± 35 (12)	16.6 ± 5.8 (6)	14.1 ± 3.4 (5)	21.6 ± 8.0 (5)	11.7 ± 5.4 (5)	1/11.4
Val	281 ± 73 (9)	304 ± 88 (8)	227 ± 45 (9)	303 ± 134 (12)	20.3 ± 3.5 (6)	21.4 ± 2.7 (5)	20.8 ± 2.6 (5)	30.5 ± 14.8 (5)	1/9.97
Cysta	6.78 ± 3.04 (9)	8.94 ± 2.44 (8)	98.9 ± 11.4 (9)	137 ± 33 (12)	19.3 ± 6.9 (6)	22.1 ± 5.4 (5)	20.4 ± 4.6 (5)	35.7 ± 26.3 (5)	1/3.85
Taurine	469 ± 167 (9)	415 ± 145 (8)	379 ± 147 (9)	271 ± 129 (12)	1,236 ± 385 (6)	1,055 ± 504 (5)	1,387 ± 735 (5)	768 ± 555 (5)	1/0.35
Citru	90.2 ± 21.1 (9)	94.3 ± 16.3 (8)	182 ± 51 (9)	258 ± 71 (12)	7.44 ± 2.4 (6)	16.0 ± 3.7 (5)	27.6 ± 10.5 (5)	32.5 ± 13.2 (5)	1/7.93
Orni	76.2 ± 29.7 (9)	111 ± 52 (8)	79.6 ± 26.4 (9)	114 ± 43 (12)	4.49 ± 2.05 (6)	5.38 ± 0.74 (5)	6.18 ± 1.13 (5)	5.92 ± 2.34 (5)	1/19.3

Data are mean $\pm$ standard deviation (S.D.) (sample numbers). L1, lactation day 1; N.D., not detected (below LOQ); t-Hcy, total homocysteine; t-Cys, total cysteine; t-GSH, total glutathione (=both oxidized and reduced forms of glutathione); Cysta, cystathionine; Citru, citrulline; and Orni, ornithine.

Fig. 1. Homocysteine Hypothesis on the Impaired Peripheral but Not CNS Oxytocin Responses in *Cth*^{-/-} Dam Mice

Amino acid levels, including total Hcy, in the serum and CSF of virgin and L1 *Cth*^{-/-} dam mice were measured in this study. The blood–brain barrier protects from circulatory Hcy levels and confers normal oxytocin responses in the CNS such as maternal and social behaviors. In contrast, a high circulatory Hcy level in *Cth*^{-/-} dam mice partially impairs milk ejection and occasionally causes neonatal death due to nutrition deficiency. *Cth*^{-/-} dam mice produced Hcy-rich milk, but this did not cause homocysteinemia in their *Cth*^{+/-} pups. Data are the mean values from Table 1 or our previous study (data with asterisks).¹²⁾ (Color figure can be accessed in the online version.)

with oxytocin, for example, by forming Hcy-oxytocin dimers *via* disulfide bonds between their Cys. Milk containing high levels of total Hcy (49.6 $\mu\text{mol/L}$) produced from homocysteinemic (111 $\mu\text{mol/L}$) *Cth*^{-/-} L14 dam mice had no influence on the growth of their *Cth*^{+/-} pups, perhaps because of its lack of impact on their blood Hcy levels.¹²⁾ Similarly, the relatively very low levels of total Hcy in the CSF of virgin or L1 *Cth*^{-/-} mice may underlie their normal oxytocin-regulated maternal and social behaviors.¹²⁾ Serum oxytocin levels were found to be comparable between wild-type and *Cth*^{-/-} mice,¹²⁾ and their CSF levels could not be measured because of its low yields.

Guiraud *et al.* reported that plasma levels of total Hcy in patients with Alzheimer's disease and their control subjects were 8.1 ± 6.9 and $5.3 \pm 1.6 \mu\text{mol/L}$, respectively, whereas both of their CSF levels were $<0.25 \mu\text{mol/L}$ (LOQ).¹⁷⁾ Oikonomidi *et al.* reported that plasma and CSF levels of total Hcy in 35 adult women were 11.9 $\mu\text{mol/L}$ and 64.4 nmol/L, respectively,¹⁸⁾ and Valentino *et al.* reported that these levels in adult patients with ALS were 12.8 and 0.45 $\mu\text{mol/L}$ (in approx. 45 men) and 11.9 and 0.49 $\mu\text{mol/L}$ (in approx. 24 women), respectively.¹⁹⁾ In our literature search, we could not find previous reports on mouse CSF Hcy levels, except our results on 2-week-old wild-type, *Cbs*^{-/-}, and *Cth*^{-/-} mice, and these levels were 1.08, 25.5, and 7.43 $\mu\text{mol/L}$, respectively, when their serum levels were 13.0, 263, and 184 $\mu\text{mol/L}$, respectively.²⁰⁾ Therefore, the blood–brain barrier may act protectively against circulatory Hcy in human and mice, and it may act more protectively in adult mice than in 2-week-old pups. It has been reported that patients with homocystinuria (*CBS*^{-/-}) displayed mental retardation¹⁾ and that *Cbs*^{-/-} mouse survivors had a learning deficit.⁹⁾ Therefore, Hcy level increases beyond the threshold in the CSF (as observed in 2-week-old *Cbs*^{-/-} pups)²⁰⁾ could be related to some CNS alterations in human and mice.

CONCLUSION

Total Hcy levels in the CSF of *Cth*^{-/-} mice were specifically much lower than those in their serum levels. We hypothesize that the blood–brain barrier could protect the CNS from circulatory high Hcy levels, which confers normal oxytocin signaling in the CNS but not peripheral tissues of *Cth*^{-/-} mice (Fig. 1). Importantly, dysfunction of the barrier could cause amino acid leakages to the CSF as observed in more homocysteinemic *Cbs*^{-/-} mice²⁰⁾ that have the CNS disorders.⁹⁾

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Conflict of Interest The authors declare no conflict of interest.

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