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***GCH1* expression in human cerebellum from healthy individuals is not gender dependant**

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Abstract

Dopa-responsive dystonia is a familial childhood-onset disease characterized by fluctuating dystonia, associated with tremor and parkinsonism in some patients. In most families the disease displays autosomal dominant inheritance due to mutations in the *GTP cyclohydrolase 1* gene (*GCH1*). Penetrance and symptom severity display strong female predominance for which gender-specific *GCH1* expression has been hypothesized. In this study, *GCH1* mRNA expression was measured in cerebellar tissue from 66 healthy human subjects (30 women), and in cerebellar and nigral tissue from 8 individuals. No significant difference was found between men and women with small effect sizes observed. Although the correlation between cerebellar and nigral *GCH1* expression remains to be further examined, this exploratory study does not support gender-specific *GCH1* expression being the basis for the skewed gender distribution observed in DRD patients.

Keywords

Dopa-responsive dystonia; DRD; *GCH1* expression; gender distribution

Dopa-responsive dystonia (DRD) is a familial childhood-onset disease characterized by fluctuating foot dystonia which tends to generalize and may lead to severe motor impairment

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[2,9–11]. Additional symptoms include tremor and parkinsonism with variable combinations of bradykinesia, tremor, rigidity and postural instability. Response to levodopa is dramatic and long-lasting. Most patients display autosomal dominant inheritance of disease with reduced penetrance, due to mutations in the *GTP cyclohydrolase 1* gene (*GCH1*; *DYT5*; MIM: 600225) on chromosome 14q22.1-q22.2 [7]. *GCH1* encodes an enzyme critical for tetrahydrobiopterin (BH4) synthesis. BH4 is a co-factor of tyrosine hydroxylase, the rate-limiting enzyme for dopamine synthesis. Mutations in *GCH1* cause DRD by reducing endogenous dopamine production.

Gender specificity appears to be a consistent, albeit enigmatic finding in a variety of disorders associated with the dopaminergic system, including DRD, Parkinson's disease and restless legs syndrome. In DRD families, the female-to-male ratio is generally around four, with disease penetrance reported to be 2.3 times higher in women than in men [3,15]. Affected women also tend to display more severe symptoms than men [16]. It has been suggested that such differences may stem from lower basal *GCH1* expression in women. Herein, we provide evidence against this hypothesis by showing there is no gender difference in *GCH1* mRNA expression in a series of cerebellar brain samples from healthy men and women. We also examine the correlation between nigral and cerebellar *GCH1* expression in a limited sample.

Sixty-six human cerebellar brain samples were collected under approved ethical committee protocols from each institution (available upon request) (Table 1). Individuals who donated their brain had no known neurological condition and did not display pathologic abnormalities suggestive of Alzheimer's disease or Lewy body disease. The project was conducted in accordance with the principles of the WMA Declaration of Helsinki. Subjects were free of neurological disease and had no personal or familial history of parkinsonism or dystonia. Total RNA was extracted from cerebellar tissue using TRIzol PureLink total RNA purification System (Invitrogen). RNA integrity was checked using the Agilent 2100 Bioanalyzer (Agilent) and expressed as a RNA quality score (RIN) from 1 to 10. Samples with a RIN < 5 are considered degraded, between 5.0 and 6.0 adequate, and > 6.5 of high quality. All samples had a RIN ≥ 5.2 and 76% had a RIN > 6.0. cDNA was synthesized from 1.5ug RNA with Applied Biosystems High Capacity cDNA Archive Kit and used as a template for relative quantitative PCR employing ABI Taqman® chemistry (Applied Biosystems). *GCH1* expression was quantified using fluorescein (6-FAM)-labeled probes for *GCH1* exon 1-exon 2 (X1X2, sequence available on request) and *GCH1* exon 4-exon 5 (X4X5) boundaries (Hs00609198_m1). Results were normalized to the geometric means of three housekeeping genes, *YWHAZ* (Hs00852925_sH), *GAPDH* (Hs99999905_m1) and *HPRT* (Hs99999909_m1). Each sample was run in replicates of four on an ABI 7900 and analysis was performed using SDS2.2.2 software (Applied Biosystems).

Numerical variables were summarized with the sample median, 25th percentile, and 75th percentile. Linear regression models adjusted for age of death and RIN were used to investigate the association between gender and *GCH1* expression. Estimated multiplicative effects and corresponding 95% confidence intervals (CI's) were calculated. Estimated multiplicative effects are interpreted as the multiplicative increase on the median *GCH1* expression, resulting from the exponentiation of the least squares estimates from a model where *GCH1* expression was considered on the natural logarithm scale due to its skewed distribution. Pearson's correlation was used to examine the correlation between *GCH1* expression in the cerebellum and substantia nigra. Statistical significance was determined at the 5% level.

The analysis adjusted for age and RIN did not show any significant influence of gender on *GCH1* expression in human cerebellar samples, with small effect sizes observed (Table 2, Figure 1). Estimated median *GCH1* X1X2 expression was 1.07 times higher (95% CI: 0.82 – 1.39, P=0.63) in men than in women, while median *GCH1* X4X5 expression was 1.05 times

higher (95% CI: 0.81 – 1.37, $P=0.69$) in men compared to women. Likewise, age of death did not modify *GCH1* expression (Table 2). There was evidence of a negative influence of the RIN on *GCH1* expression (Table 2). Thus it was of interest to consider very high quality samples separately and perform a secondary analysis only in the 41 samples with a $RIN \geq 6.5$, using linear regression models adjusted only for age of death. Lack of association between gender and *GCH1* expression was also observed in this subgroup of subjects for *GCH1* X1X2 ($P=0.77$) and *GCH1* X4X5 ($P=0.89$), with similar effect sizes (1.05 and 1.02, respectively). Also, although our analysis was adjusted for age, there was a significant difference in age at death between men and women (Table 1). Therefore, we performed a sensitivity analysis using a subset of 38 individuals (19 men, 19 women) matched for age (± 3 years), and found no gender-specific difference in *GCH1* expression (data not shown).

Nigral samples (tissue prepared from the substantia nigra exclusively) were available in a subset of eight individuals (two women; median age: 82 years, 25th–75th percentiles: 80–85 years; median RIN for cerebellum: 6.0, 25th–75th percentiles: 5.6–6.8; median RIN for nigra: 6.3, 25th–75th percentiles: 5.5–6.6). Exploratory analysis in these subjects showed cerebellar expression of *GCH1* is representative of *GCH1* expression in the substantia nigra, with similar results for *GCH1* X1X2 (correlation coefficient: 0.71, $P=0.12$) (not shown) and *GCH1* X4X5 (correlation coefficient: 0.69, $P=0.059$) (Figure 2). Despite relatively high correlation coefficients, P -values did not reach statistical significance in this small sample with low power to detect such correlations.

Animal studies have shown *GCH1* mRNA expression [8,14] and GCH1 protein levels [6] are low in nigrostriatal neurons compared to other monoaminergic neurons in rats [6,8] and in mice [14]. Similar low expression of *GCH1* mRNA in nigrostriatal neurons has been reported in humans [5]. Furthermore, in monoaminergic neurons, basal *GCH1* mRNA expression was lower in female mice compared to males, which has led to the hypothesis that gender-specific basal *GCH1* expression in humans may explain higher female susceptibility to develop DRD symptoms [14]. Possible mechanisms include estrogen-mediated regulation of *GCH1* transcription, as estradiol was shown to modulate *GCH1* mRNA levels in rats [13] and to attenuate the *GCH1* promoter response to cyclic AMP in PC12 cells [12]. However, the present study does not support this hypothesis, as cerebellar *GCH1* mRNA expression was similar in healthy men and women, with no significant difference observed. These results are in agreement with data from GCH1 enzymatic activity in stimulated mononuclear blood cells from healthy controls showing no gender-related difference [4].

Although our analysis included adjustment for age, all our samples came from individuals over age 49. Age-related changes in GCH1 activity in stimulated mononuclear blood cells have been reported in healthy controls [4]. Likewise, a study found a significant age-related reduction of the number of GCH1-immunoreactive nigral neurons in aged humans (81.5%) and non-human primates (67.4%) compared to young cohorts [1]. Therefore, our study may have overlooked *GCH1* expression differences in younger individuals, particularly in women given estrogen influence over *GCH1* transcription. However, a putative age-dependent gender specificity of *GCH1* expression would not account for susceptibility to DRD in which symptoms worsen or are stable over time. An additional caveat may be the use of cerebellar tissue, as cerebellar *GCH1* expression may not adequately reflect that of the substantia nigra. However, exploratory data in a limited number of samples indicates cerebellar *GCH1* expression may be representative of nigral *GCH1* expression; therefore use of cerebellar samples would appear to be a reasonable surrogate. Finally, sample quality is unlikely to have influenced the results as data analysis included adjustment for RNA quality, and a secondary analysis was performed only in samples with the highest RNA quality. The present study did not identify gender-specific differences in *GCH1* expression; however, other mechanisms may explain the skewed gender distribution of DRD among *GCH1* mutation carriers. Variants in *GCH1* un-transcribed

regulatory regions may induce post-transcriptional *GCHI* mRNA modifications, thereby reducing GCH1 protein levels. Alternatively, genetic susceptibility may stem from differential expression of other genes implicated in the dopaminergic system by means of gene-gene, gene-mRNA or gene-protein interactions. Gender-specific striatal sensitivity to dopamine depletion or differences in dopaminergic system compensatory mechanisms may play a role, although this would likely also alter the risk of other conditions such as Parkinson's disease.

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Figure 1.

GCH1 mRNA expression is similar in men and women when probed for the exon 1-exon 2 (X1X2, *left*) and the exon 4-exon 5 (X4X5, *right*) boundaries. See text for measurement methods.

Figure 2. *GCH1* expression in cerebellum and substantia nigra from 8 normal controls (probed for the exon 4-exon 5 boundary). Pearson's correlation coefficient: 0.69, $P=0.059$.

Table 1

Age, RNA quality and institution of origin

Variable	Males (N=36)	Females (N=30)	P-value
<i>Age of death</i>	67 (63 – 83)	84 (76 – 92)	0.002
<i>RIN</i>	6.8 (6.2 – 7.0)	6.6 (5.9 – 7.3)	0.48
<i>Mayo Clinic Jacksonville</i>	6	1	NA
<i>University of Miami</i>	29	26	NA
<i>University of Saskatchewan</i>	1	3	NA

Legend. The sample median (25th percentile – 75th percentile) is given. P-values result from a Wilcoxon rank sum test. NA, not applicable. RIN, RNA quality score.

Table 2
Association of *GCH1* expression with gender, age of death, and RNA quality (RIN)

Variable/marker	Estimated multiplicative effect (95% CI)	P-value
X1X2		
Age of death (10 year increase)	0.96 (0.87 – 1.06)	0.41
Sex (Male)	1.07 (0.82 – 1.39)	0.63
RIN (1 unit increase)	0.75 (0.62 – 0.91)	0.004
X4X5		
Age of death (10 year increase)	0.97 (0.88 – 1.07)	0.52
Sex (Male)	1.05 (0.81 – 1.37)	0.69
RIN (1 unit increase)	0.75 (0.62 – 0.9)	0.003

Legend. P-values result from linear regression models adjusted for age of death and RIN number. Estimated multiplicative effects are interpreted as the multiplicative increase on median *GCH1* exon 1-exon 2 (X1X2) and exon 4-exon 5 (X4X5) expression with the increase given in parenthesis (numerical variables) or presence of the trait given in parenthesis (categorical variables).