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Tryptophan Hydroxylase 2 Genotype Determines Brain Serotonin Synthesis but Not Tissue Content in C57Bl/6 and BALB/c Congenic Mice

William B. Siesser[1], **Xiaodong Zhang**[1,2], **Jacob P.R. Jacobsen**[1], **Tatyana D. Sotnikova**[1,3], **Raul R. Gainetdinov**[1,3], and **Marc G. Caron**[1,4]

[1] Department of Cell Biology, Duke University, Durham, NC 27710, USA

[2] Duke–National University of Singapore Graduate Medical School, Singapore

[3] Department of Neuroscience and Brain Technologies, Italian Institute of Technology (IIT), Via Morego 30, Genova, 16163 Italy

[4] Department of Neurobiology, Duke University Medical Center, Durham, NC 27710, USA

Abstract

Tryptophan hydroxylase 2 (TPH2) catalyzes the rate-limiting step in the synthesis of brain serotonin (5-HT). In a previous report, a single nucleotide polymorphism in *mTph2* (C1473G) reduced 5-HT synthesis by 55%. Mouse strains expressing the 1473C allele, such as C57Bl/6, have higher 5-HT synthesis rates than strains expressing the 1473G allele, such as BALB/c. Many studies have attributed strain differences to *Tph2* genotype without ruling out the potential role of alterations in other genes. To test the role of the C1473G polymorphism in strain differences, we generated C57Bl/6 and BALB/c mice congenic for the *Tph2* locus. We found that the 1473G allele reduced 5-HT synthesis in C57Bl/6 mice but had no effect on 5-HT tissue content except for a slight reduction (15%) in the frontal cortex. In BALB/c mice, the 1473C allele increased 5-HT synthesis but again did not affect 5-HT tissue content. At the same time, 5-hydroxyindoleacetic acid (5-HIAA) was significantly elevated in BALB/c congenic mice. In C57Bl/6 mice, there was no effect of genotype on 5-HIAA levels. BALB/c mice had lower expression of monoamine oxidase A and B than C57Bl/6 mice, but there was no effect of *Tph2* genotype. On the tail suspension test, escitalopram treatment reduced immobility regardless of genotype. These data demonstrate that the C1473G polymorphism determines differences in 5-HT synthesis rates among strains but only minimally affects 5-HT tissue levels.

Keywords

tryptophan hydroxylase 2; serotonin; 5-hydroxyindoleacetic acid; mouse strains; congenic mice; monoamine oxidase

Tryptophan hydroxylase is the rate-limiting step in the synthesis of serotonin (5-HT). Two isoforms of tryptophan hydroxylase have been identified: one controlling 5-HT synthesis in

Corresponding author: Marc G. Caron, m.caron@cellbio.duke.edu, 487 CARL Building, Box 3287, Duke University Medical Center, Durham, NC 27710, 919-684-5433, Fax: 919-681-8641.

the periphery (TPH1) and the other in the brain [TPH2; 8, 27, 30]. In the mouse tryptophan hydroxylase 2 (*mTph2*) gene, a functional single nucleotide polymorphism (SNP; C1473G) reduces 5-HT synthesis by approximately 55% in PC-12 cells expressing the 1473G allele. The C57Bl/6 and 129X1/SvJ strains of mice are homozygous for the 1473C allele, whereas the BALB/c and DBA/2 strains are homozygous for the 1473G allele. In strains expressing the 1473G allele, 5-HT synthesis is 45 to 70% lower than in strains expressing the 1473C allele; furthermore, 5-HT tissue content in the frontal cortex and striatum is 40% lower in strains expressing the 1473G allele than in strains expressing the 1473C allele [30]. However, it is unknown whether the strain differences in 5-HT synthesis rates and tissue content are the consequences of the *mTph2* SNP or other genetic differences between the strains.

Previous investigators have described multiple behavioral, neurochemical, and biochemical differences between mouse strains that differ at the *mTph2* locus. Strains with the 1473G allele have less aggression, more anxiety, and a higher editing profile of the 5HT2C receptor mRNA than strains expressing the 1473C allele [7,11,16,17]. Several groups have shown that 1473G-carrying strains have reduced responsiveness to selective serotonin reuptake inhibitors (SSRIs) on behavioral measures of antidepressant function; however, the results are often conflicting [4,5,10,12,14,18,19,22,25]. In addition, strains carrying the 1473G SNP have reduced 5-HT release in the medial prefrontal cortex and hippocampus and reduced 5-HT overflow after SSRI treatment than strains expressing the 1473C SNP [3,13]. The strains also differ in the structure and function of the enteric serotonergic system [20]. Although the strain differences are sometimes attributed to the C1473G polymorphism, none of these studies have ruled out the possibility that the strain differences are the result of variation in other genes. To address this problem, Zhang et al. [29] proposed the generation of congenic mice lines in which the 1473C and 1473G alleles would be backcrossed onto strains that normally express the converse SNP. A previous study backcrossed the 1473G allele onto the C57Bl/6 strain for 3 generations [21]. Mice carrying the 1473G allele had lower 5-HT synthesis in the midbrain and were less aggressive. However, after only 3 generations of backcrossing, the genomes were still significantly mixed so other genetic factors could have been responsible for the observed differences. Another study backcrossed the 1473G allele onto the C57Bl/6 strain for 8 generations but found no effects of the SNP on 5-HT synthesis rates or tissue content [26]. However, the measurement of 5-HT synthesis rate was performed with an *in vitro* assay, in which brain tissue from the congenic mice was homogenized, and then 5-HT synthesis was assayed by addition of exogenous tryptophan, *m*-hydroxybenzylhydrazine, and cofactors. It is unclear whether such an assay would accurately measure the activity of TPH2 *in vivo*. As a result, it still remains to be established whether the C1473G SNP is responsible for the differences in 5-HT synthesis between the C57Bl/6 and BALB/c strains. In the present study, we backcrossed the 1473G allele onto C57Bl/6 mice and the 1473C allele onto BALB/c mice for 12 generations. We found that 5-HT synthesis was significantly reduced in C57Bl/6 mice carrying the 1473G allele and significantly elevated in BALB/c mice congenic for the 1473C allele. However, 5-HT tissue levels were unchanged except for a 15% reduction in the frontal cortex of congenic C57BL/6 mice and an elevation in the 5-HT metabolite, 5-hydroxyindoleacetic acid (5-HIAA), in the BALB/c congenic mice.

Mice were housed 2 to 5 per cage in an AALAC-approved vivarium maintained at 23 ± 2° C on a 12-hour light-dark cycle with onset of lights at 8AM. In order to generate congenic mice, C57Bl/6 mice were crossed with BALB/c mice to generate an F1 hybrid, heterozygous for the C1473G SNP. The heterozygous mice were then backcrossed for 12 generations onto either the C57Bl/6 or BALB/c strain. Finally, the backcrossed heterozygous progeny were bred together to produce C57Bl/6 or BALB/c mice congenic at the *mTph2* locus, which was

confirmed by genotyping. All experiments were approved by the Duke University and Medical Center Institutional Animal Care & Use Committee.

For the analysis of monoamine levels (n=6–7 per genotype), frontal cortex, hippocampus, and striatum were rapidly dissected and frozen on liquid nitrogen. Monoamine levels were analyzed using HPLC by electrochemical detection as described previously [28,30]. Serotonin synthesis rates (n=6–9 per genotype) were measured as described previously [30]. Briefly, mice were treated with *m*-hydroxybenzylhydrazine (100 mg/kg, *i.p.*) for 1h, and then the frontal cortex, hippocampus, and striatum were rapidly dissected, frozen, and analyzed for levels of 5-hydroxytryptophan using HPLC by electrochemical detection. Statistical analyses of differences between genotypes were made using two-tailed Student's *t*-tests. The α-level was .05. Outliers were detected by Grubb's Test and removed from analysis. GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, CA) was used for all analyses.

BALB/c congenic mice were created by backcrossing the 1473C allele onto the BALB/c background for 12 generations. The 1473C allele increased 5-HT synthesis 93% in the frontal cortex ($p<.0001$), 67% in the hippocampus ($p<.0001$), and 57% in the striatum ($p=.01$; Fig 1A-C). Likewise, the C57Bl/6 congenic mice were created by backcrossing the 1473G allele onto the C57Bl/6 background for 12 generations. In these mice, 5-HT synthesis was significantly reduced in the frontal cortex (48%; $p=.02$) and hippocampus (42%; $p=.02$) (Fig 1D-E). There was no effect of genotype in the striatum (Fig 1F). Tissue content of 5-HT was measured in the frontal cortex, hippocampus, and striatum in both lines of congenics (Fig 2). Despite the increase in 5-HT synthesis, BALB/c congenic mice had normal 5-HT tissue levels in all brain regions tested (Fig 2A-C). There was a non-significant trend towards higher 5-HT tissue levels, but the difference was much smaller than would be expected on the basis of the differences in 5-HT synthesis. In the frontal cortex of C57Bl/6 congenic mice, there was a slight reduction in 5-HT levels (15%) in the C57Bl6/J G/G mice ($p=.03$; Fig 2D). However, there were no significant differences between genotypes in the hippocampus and striatum (Fig 2E, 2F). Taken together, these data demonstrate that the C1473G SNP is responsible for the differences in 5-HT synthesis between the strains, but other processes contribute to the differences in 5-HT tissue content.

The absence of dramatic changes in 5-HT tissue content suggested that other mechanisms were compensating for the changes in 5-HT synthesis. To assess the role of 5-HT metabolism, we measured the 5-HT metabolite, 5-HIAA. In BALB/c congenic mice, 5-HIAA was significantly elevated in the frontal cortex ($p=.04$) and hippocampus ($p=.02$) but not in the striatum (Fig 3A-C). There were no significant genotype differences in 5-HIAA levels in C57Bl/6 congenics in any of the brain regions tested (Fig 3D-F). In order to determine the contribution of monoamine oxidase (MAO) to 5-HT and 5-HIAA tissue content, MAO expression and activity were measured in the frontal cortex and hippocampus (see supplementary Materials and Methods). The results of quantitative real time PCR indicated no effect of *mTph2* genotype on MAO-A or MAO-B expression in either strain (Fig S1A-D). However, MAO-B expression was significantly lower in BALB/c mice than in C57Bl/6 mice (frontal cortex and hippocampus, $p<.0001$; Fig S1B, S1D). MAO-A expression was also slightly, but significantly, lower in the frontal cortex of BALB/c mice than in the frontal cortex of C57Bl/6 mice ($p<.01$; Fig S1A). There was no effect of strain on MAO-A expression in the hippocampus (Fig S1C). Despite these changes in MAO expression, MAO activity in the frontal cortex was not affected by strain or genotype (Fig S1E-G). Exogenously applied 5-HT was broken down at the same rate in the frontal cortex in BALB/c mice as in C57Bl/6 mice (Fig S1E). Likewise, there was no effect of *mTph2* genotype within strains on 5-HT metabolism (Fig S1F, S1G).

Previous studies have suggested that strain differences in antidepressant response are the result of the C1473G SNP. To assess the contribution of the SNP to SSRI response, we tested both lines of congenic mice for differences in immobility on the tail suspension test (see supplementary Materials and Methods). Mice that climbed their tails during testing were excluded from analysis (15% of C57Bl/6 and 1% of BALB/c mice). There was no genotype effect on immobility among vehicle treated BALB/c mice (Fig S2A) or C57Bl/6 mice (Fig S2B). Escitalopram significantly reduced immobility time in both strains but to a greater extent in BALB/c mice than in C57Bl6/J mice (Strain x Drug, p=.039; BALB/c, Drug: p<.0001, Fig S2A; C57Bl6/J, Drug: p=.02, Fig S2B). However, genotype did not affect the response to escitalopram in either strain (BALB/c: Genotype x Drug, p=.27; C57Bl6/J: Genotype x Drug, p=.24).

The finding that 5-HT tissue content is reduced in only the frontal cortex of C57Bl/6 G/G mice suggests that 5-HT synthesis is regulated differently across brain regions and the frontal cortex is more sensitive to partial reductions in the 5-HT synthesis rate. This result is similar to the tissue content profile observed in the R439H TPH2 heterozygous mice [2]. The R439H polymorphism in TPH2 was identified originally at the analogous locus in human *TPH2* at a site near to the C1473G SNP. In mice homozygous for the R439H mutation, both 5-HT synthesis and tissue content were reduced approximately 80%. However, in mice heterozygous for the R439H mutation, 5-HT synthesis was reduced only 50%, and 5-HT tissue content was unchanged, except for a partial reduction in the frontal cortex. In the *Tph2* knock-out homozygous mice, 5-HT levels were reduced to low levels in the raphe nuclei, frontal cortex, hippocampus, and other brain regions [1,9,24]. However, in the heterozygous *Tph2* knock-out mice, 5-HT tissue levels were unchanged [1]. These data in combination with those of the present study suggest that the 5-HT system can compensate for partial (~50%) reductions in 5-HT synthesis under normal conditions. Similar observations have been made in mice heterozygous for deletion of the tyrosine hydroxylase gene; the dopamine synthesis rate was partially reduced, but dopamine tissue content was unaffected [15].

The finding of altered synthesis in the congenic mouse strains conflicts with the findings of an earlier report characterizing C57Bl/6 congenic mice [26]. In this earlier study, no differences in 5-HT synthesis were observed in mice carrying the 1473G allele. However, synthesis was measured using an *in vitro* method that is unlikely to accurately represent TPH2 activity *in vivo*. Furthermore, whereas both studies are consistent with regards to the unchanged 5-HT tissue content in the hippocampus and striatum, we demonstrate here that 5-HT tissue content is slightly reduced in the frontal cortex of C57Bl/6 congenics carrying the 1473G allele. The differences between C57Bl/6 and BALB/c mice in 5-HT synthesis rates in this study were somewhat smaller than those reported in the previous study [30]. The slightly smaller difference may be due to genetic drift among the strains bred for multiple generations in our laboratory.

The higher level of 5-HIAA in the BALB/c congenics indicates increased breakdown of 5-HT by MAO-A. However, MAO-A expression and MAO activity did not differ on the basis of *mTph2* genotype. Therefore, a likely explanation for the increased 5-HIAA levels is an increase in 5-HT release. Such increased release of 5-HT in turn would lead to higher 5-HT reuptake and breakdown by MAO. The MAO activity assay would not detect this increased breakdown, because it measures the breakdown of exogenously applied 5-HT. In the C57Bl/6 congenic mice, the normal 5-HT levels in combination with reduced 5-HT synthesis could be the result of elevated 5-HT vesicular packaging. This putative compensatory mechanism would maintain normal levels of 5-HT, despite lower levels of synthesis. Although there was no genotype effect on MAO-B expression, BALB/c wild types had 50% lower MAO-B expression than C57Bl/6 wild types. MAO-A expression was also slightly lower in BALB/c

mice than in wild-type mice. MAO-B has a lower affinity for 5-HT than MAO-A and metabolizes mainly dopamine. The enzyme's lower level of expression in BALB/c mice could potentially be another cause of phenotypic differences between BALB/c and C57Bl/6 mice. Future studies with these mouse strains should explore the effects of MAO-B expression on the dopamine system and its role in their behavioral differences. Although the C1473G SNP is sometimes suggested to be responsible for strain differences in antidepressant response on the tail suspension test, we found no differences between genotypes in the effects of escitalopram on either strain. Likewise, there was no effect of *mTph2* genotype on baseline immobility. It is thus likely that strain differences observed on this behavioral test are the consequence of variations in multiple genes, rather than being determined solely by the variation in *mTph2*. However, the possibility that these congenic strains may differ on other behavioral tests more sensitive to 5-HT deficiency cannot be excluded.

The lack of a change in 5-HT tissue content suggests that, under normal conditions, the 5-HT system compensates for perturbations caused by the C1473G SNP. The observation of changes in 5-HT synthesis complement our previous findings [30] by demonstrating that the C1473G SNP in *mTph2* is responsible for the differences in synthesis between mouse strains; however, other genetic factors determine the final 5-HT tissue content. Because tissue neurotransmitter content is usually a reasonable predictor of functional levels of released neurotransmitters, it is unlikely that differences in the *mTph2* genotypes between the C57Bl/6 and BALB/c mouse lines can be the only determinant of behavioral, neurochemical, and biochemical differences between these lines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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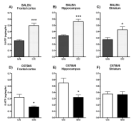

Figure 1.

Rate of 5-HT synthesis in BALB/c and C57Bl/6 mice. The C1473G single nucleotide polymorphism in *mTph2* determined the rate of 5-HT synthesis in both strains. Levels of 5-hydroxytryptophan (5-HTP) were measured 1h after treatment with *m*-hydroxybenzylhydrazine (100 mg/kg) in BALB/c and C57Bl/6 mice congenic at the C1473G locus. In the BALB/c congenic mice (top row), 5-HT synthesis was significantly higher in C/C mice than in wild-type BALB/c mice in the A) frontal cortex, B) hippocampus, and C) striatum. In the C57Bl/6 congenic mice (bottom row), the 5-HT synthesis rate was lower than in wild-type C57Bl/6 mice in the D) frontal cortex, E) hippocampus, but not F) striatum. Data are presented as ng of 5-HTP per mg of wet tissue weight. N=6–9. *, p<.05; ***, p<.001.

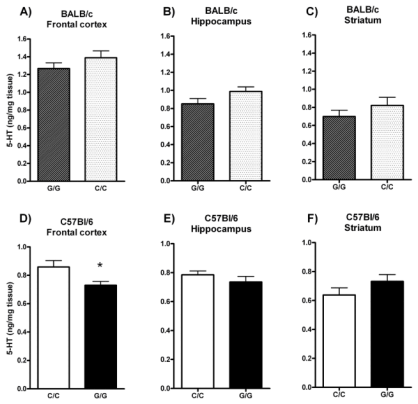

Figure 2.

Tissue content of 5-HT in BALB/c and C57Bl/6 mice. Tissue content of 5-HT was not significantly affected by the C1473G single nucleotide polymorphism. BALB/c congenic mice (top row) did not have significantly changed levels of 5-HT in the A) frontal cortex, B) hippocampus, or C) striatum. C57Bl/6 mice congenic for the 1473G allele had a 15% reduction in 5-HT levels in the D) frontal cortex, but no changes in 5-HT tissue content in the E) hippocampus or F) striatum. Data are expressed in ng / mg wet tissue for BALB/c G/G (top row) or C57Bl/6 C/C (bottom row) and presented as mean ± SEM. N=6–7. *, $p<.05$.

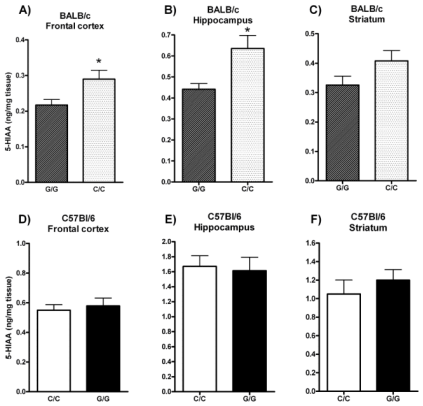

Figure 3.

5-hydroxyindoleacetic acid (5-HIAA) in *Tph2* congenic mice. BALB/c congenic mice (top row) had elevated levels of 5-HIAA in the A) frontal cortex and B) hippocampus, but no significant increase in C) striatum. In the C57Bl/6 mice (bottom row), there were no significant changes in 5-HIAA levels in the D) frontal cortex, E) hippocampus, or F) striatum. Data are expressed in ng / mg wet tissue for BALB/c G/G (top row) or C57Bl/6 C/C (bottom row) and presented as mean ± SEM. N=6–7. *, $p<.05$.