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A Functional *Tph2* C1473G Polymorphism Causes an Anxiety Phenotype via Compensatory Changes in the Serotonergic System

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The association of single-nucleotide polymorphisms (SNPs) in the human tryptophan hydroxylase 2 (*TPH2*) gene with anxiety traits and depression has been inconclusive. Observed inconsistencies might result from the fact that *TPH2* polymorphisms have been studied in a genetically heterogeneous human population. A defined genetic background, control over environmental factors, and the ability to analyze the molecular and neurochemical consequences of introduced genetic alterations constitute major advantages of investigating SNPs in inbred laboratory mouse strains. To investigate the behavioral and neurochemical consequences of a functional C1473G SNP in the mouse *Tph2* gene, we generated congenic C57BL/6N mice homozygous for the *Tph2* 1473G allele. The Arg^{447} substitution in the TPH2 enzyme resulted in a significant reduction of the brain serotonin (5-HT) *in vivo* synthesis rate. Despite decreased 5-HT synthesis, we could detect neither a reduction of brain region-specific 5-HT concentrations nor changes in baseline and stress-induced 5-HT release using a microdialysis approach. However, using a [^{35}S]GTP-γ-S binding assay and 5-HT_{1A} receptor autoradiography, a functional desensitization of 5-HT_{1A} autoreceptors could be identified. Furthermore, behavioral analysis revealed a distinct anxiety phenotype in homozygous *Tph2* 1473G mice, which could be reversed with chronic escitalopram treatment. Alterations in depressive-like behavior could not be detected under baseline conditions or after chronic mild stress. These findings provide evidence for an involvement of functional *Tph2* polymorphisms in anxiety-related behaviors, which are likely not caused directly by alterations in 5-HT content or release but are rather due to compensatory changes during development involving functional desensitization of 5-HT_{1A} autoreceptors.
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INTRODUCTION

Dysfunction within central serotonergic neurotransmission is regarded as a major factor implicated in many neuropsychiatric diseases, such as anxiety and depressive disorders (Ressler and Nemeroff, 2000). Standard therapy for these disorders involves substances targeting the serotonergic system (Blier and de Montigny, 1999) and association studies have linked single-nucleotide polymorphisms (SNPs) in genes modulating serotonergic neurotransmission with these disorders (Anguelova *et al*, 2003; Murphy and Lesch, 2008; Yu *et al*, 2005).

Since the discovery of the tryptophan hydroxylase 2 (TPH2) isoenzyme (Walther *et al*, 2003)—the rate-limiting enzyme of 5-HT synthesis in the brain—*TPH2* polymorphisms in humans have been associated with anxiety traits, depressive disorders, and suicidality (Gutknecht *et al*, 2007; Reuter *et al*, 2007; Van Den Bogaert *et al*, 2006; Zhang *et al*, 2005; Zill *et al*, 2004a, b) yet these results have not been supported by other studies (De Luca *et al*, 2006; Garriock

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et al, 2005; Juhasz *et al*, 2010; Lopez *et al*, 2007; Mann *et al*, 2008; Middeldorp *et al*, 2010). These discrepancies might be partially attributed to the fact that different SNPs in the *TPH2* gene were analyzed. Most of the reported polymorphisms have not been characterized regarding their functional consequences on *TPH2* transcription or 5-HT synthesis *in vivo*. Also, environmental factors like stress or genetic diversity are confounding variables that need to be taken into account in human studies.

In studies with rodents, control over environmental factors and a defined genetic background constitute major advantages for investigating the contribution of single-nucleotide alterations on the organism. For the mouse *Tph2* gene, a functional SNP has been identified (C1473G) among different inbred mouse strains, which results in the substitution of Pro^{447} (1473C allele) with Arg^{447} (1473G allele). In mouse strains homozygous for the 1473G allele (G/G), the enzymatic activity of TPH2 was reduced by 50% and correspondingly 5-HT concentrations were found to be decreased in several brain regions (Zhang *et al*, 2004). Inconsistent results were reported in subsequent studies, which tried to correlate the *Tph2* C1473G polymorphism with behavioral differences in mouse strains homozygous for either the G/G allele (DBA/2 and BALB/c) or C/C allele (C57BL/6 and 129) (Cervo *et al*, 2005; Crowley *et al*, 2005). However, the divergent genetic background among different inbred mouse strains may have contributed to the inconsistencies found in the behavioral analysis of the *Tph2* C1473G SNP.

The strategy of choice to investigate the impact of single gene modifications on complex physiological and behavioral traits is the introduction of a particular genetic variation into the genome of an animal with a suitable inbred genetic background. With this approach, a comparative functional analysis of a specific SNP on an identical genetic background is feasible. This can either be achieved by a knock-in strategy in embryonic stem cells (Beaulieu *et al*, 2008) or alternatively by generating congenic strains by a backcrossing strategy in which one inbred strain is mated to another recipient inbred strain (Tenner *et al*, 2008). A 'knock-in' approach was applied to study the consequences of the human *TPH2* SNP G1463A (Beaulieu *et al*, 2008), which previously had been shown to be associated with unipolar depressive disorder (Zhang *et al*, 2005). The respective polymorphism (G1449A) in the mouse *Tph2* gene reduced the enzymatic activity of TPH2 by 80% and led to a highly significant reduction of extracellular 5-HT concentration in several brain regions (Jacobsen *et al*, 2011). This correlated with an increase in anxiety- and depression-related behavior. In contrast, congenic C57BL/6 mice homozygous for the 1473G—generated by a backcrossing strategy—showed no differences in either brain stem TPH2 activity *in vitro* or brain region-specific 5-HT concentrations compared with littermates homozygous for the 1473C allele (Tenner *et al*, 2008). Also, anxiety and depressive-like behavior were undistinguishable between respective mice.

Here, we provide a detailed comparative neurochemical, molecular, and behavioral characterization of C57BL/6N mice homozygous for either the *Tph2* 1473G or 1473C allele. On an otherwise identical genetic background, we show that the *Tph2* 1473G/G allele alone leads to a reduced *in vivo* 5-HT synthesis rate. However, the distinct and pharmacologically reversible anxiety phenotype in 1473G/G mice is not the result of reduced 5-HT tissue content or 5-HT neurotransmission but is likely mediated via compensatory homeostatic changes involving a functional desensitization of 5-HT_{1A}-autoreceptors.

MATERIALS AND METHODS

Animals

Congenic C57BL/6N animals, homozygous for the *Tph2* 1473G allele (1473G/G mice), were generated using a backcrossing breeding strategy. DBA/2N inbred mice, which are homozygous for the 1473G allele, were backcrossed to a C57BL/6N genetic background for 10 generations. Congenic C1473G C57BL/6N mice were then bred to homozygosity. Inbred C57BL/6N mice, homozygous for the *Tph2* 1473C allele (1473C/C mice) were purchased from Charles Rivers Laboratories (Sulzfeld, Germany). Breedings and litter sizes of 1473G/G mice were normal and genotypes followed Mendelian distribution. For genotyping of the *Tph2* 1473G and 1473C allele and housing condition of the animals, see Supplementary section for details.

Neurochemistry

The whole brain from experimentally naive male 1473 C/C and 1473 G/G mice was cut sagitally and each brain hemisphere was manually microdissected into a forebrain section (containing projections of serotonergic neurons) and a midbrain/brainstem section (containing the cell bodies of serotonergic neurons from median and dorsal raphe nuclei (DRN)). For the determination of the synthesis rate of TPH2, mice were injected with the L-aromatic acid decarboxylase inhibitor NSD 1015 (*m*-hydroxybenzyl-hydrazine; 100 mg/kg i.p.) 30 min before brain dissection. Dissected brain regions were homogenized in an extraction solution (0.1 M perchloric acid, 1 mM EDTA) using a tissue homogenizer Mixer Mill (Qiagen, Hilden, Germany) and yielded solutions subsequently centrifuged at 15000 *g* for 10 min at 4 °C. In all, 10 µl of the spun sample was loaded on a HPLC system with electrochemical detection (see Supplementary section for details). Brain region-specific accumulation of 5-hydroxytryptophan (5-HTP) and L-DOPA—serotonin and dopamine precursor molecules—or of 5-HT and 5-HIAA tissue concentration was determined by normalizing the quantified neurotransmitter amounts to the respective weight of the tissue sample.

Microdialysis

Three-month old, experimentally naive male 1473 C/C and 1473 G/G mice were used. Surgical procedures, sampling of microdialysates, and quantification of extracellular 5-HT was performed as previously described (Engblom *et al*, 2008) (see Supplementary section for details).

Quantitative Autoradiography of 5-HT_{1A}-Mediated [^{35}S]GTP-γ-S Binding and [^{3}H]-WAY100635 Binding

Six-month old, experimentally naive male 1473 C/C and 1473 G/G mice were used for autoradiographic measurements of

5-HT_{1A} receptor-stimulated [^{35}S]GTP-γ-S binding in the DRN and the CA1 area of the hippocampus as previously described (Froger *et al*, 2004). In a separate group of 3-month old, experimentally naive male mice of both *Tph2* genotypes, [^{3}H]-WAY100635 binding was performed on sections containing the DRN as previously described (Khawaja, 1995) (see Supplementary section for details).

Plasma Corticosterone Concentrations

Blood was collected from the saphenous vein according to Hoff (2000) using an EDTA-coated Microvette CB300 (Sarstedt, Nürnbrecht, Germany) and yielded serum was stored at $-20\,^{\circ}$C. Corticosterone was quantified by a specific in-house radioimmunoassay (RIA) established at the Steroid Laboratory of the Department of Pharmacology, University of Heidelberg as previously described (Bielohuby *et al*, 2007). A recovery-corrected extraction was performed before each RIA. Intra-assay and inter-assay coefficients of variance were $<10\%$ and $<15\%$, respectively.

Behavioral Analysis

The animal numbers and the order of the behavioral experiments are displayed in Supplementary Table S1. The experimental protocols used in this study complied with national and international ethical guidelines, and were performed in compliance with the German Animal Welfare Act and approved by the Animal Welfare Commission of the Regierungspräsidium Karlsruhe, Germany (35-9185.81/G-182/09). All experiments were conducted only with male mice during the 'dark' period of the day, that is, in the animals' active phase. Experimental procedures of the open field test and hotplate test are described in the Supplementary section.

Elevated Plus Maze

The elevated plus maze is a widely used test to determine the state anxiety of an animal. The apparatus (see Supplementary section for details) consisting of two open and two enclosed arms was illuminated indirectly by an overhead lamp with an intensity of 25 lux. For testing, mice were individually placed on the center square facing an enclosed arm, and allowed to freely explore the maze for 5 min. Their behavior was recorded and analyzed by the video tracking software EthoVision 3.0 (Noldus, Wageningen, The Netherlands). Parameters assessed were time spent in open or closed arms, number of exits into the open arms, number of head dips, number of transitions between arms and total distance moved.

Elevated Zero-Maze

The elevated zero-maze inflicts an approach–avoidance conflict on the mice, measuring anxiety by their aversion to enter the elevated, exposed sections of a round maze. The experiment was performed as previously described (Fuss *et al*, 2010). The following parameters were analyzed: latency to first exit, number of exits to, and total time spent in the open compartments.

Light–Dark Exploration Test

The light–dark exploration test examines the anxiety-related behavior to an aversively, brightly lit compartment. The test apparatus ('dark–light box') consisted of two plastic chambers, connected by a tunnel of $5 \times 7 \times 10$ cm ($l \times w \times h$). The 'dark' chamber (black plastic) measured $20 \times 15 \times 30$ cm ($l \times w \times h$) and was covered by a lid. The lit chamber, $30 \times 15 \times 30$ cm ($l \times w \times h$), made of white plastic, was brightly illuminated from above with tubular fluorescent lamps (1000 lux). Mice were individually placed into the dark compartment and their behavior was monitored by the video tracking software EthoVision 3.0 (Noldus) for 5 min. Analyzed parameters included: latency of first exit and number of exits into the lit compartment, time and distance moved in the lit compartment, risk assessments (head dips from the dark compartment), and time of end-exploration.

Novelty-Induced Hypophagia

The novelty-induced hypophagia test is an animal model for anxiety-related behavior, which is sensitive to chronic but not acute treatment of the animal with serotonin reuptake inhibitors (Dulawa and Hen, 2005). Mice are first trained to consume sweetened condensed milk (1:3 dilution of La Lechera (Nestlé, Frankfurt, Germany) with water) in their home cage once a day for 1 h. A trained observer scored the mice for their latency to start consuming milk and for their consumption of milk within the first 10 min and the whole training session (60 min). Training was continued until the latency to start consuming milk was below 20 s. Mice that never consumed milk during home cage testing (1473C/C: $n = 1$) were eliminated from the experiment. The next day following training, the sweetened condensed milk was presented to each individual mouse within a novel environment (new cage without bedding or a new cage with a metal floor) for 10 min and the applicable parameters were scored by the observer. For escitalopram treatment, see Supplementary section for details.

Tail Suspension Test

The tail suspension test determines despair behavior (failure in the persistence of escape-directed behavior toward a stressor) of the animal. Here, mice were individually suspended by their tail taped to a metal hook connected to a strain gauge, which is part of a computer-assisted tail suspension test device (Bioseb, Chaville, France). Within the 5 min of testing, all movements of the mice were automatically recorded. The parameter of the test is the total duration of immobility.

Forced Swimming Test

To quantify depression-related despair behavior, mice were placed into a glass cylinder (23 cm height, 13 cm diameter), which was filled with water (22 $^{\circ}$C) up to a height of 10 cm. Within the testing period of 6 min, the activity of each mouse was recorded from the side by the video tracking software EthoVision 3.0 (Noldus). Immobility was defined

as motionless floating in water, only allowing movements necessary for the animal to keep its head above the water.

Learned Helplessness

The learned helplessness paradigm is a depression model in which an animal is exposed to unpredictable and uncontrollable stressors. This subsequently leads to the development of coping deficits in aversive but escapable situations. The experimental procedure was used as previously described (Chourbaji *et al*, 2005) (see Supplementary section for details).

Chronic Mild Stress Procedure

Chronic mild stress applied to rodents is considered as an etiological model of depression (Willner, 1997). The procedure applied herein was performed as described by Pothion *et al* (2004) with some modifications (see Supplementary section for details).

Sucrose Preference Using a 'Matching Law' Approach

Anhedonia, the loss of interest in pleasurable activities, is a core symptom of depressive disorders (APA, 2000). To detect hedonic alterations in rodents, the reinforcing properties of sucrose were assessed by a free choice, two-bottle sucrose consumption paradigm using the principles of the matching law, which provides a quantitative index of the steady, internal evaluation of the reward (Herrnstein, 1961). The testing procedure used herein was adapted from Sanchis-Segura *et al* (2004) (see Supplementary section and Supplementary Table 2 for details).

Statistical Analysis

Statistical analyses were performed using either *t*-test or univariate or multivariate analysis of variance (ANOVA) with repeated or independent measures (factors included: genotype, stress, cage environment, and time), followed by either a Bonferroni or Dunnett *post hoc* test. Respective F- and *p*-values were calculated using GraphPad Prism 5.0 or SPSS Version 19. All data are presented as either mean + SEM or mean ± SEM. $p<0.05$ was considered statistically significant.

RESULTS

In vivo 5-HT Synthesis Rate in Homozygous *Tph2* 1473G Mice

We first analyzed the *in vivo* 5-HT synthesis rate in mice homozygous for either the 1473G or 1473C allele. After injection of the L-aromatic amino-acid decarboxylase inhibitor NSD 1015, the accumulation of the 5-HT precursor 5-HTP and the dopamine precursor L-DOPA was quantified in both the 5-HT neuronal projection areas in the forebrain and in the midbrain/brain stem where 5-HT neurons reside in the raphe nuclei (Table 1). As expected, the dopamine synthesis rate, reflected by accumulation of L-DOPA, was unaffected by the C1473G SNP. In contrast, mice homozygous for the 1473G allele showed a highly significant 30% reduction of total brain 5-HT synthesis relative to 1473C/C mice. There were brain regional differences with forebrain 5-HTP accumulation significantly reduced by 40% whereas 5-HT synthesis rate in the raphe nuclei of midbrain/brain stem punches showed only a small and nonsignificant decrease.

5-HT Concentration, Metabolism, and Synaptic Release in Homozygous *Tph2* 1473G Mice

We next analyzed the impact of reduced 5-HT synthesis on brain 5-HT concentration and metabolism. Tissue concentrations of 5-HT and 5-HIAA were determined from forebrain and midbrain/brain stem sections by HPLC with electrochemical detection.

Despite a significant reduction of *in vivo* 5-HT synthesis, there was no concomitant reduction in tissue 5-HT. Thus, forebrain 5-HT tissue concentration in 1473G/G mice was undistinguishable from homozygous 1473C/C mice (Table 2), while in midbrain/brain stem samples, there was a small 15% increase in 5-HT concentration in 1473G/G mice relative to 1473C/C mice. When total brain 5-HT concentration was calculated, no significant differences were detected in mice of both *Tph2* alleles.

Compensation for reduced 5-HT synthesis could occur via decreased 5-HT metabolism. Indeed, the 5-HIAA concentration in the forebrain of 1473G/G mice was significantly 15% lower than in 1473C/C mice (Table 2). This resulted in a significant reduction of the 5-HT turnover rate (5-HIAA/5-HT ratio; Table 2) in the forebrain. In contrast, within the

Table 1 Serotonin and Dopamine Synthesis Rate in Homozygous 1473G and 1473C Mice

	DOPA	5-HTP
Forebrain		
1473C/C	1.30 ± 0.26	0.79 ± 0.04
1473G/G	1.26 ± 0.25	0.48 ± 0.02***
Midbrain/brain stem		
1473C/C	0.78 ± 0.16	1.45 ± 0.07
1473G/G	0.94 ± 0.17	1.27 ± 0.09
Total		
1473C/C	1.18 ± 0.24	0.94 ± 0.04
1473G/G	1.19 ± 0.23	0.64 ± 0.03***

Brain tissue concentrations of 5-hydroxytryptophan (5-HTP; serotonin precursor) and dihydroxyphenylalanin (DOPA; dopamine precursor) were determined in experimentally naive male 1473G/G ($n=5$) and 1473C/C mice ($n=5$) that had been treated with the L-aromatic amino acid decarboxylase inhibitor NSD 1015 30 min before analysis. Concentrations of accumulated DOPA, representing dopamine synthesis, were undistinguishable between homozygous mice of both *Tph2* alleles. Accumulated 5-HTP concentrations representing 5-HT synthesis were significantly lower by 40% in the forebrain ($p<0.001$) and insignificantly lower by 15% in the midbrain/brain stem of 1473G/G mice. The total 5-HTP brain content was significantly reduced by 30% ($p<0.001$). All data are presented as mean values ± SEM. All stars represent *p*-values of significances between genotypes from unpaired *t*-tests.
***$p<0.001$.

Table 2 5-HT and 5-HIAA Tissue Concentrations in Homozygous 1473G/G and 1473C/C Mice

	5-HT	5-HIAA	5-HIAA/5-HT
Forebrain			
1473C/C	3.98 ± 0.18	1.12 ± 0.06	0.282 ± 0.010
1473G/G	3.89 ± 0.10	0.95 ± 0.03*	0.246 ± 0.009*
Midbrain/brain stem			
1473C/C	5.11 ± 0.10	2.29 ± 0.10	0.449 ± 0.019
1473G/G	5.76 ± 0.23*	2.44 ± 0.09	0.425 ± 0.013
Total			
1473C/C	4.24 ± 0.13	1.38 ± 0.07	0.327 ± 0.012
1473G/G	4.28 ± 0.10	1.27 ± 0.04	0.297 ± 0.011

Tissue concentrations of 5-HT and 5-HIAA were determined in the forebrain and midbrain/brain stem of experimentally naive male 1473G/G ($n = 7$) and 1473C/C mice ($n = 8$). 5-HT turnover (5-HIAA/5-HT), representing metabolism rate, was calculated from the measured concentrations. In the forebrain, 5-HT concentrations were similar in homozygous mice of both *Tph2* alleles but 5-HIAA concentration ($p = 0.032$) and 5-HT turnover ($p = 0.022$) were significantly reduced by 15% in 1473G/G mice. In the midbrain/brain stem, 1473G/G mice showed a significantly elevated 5-HT concentration by 13% ($p = 0.020$). However, 5-HIAA and turnover rate were similar between homozygous *Tph2* 1473G and 1473C mice in this brain region. For the total brain, 5-HT and 5-HIAA concentrations were similar in both groups. All data is presented as mean values ± SEM. All symbols represent *p*-values of significances between genotypes from unpaired *t*-tests.
*$p < 0.05$.

midbrain/brain stem section no difference in 5-HIAA concentrations or 5-HT turnover rate could be detected between 1473G/G and 1473C/C mice.

As no major alterations in brain 5-HT content could be identified, we next determined the impact of reduced 5-HT synthesis on basal and stress-induced 5-HT release. Microdialysis in freely moving animals was performed in the ventral hippocampus and the prefrontal cortex, two brain regions innervated by serotonergic projections originating from dorsal and median raphe nuclei (Azmitia and Segal, 1978). Microdialysis samples were taken every 20 min, first under basal conditions for 100 min, thereafter during restraint stress for 1 h and finally after termination of stress (post-stress) for 1 h. Basal extracellular 5-HT concentrations were indistinguishable between the G/G and C/C mice in the prefrontal cortex (Figure 1b) as well as in the ventral hippocampus (Figure 1d). Furthermore, both the stress-induced increase in extracellular 5-HT and the persistence of elevated post-stress 5-HT concentrations were not significantly different in homozygous 1473C and 1473G mice in either brain region investigated (Figures 1a and c).

Functional Desensitization of 5-HT_{1A} Autoreceptors in the DRN of Homozygous *Tph2* 1473G Mice

Despite reduced 5-HT synthesis, we could not detect lower 5-HT brain concentrations or decreased 5-HT release. These findings suggest that developmental changes have led to adult 5-HT homeostasis, potentially involving 5-HT_{1A}-autoreceptors. 5-HT_{1A} receptors can be found somatoden-

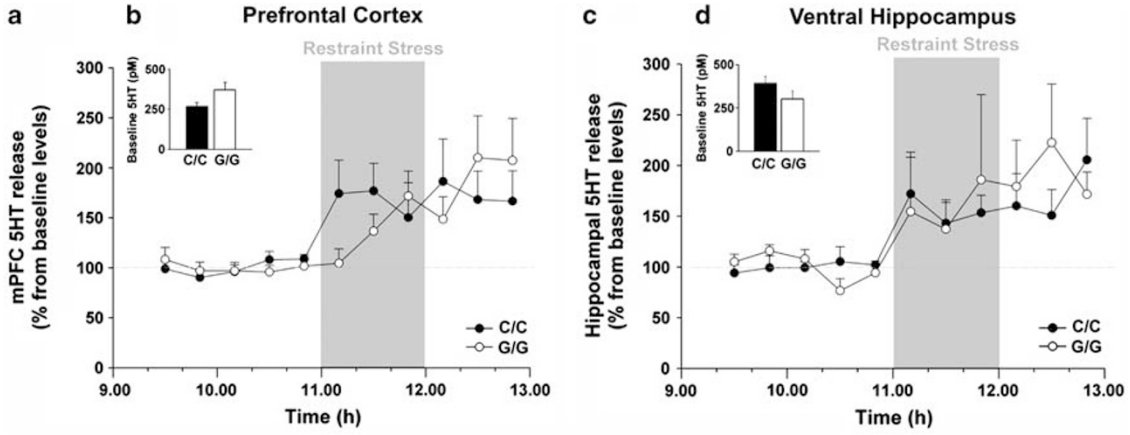

Figure 1 Basal and stress-induced extracellular 5-HT concentrations in the prefrontal cortex and ventral hippocampus of *Tph2* 1473G/G and 1473C/C mice. (a, c) Percentage increase in 5-HT release in the prefrontal cortex (PFC) (a) and in the ventral hippocampus (c) compared with baseline levels. (b, d) Basal concentration of extracellular 5-HT measured in the PFC (b) and ventral hippocampus (d) of experimentally naive male 1473C/C (PFC: $n = 8$; ventral hippocampus: $n = 5$) and 1473G/G mice (PFC: $n = 9$; ventral hippocampus: $n = 6$). Dialysates were sampled in 20-min intervals. Pre-stress, basal dialysates were taken over 100 min, samples under acute restraint stress over 60 min (gray area) and post-stress sampling was conducted for another 60 min in both 1473C/C and 1473G/G mice. (b, d) Mean basal 5-HT concentration (averaged from the first five samples collected) in the PFC and ventral hippocampus were not significantly different between 1473C/C and 1473G/G mice (PFC: $p = 0.14$; ventral hippocampus $p = 0.12$). (a, c) In mice of both *Tph2* genotypes, restraint stress increased the release of serotonin in the PFC ($F_{Treatment}$ (10,150) = 6.60; $p < 0.001$) (a) and ventral hippocampus ($F_{Treatment}$ (10,90) = 3.25; $p < 0.0013$) (c). However, no significant difference could be detected between 1473C/C and 1473G/G mice in the prefrontal cortex ($F_{Genotype}$ (1,150) = 0.004; $p = 0.95$) and the ventral hippocampus ($F_{Genotype}$ (10,90) = 0.25; $p = 0.63$). Furthermore, mice of both genotypes did not react differently to restraint stress (PFC: $F_{Treatment*Genotype}$ (10,150) = 1.47; $p = 0.155$ and ventral hippocampus $F_{Treatment*Genotype}$ (10,90) = 0.83; $p = 0.70$). An additional 'area under the curve' analysis for the time points under restraint stress for the PFC also showed no significant differences between 1473C/C and 1473G/G mice (AUC: 6891.43 ± 1009.55 (1473C/C); 5328.45 ± 526.52 (1473G/G)) ($p = 0{:}18$). All data presented are mean values + SEM. Statistical analysis was performed using unpaired *t*-tests and two-way ANOVA of repeated measures.

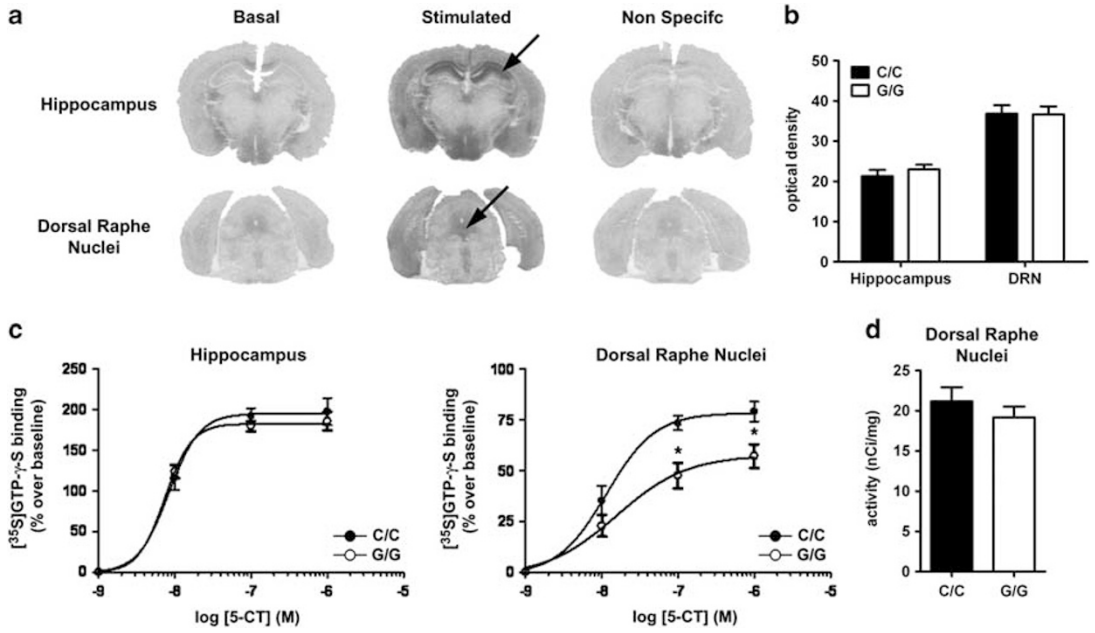

Figure 2 5-HT_{1A} receptor mediated [^{35}S]GTP-γ-S binding (a–c) and 5-HT_{1A} receptor labeling (d) in experimentally naive 1473C/C and 1473G/G mice. (a) Representative autoradiograms of brain sections from 1473 C/C mice at the level of the hippocampus and the DRN, labeled by [35S]GTP-γ-S in the absence (basal) or the presence of 10^{-6}M 5-CT (stimulated). Nonspecific labeling was obtained from adjacent sections exposed to 5-CT 10^{-6}M plus 10^{-5}M WAY 100635. Arrows indicate investigated brain regions. (b) Basal [^{35}S]GTP-γ-S binding is highly similar between mice of both genotypes in the hippocampus and the DRN. (c) In the hippocampus, 5-CT-stimulated [^{35}S]GTP-γ-S binding ($F_{Treatment}$ (3,32) = 148.20, $p<0.0001$; two-way ANOVA of repeated measures) was not significantly different in 1473G/G ($n=5$) and 1473C/C mice ($n=5$) ($F_{Genotype}$ (1,32) = 0.35, $p=0.55$). However, the 5-CT induced increase in [^{35}S]GTP-γ-S binding in the DRN ($F_{Treatment}$ (3,32) = 81.35, $p<0.0001$) was significantly lower in 1473G/G mice ($n=5$) than in 1473C/C mice ($n=5$) ($F_{Genotype}$ (1,21) = 18.73; $p=0.0001$). (d) 5-HT_{1A} receptor labeling with the selective radioligand [^{3}H]WAY 100635 in the DRN was similar in both 1473C/C ($n=10$) and 1473G/G mice ($n=10$). All data are presented as mean values ± SEM or as mean values + SEM. Stars represent p-values of significances between genotypes obtained from Bonferroni *post hoc* testing following two-way ANOVA of repeated measures: *$p<0.05$.

dritically as autoreceptors on serotonergic neurons and as heteroreceptors on postsynaptic non-5-HT neurons. In both cases, they are involved in 5-HT regulated inhibition of neuronal firing (Lanfumey and Hamon, 2000). 5-HT-induced 5-HT_{1A} receptor signaling was determined using a [^{35}S]-GTP-γ-S binding assay. Under basal conditions, that is, in the absence of the non-selective 5-HT_{1A} receptor agonist 5-carboxamido-tryptamine (5-CT), [^{35}S]GTP-γ-S labeling within both the DRN and the hippocampus did not differ between 1473G/G and 1473C/C mice (Figures 2a and b). In both groups of mice, 5-CT induced a concentration-dependent increase in [^{35}S]GTP-γ-S labeling in the DRN and in the hippocampus (Figures 2a and c), which could be prevented by the selective 5-HT_{1A} receptor antagonist WAY 100635 (10 μM) ('nonspecific' condition) (Figure 2a). 5-CT-stimulated [^{35}S]GTP-γ-S binding in the hippocampus was not significantly different in mice of both *Tph2* alleles (Figure 2c). However, the 5-CT-induced increase in [^{35}S]GTP-γ-S binding within the DRN was significantly lower in 1473G/G mice than in 1473C/C mice indicating a functional desensitization of 5-HT_{1A} autoreceptors on 5-HT neurons of the DRN (Figure 2c). To explore the possibility that the observed reduction in [^{35}S]-GTP-γ-S binding in the DRN results from a reduction of 5-HT_{1A} autoreceptor expression, a receptor binding assay with the specific radioligand [^{3}H]WAY 100635 was performed. Quantification of 5-HT_{1A} autoreceptors in DRN showed no difference in radioligand binding between the mice of both *Tph2* genotypes (Figure 2d). Taken together, these findings demonstrate that postsynaptic 5-HT_{1A} receptor signaling in 1473G/G mice is unchanged and functional desensitization selectively affects 5-HT_{1A} autoreceptors without changing its somatodendritic density on 5-HT neurons.

Anxiety-Related Behavior in Homozygous *Tph2* 1473G Mice

Following the biochemical characterization of *Tph2* C1473G allele-dependent changes, three cohorts of animals were used to determine SNP-related alterations in general and emotional behavior (Supplementary Table S1).

Baseline parameters like locomotor activity and rearings (Supplementary Figures S1 A–C), body weight (Supplementary Figure S1 D), and nociception (Supplementary Figure S1 E) were similar in 1473G/G and 1473C/C mice. In mice carrying the *Tph2* 1473G/G SNP responsible for reduced TPH2 enzymatic efficiency, a significant increase in anxiety-like behavior was observed in all four applied paradigms (Figure 3). Three of the tasks are based on a natural approach–avoidance conflict of mice in mazes that consist of sheltered (closed, dark) and unsheltered (open, lit) compartments. Here, an increased avoidance of the unsheltered compartment, which is regarded as an elevation in anxiety-related behavior, was observed in the elevated plus maze (Figures 3a–c), the light–dark exploration test (Figure 3d–f) and the elevated zero maze task (Figures 3i–j). The fourth paradigm used was the novelty-induced hypohagia test (Dulawa and Hen, 2005), in which anxiety-related behavior

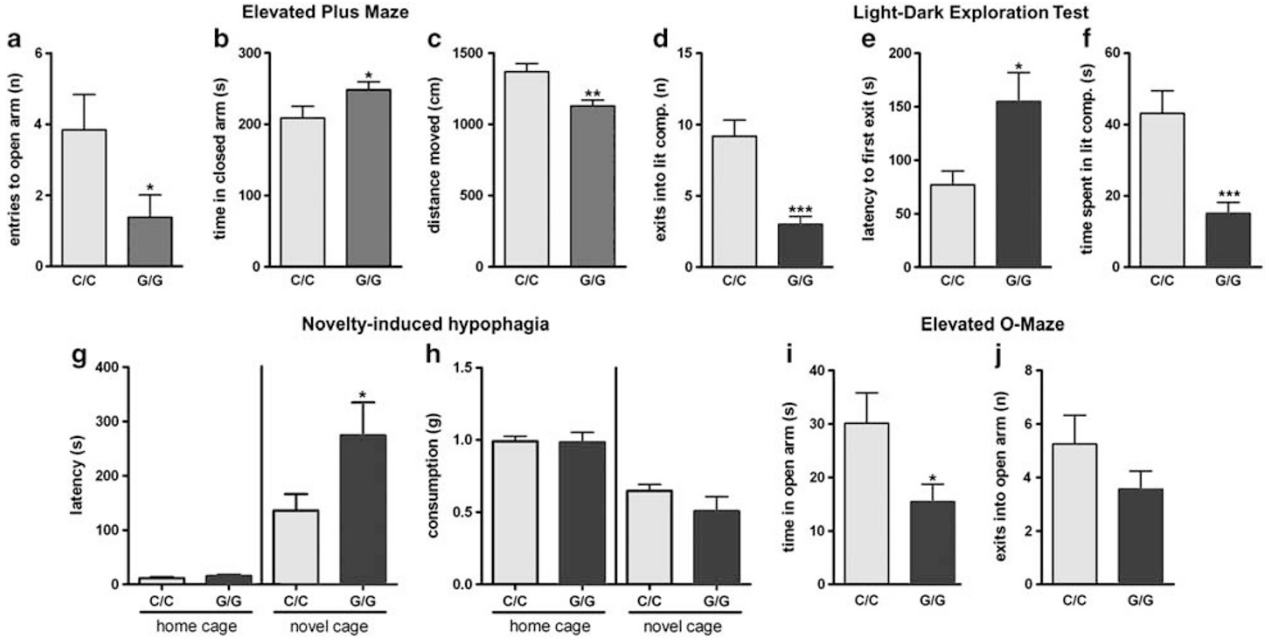

Figure 3 C57BL/6N mice homozygous for the *Tph2* 1473G allele display increased anxiety-related behavior. 1473G/G and 1473C/C mice were subjected to behavioral tests assessing anxiety-like behavior (for animal numbers and order of behavioral experiments see Supplementary Table S1). (a–c) Elevated plus maze. 1473G/G mice made significantly fewer entries into the open arms ($p = 0.041$) (a) and spent significantly more time in the closed arms of the maze ($p = 0.036$) (b). In addition they displayed a significantly reduced locomotor activity, determined by the distance moved on the maze ($p = 0.002$) (c). (d–f) Light–dark exploration test. 1473G/G mice made significantly fewer entries ($p < 0.001$) (d), had a significantly higher latency for their first exit ($p = 0.016$) (e), and spent significantly less time in the lit compartment ($p < 0.001$) (f). (g, h) Novelty-induced hypophagia. In the novel cage, mice of both genotypes showed a significantly increased latency to consume milk ($F_{Environment}$ (1,27) = 30.72, $p < 0.0001$) and a highly significant reduction in the amount of consumed milk ($F_{Environment}$ (1,27) = 48.46, $p < 0.0001$). There was a significant effect of genotype on the latency to consume milk ($F_{Genotype}$ (1,27) = 4.26, $p = 0.049$). *Post hoc* analysis revealed a higher latency of 1473G/G mice to start consuming sweetened condensed milk in the novel environment ($p < 0.05$) (g). However, the consumption of milk within the 10 min of testing was not different between the two genotypes ($F_{Genotype}$ (1,27) = 0.92, $p = 0.346$) and not altered by the novel environment ($F_{Environment*Genotype}$ (1,27) = 1.31, $p = 0.263$) (h). (I, j) Elevated O-maze. Mice homozygous for the 1473G allele displayed increased anxiety-like behavior, as indicated by a significant decrease in the time spent in the open arms ($p = 0.035$) (i) and a nonsignificant reduction of the number of exits into the open arms ($p = 0.194$) (j). All data presented are mean values + SEM. Stars represent *p*-values obtained by comparing 1473G/G and 1473C/C mice with either unpaired *t*-test or Bonferroni *post-hoc* test following two-way ANOVA of repeated measures: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

of an animal is reflected by its consumatory behavior toward a known, highly palatable food snack in a potentially dangerous, novel environment. Again, 1473G/G mice displayed elevated anxiety-related behavior by showing an increased latency to consume sweetened condensed milk in the novel environment (Figure 3g). However, despite the increased latency to consume milk, the reduction of milk intake in the new environment was not different between mice of both genotypes (Figure 3h).

No Depressive-Like Behavior Under Basal Conditions and After Chronic Mild Stress in Homozygous *Tph2* 1473G Mice

Next, we assessed depression-related behaviors in different animal models of depression. Two of these tests, the tail suspension test (Figure 4a) and the forced swimming test (Figure 4b), investigate 'behavioral despair' of an animal by quantifying its escape-oriented behaviors in an inescapable, threatening situation. Differences between genotypes in the immobility time, representing the failure to actively cope with the stressful condition, could not be detected in either test (Figures 4a–b). In the learned helplessness paradigm, the animal's predisposition to develop a helpless behavior after exposure to inescapable electric foot-shocks is assessed. Again, no difference could be observed between mice homozygous for the 1473G and 1473C alleles (Figures 4d–f). Also, mice of both genotype failed to reach criteria for helpless behavior (Chourbaji *et al*, 2005) (see figure legend for details). Finally, no differences could be found between homozygous 1473G and 1473C mice when tested for anhedonia in a sucrose preference test (Figure 4c).

In the absence of any basal depressive-like symptoms in 1473G/G mice, we assessed whether the 1473G polymorphism within the C57BL/6N genetic background is associated with an increased vulnerability to chronic stress. Mice were subjected to a chronic mild stress protocol, in which the animals were exposed to three unpredictable mild stressors daily for a total of 6 weeks. During the chronic mild stress procedure, mice of both genotypes reacted to stress with a significant but transient loss of body weight (Supplementary Figure S2 A). Here, the effect of stress was significantly more pronounced in 1473G/G animals. One week after termination of stress, mice were tested for locomotor activity as well as for anxiety- and depressive-like behaviors. As a consequence of the chronic mild stress procedure, mice of both genotypes displayed a similar increase in locomotion in the open field test (Supplementary Figure S2 B) and in the elevated plus maze (Supplementary Figure S2 D). A stress-induced increase in immobility was also observed in the tail suspension test and in the forced swimming test,

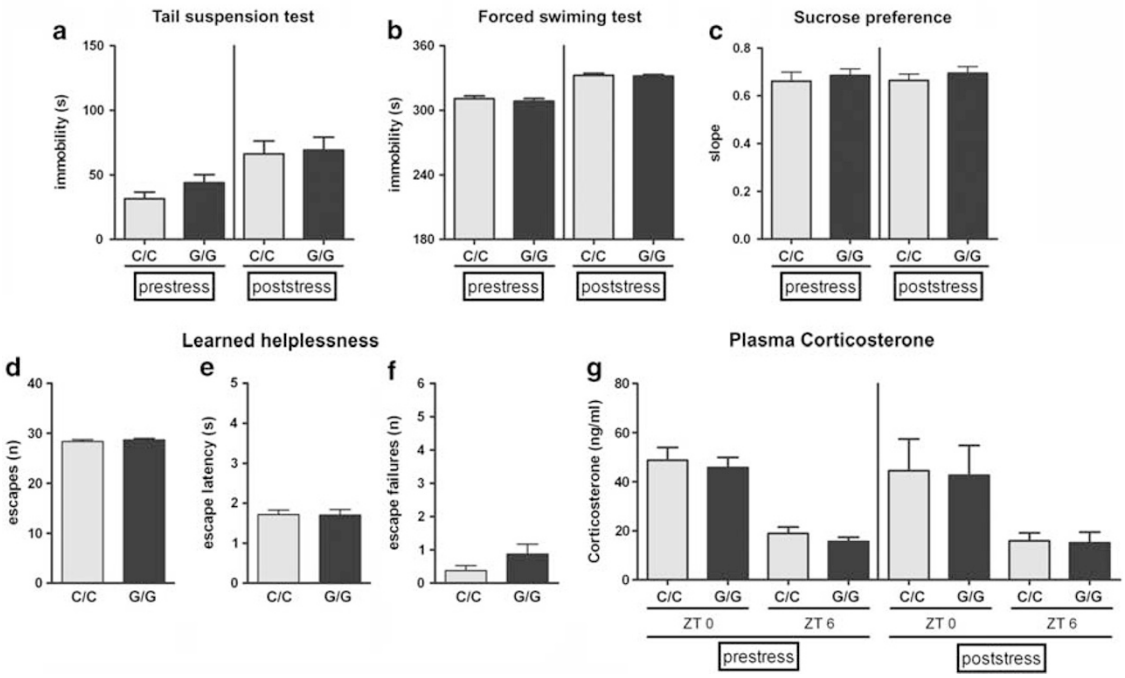

Figure 4 C57BL/6N mice homozygous for the *Tph2* 1473G allele do not display depressive-like behavior under basal conditions and after chronic mild stress. (a) Tail suspension test (TST) and (b) forced swimming test (FST) (for animal numbers and order of behavioral experiments see Supplementary Table S1). In both behavioral paradigms, mice of both *Tph2* 1473 alleles showed no differences in immobility times, which is a measure of behavioral despair, under unstressed conditions (pre-stress) and following a chronic mild stress procedure (CMS; post-stress) (TST: $F_{Genotype}$ (1,28) = 0.68, $p = 0.417$; FST: $F_{Genotype}$ (1,28) = 0.33, $p = 0.573$; two-way ANOVA of repeated measures). Although immobility times are significantly higher when measured after the CMS procedure (TST: F_{Stress} (1,28) = 18.69, $p = 0.0002$; FST: F_{Stress} (1,28) = 237.9, $p < 0.0001$), this is due to the condition of the second testing as a direct comparison with unstressed wild-type C57BL/6N mice revealed lower immobility of stressed mice of both genotypes (Supplementary Figure S2 F+G). (c) Sucrose preference test. Preference to sucrose-containing solutions, determined with a matching law procedure, is undistinguishable in *Tph2* 1473G/G and 1473C/C mice both under pre-stress and post-stress conditions ($F_{Genotype}$ (1,28) = 0.78, $p = 0.384$). Also, sucrose consumption is not affected by stress (F_{Stress} (1,28) = 0.04, $p = 0.844$). (d–f) Learned helplessness paradigm. 1473G/G mice showed a similar number of escapes (d), escape latencies (e), and escape failures (f) in comparison with 1473C/C mice after two sessions of inescapable foot shocks. Mice of both genotypes failed to reach criteria for helpless behavior, which are >6 failures out of 30 escape trials and a mean escape latency above 4.75 s (Chourbaji *et al*, 2005). (g) Plasma corticosterone concentrations. Venous blood was taken from the animals within the first hour of the dark cycle (peak corticosterone; ZT = 0) and 6h later (basal corticosterone; ZT = 6). Plasma corticosterone concentrations were determined both before (pre-stress) and after (post-stress) mice were subjected to the chronic mild stress procedure. Under both conditions, peak concentrations of corticosterone (ZT = 0) are significantly higher in comparison with basal concentrations (ZT = 6) (F_{ZT} (1,28) = 32.31, $p < 0.001$; three-way ANOVA of repeated measures). However, neither stress no genotype had a significant effect on this rhythmicity ($F_{ZT*Stress}$ (1,28) = 0.04, $p = 0.836$; $F_{ZT*Genotype}$ (1,28) = 0.001, $p = 0.975$). All data presented are mean values + SEM. Statistical analysis was performed using unpaired *t*-tests or two- and three-way ANOVA of repeated measures, respectively.

when stressed 1473G/G and 1473C/C mice were compared with a group of unstressed C57Bl/6N mice (Supplementary Figures S2 F and G). However, neither 1473G/G nor 1473C/C mice showed post-stress depression-related behaviors in all paradigms tested (Figures 4a–c). In addition, peak and basal plasma corticosterone concentrations were unaffected by chronic mild stress in mice of both *Tph2* alleles (Figure 4g). Interestingly, the difference in pre-stress anxiety-related behavior between 1473G/G and 1473C/C mice persisted unchanged after stress (Supplementary Figures S2 C–E).

Pharmacological Treatment of Elevated Anxiety with Chronic Escitalopram in Homozygous *Tph2* 1473G Mice

Selective serotonin reuptake inhibitors (SSRIs) are the treatment of choice for anxiety disorders (Rickels and Rynn, 2002; Sheehan *et al*, 1993). The novelty-induced hypophagia test is currently the only behavioral paradigm in mice sensitive to the selective anxiolytic effect of chronic, but not acute, antidepressant treatment (Dulawa and Hen, 2005). Mice that had been tested in the novelty-induced hypophagia test were treated with 0.225 mg/ml escitalopram in the drinking water for 6 weeks (for details, see Supplementary Materials and Methods and Supplementary Figure S3), which resulted in an average dosage of 30 mg/kg/d. In mice of both *Tph2* genotypes, escitalopram treatment resulted in a significant decrease in the latency to consume condensed milk (Figure 5a) and a significant reversal of the suppression of milk consumption in the novel environment (Figure 5b). In addition, the SSRI treatment was considerably more effective in the anxious 1473G/G mice, as shown by the significantly more pronounced reversal of milk consumption under chronic escitalopram administration (Figure 5b). Also, the treatment effect of escitalopram in reducing the latency for milk consumption in the novel cage was appreciably greater although not statistically significant in 1473G/G mice (Figure 5a). More appetite for sweetened condensed milk as the reason for the observed behavior can be excluded, as escitalopram treatment even reduced the total amount of milk intake during home cage training by 13% in mice of both genotypes (Figure 5c).

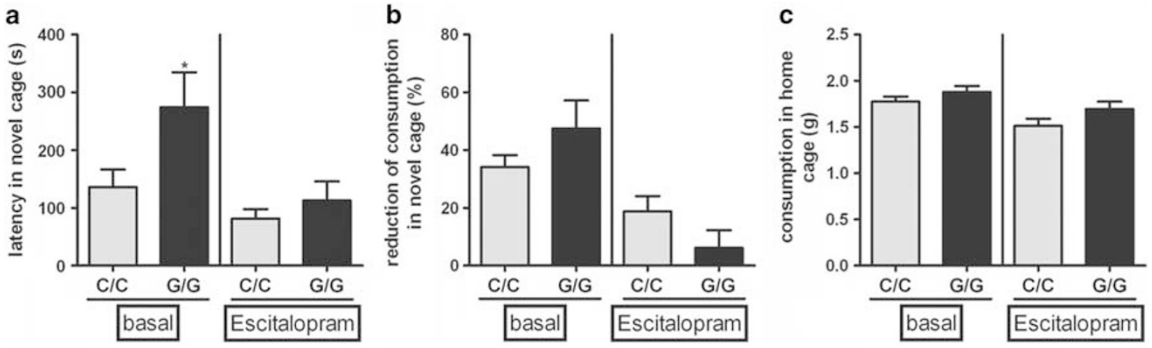

Figure 5 Chronic treatment with escitalopram alleviates anxiety-related behavior in homozygous *Tph2* 1473G and 1473C mice. The novelty-induced hypophagia paradigm was applied in C57BL/6N mice with the 1473G/G or 1473C/C allele before (basal) and after oral escitalopram treatment (0.225 mg/ml for 6 weeks). The animal numbers and the order of the behavioral experiments are displayed in Supplementary Table S1. (a) Latency to start consuming sweetened condensed milk in the novel cage. Escitalopram significantly reduced latencies ($F_{Treatment}$ (1,27) = 12.44, $p = 0.0015$) in mice of both genotypes. Also, a trend of significance was observed between 1473C/C and 1473G/G mice ($F_{Genotype}$ (1,27) = 3.35, $p = 0.078$). Bonferroni *post hoc* testing showed that this is due to a significantly higher latency of untreated 1473G/G mice compared with 1473C/C mice (*$p < 0.05$), which is no longer present under escitalopram treatment. Furthermore, there was a trend of significance for the effect of escitalopram on latency reduction ($F_{Treatment*Genotype}$ (1,27) = 3.02, $p = 0.094$). (b) Reduction of milk consumption during novel cage testing (for details, see Supplementary Materials and Methods). Escitalopram significantly attenuated the reduction in milk consumption observed in mice of both genotypes in a novel environment ($F_{Treatment}$ (1,27) = 45.05, $p < 0.001$). The rescue of milk consumption because of chronic escitalopram treatment was significantly more pronounced in 1473G/G mice compared with 1473C/C mice ($F_{Treatment*Genotype}$ (1,27) = 9.52, $p = 0.0047$). However, there was no significant differences detected between mice of both genotypes ($F_{Genotype}$ (1,27) = 0.002, $p = 0.963$). (c) Milk consumption during home cage training. Escitalopram reduced mean milk consumption during home cage training by 13% on average ($F_{Treatment}$ (1,27) = 16.23, $p < 0.001$). The reduction of consumption was similar in mice of both genotypes ($F_{Treatment*Genotype}$ (1,27) = 0.52, $p = 0.479$). All data presented are mean values + SEM. Stars represent *p*-values obtained by comparing 1473G/G and 1473C/C mice with Bonferroni *post-hoc* analysis following two-way ANOVA of repeated measures: *$p < 0.05$.

DISCUSSION

C1473G *Tph2* SNP in Different Inbred Mouse Strains

The inbred mouse strains DBA/2 and BALB/c (both homozygous for the *Tph2* allele 1473G) show increased anxiety-like behavior in comparison with C57BL/6, which are homozygous for the *Tph2* 1473C allele (Bouwknecht and Paylor, 2002; Griebel *et al*, 2000; Mozhui *et al*, 2010). Both DBA/2 and BALB/c strains are regarded as more stress-sensitive compared with C57BL/6 because they show higher stress-induced increases in corticosterone concentration and increased anxiety-related behavior following chronic stress (Brinks *et al*, 2007; Mozhui *et al*, 2010; Shanks and Anisman, 1988). In addition, 5-HT synthesis, 5-HT tissue concentrations, and extracellular 5-HT levels were shown to be reduced in DBA/2 and BALB/c mice compared with C57Bl6/N (Calcagno *et al*, 2007; Isles *et al*, 2005; Jacobsen *et al*, 2008; Zhang *et al*, 2004). In our study, the introduction of the 1473G/G SNP into a C57BL/6N genetic background resulted in a defined anxiety-phenotype and reduced 5-HT synthesis rate. These changes occurred despite unaltered tissue and extracellular 5-HT concentrations, and without changes in depression-related behavior or in plasma corticosterone concentrations in naive animals or chronically stressed 1473G/G and 1473C/C C57BL/6N mice. Thus, apart from the anxiety phenotype and the reduced *in vivo* 5-HT synthesis rate, our findings suggest that genetic variability outside the C1473G *Tph2* SNP is responsible for the behavioral and neurochemical differences reported for these different mouse strains.

Mouse *Tph2* Polymorphisms in Defined Genetic Backgrounds

In a previous report (Tenner *et al*, 2008), the *Tph2* 1473G allele from DBA/2 mice was bred over eight generations to a C57BL/6J background to obtain congenic mice carrying the same 1473C/C or 1473G/G alleles as in our study. Using an *in vitro* TPH2 activity assay with brain stem lysates, the authors did not show any difference in the TPH2 activity of homozygous 1473G and 1473C mice. This finding contradicts other reports that detected a reduction of TPH2 activity with the 1473G polymorphism (Sakowski *et al*, 2006; Zhang *et al*, 2004). In our study, we measured the *in vivo* 5-HT synthesis rate in freely behaving animals. For this purpose, we treated live mice with m-hydroxybenzylhydrazine to determine the accumulation of the 5-HT precursor, 5-HTP. Similar to others (Siesser *et al*, 2010), we found a significant reduction of the *in vivo* 5-HT synthesis rate in the 1473G/G mice. Despite the reduced 5-HT synthesis rate, we could not detect alterations in total 5-HT content and metabolism, which is in accordance with Tenner *et al* (2008). However, while they argued that these findings reflect the unchanged TPH2 activity in 1473G/G mice, here we show that brain region-specific 5-HT concentrations are not reduced despite a significantly decreased *in vivo* 5-HT synthesis rate in 1473G/G mice. This is in accordance with a previous report (Siesser *et al*, 2010). We extend these findings using microdialysis, showing that not only the tissue concentration of 5-HT but also basal extracellular 5-HT and the stress-induced increase in 5-HT release is unaltered in 1473G/G mice. At last, Tenner *et al* (2008) conclude that the *Tph2* C1473G SNP is not responsible for behavioral differences between different inbred mouse strains. This assumption is based solely on two behavioral tests in which they could not find any differences in anxiety- and depressive-like behavior. In our study, two independent behavioral research teams performed several test batteries to assess depressive- and anxiety-like behavior. We demonstrate that 1473G/G mice show a distinct anxiety phenotype, which could be observed in four different anxiety tests.

A rare human G1463A *TPH2* SNP was analyzed via a knock-in approach in transgenic mice (Beaulieu *et al*, 2008; Jacobsen *et al*, 2011). Mice homozygous for the corresponding mouse *Tph2* allele 1449A showed an 80% reduction of 5-HT synthesis and a strong decrease in total and extracellular 5-HT concentrations. In addition, frontal 5-HT_{2A} receptor function was upregulated and the 5-HT_{1A}-receptor agonist-induced hypothermia was blunted (Jacobsen *et al*, 2011), indicating 5-HT_{1A} autoreceptor desensitization or downregulation in serotonergic neurons of the raphe nuclei (Gross *et al*, 2002; Martin *et al*, 1992). Despite these findings and in contrast to our results, Jacobsen *et al* (2011) did not find altered 5-HT_{1A} G-protein coupling in the dorsal raphe.

Most relevant to our study are results from their heterozygous 1449A mice, in which 5-HT synthesis was only reduced by 40% (Beaulieu *et al*, 2008). This decrease is comparable to the reduction of 5-HT synthesis detected in our 1473G/G mice. Identical to our homozygous 1473G mice, these heterozygous 1449A mice also showed unchanged 5-HT and 5-HIAA tissue concentrations and displayed anxiety-like behavior (Beaulieu *et al*, 2008). These results suggest that the anxiety phenotype is not directly related to a reduced 5-HT concentration in the adult brain. In contrast to our results, both homozygous and heterozygous 1449A mice show depressive-like behavior (Beaulieu *et al*, 2008). This finding could be attributed to reduced 5-HT neurotransmission in these mice. Although this has been confirmed by microdialysis in homozygous 1449A mice only (Jacobsen *et al*, 2011), to date it is unclear whether extracellular 5-HT concentration is also decreased in heterozygous 1449A mice. The mixed genetic background (F1 generation from matings of 129S6/SvEv mice with C57BL/6 mice) used in the study could contribute to the depressive phenotype as well. This was shown for serotonin transporter (5-HTT, *Slc6A4*) knockout mice, in which depressive behavior was only found when the genetic background was not 100% C57BL/6 (Kalueff *et al*, 2010). Together with our findings, this might suggest that additional genetic modifications absent in the C57BL/6 genetic background are necessary for eliciting depression-related symptoms in rodents.

In conclusion, we and others (Beaulieu *et al*, 2008; Siesser *et al*, 2010) describe two different functional *Tph2* SNPs, which are responsible for a reduced 5-HT synthesis rate. Small reductions in 5-HT synthesis during development can likely be compensated for, which suggests that reductions of 5-HT concentration or 5-HT release are not responsible for the anxiogenic behavior in adult mice, but rather adaptive changes that occurred during the animal's development. One of the potential mechanisms during development to regain 5-HT equilibrium would be the identified functional desensitization of 5-HT_{1A} autoreceptors leading to a stabilization of synaptic 5-HT release.

The 5-HT Neuronal System During Development

Studies that examined key molecules involved in serotonergic neurotransmission during development report interesting parallels. In *Tph2* knockout mice, which are almost completely deficient of 5-HT in the central nervous system, a highly significant growth retardation during postnatal development that subsided around P64 could be shown (Alenina *et al*, 2009). However, we did not observe any overt growth alterations in 1473G/G mice. Recently, changes in emotional behavior accompanied with adaptations in 5-HT_{1A} receptors and corresponding signaling pathways were proposed for *Tph2* knockout mice (Waider *et al*, 2011).

Recent studies investigating 5-HT_{1A} autoreceptors in relation to emotional behaviors revealed interesting similarities to our results. Richardson-Jones *et al* (2011) show that the reduction of 5-HT_{1A} autoreceptors expression during embryonic development led to an elevation of extracellular 5-HT concentration and to increased anxiety in adult animals, but not to depressive-like behavior. In contrast, downregulation of 5-HT_{1A} autoreceptors in adult mice did not induce similar neurochemical and behavioral changes (Richardson-Jones *et al*, 2010). In fact, it has been repeatedly shown that the second and third postnatal week is a critical period for the development of brain circuits mediating anxiety in mice. In this period, serotonergic innervations to the hippocampus and cortical areas are formed and perturbances in serotonergic signaling in this period affect anxiety in adulthood (Leonardo and Hen, 2008).

Similarly to 1473G/G mice, *Slc6a4* knockout mice on a C57BL/6 background displayed elevated anxiety without depression-like alterations (Kalueff *et al*, 2010) and showed a lower 5-HT synthesis rate *in vivo* and a desensitization of 5-HT_{1A} autoreceptors (Fabre *et al*, 2000). However, while in *Slc6a4* knockout mice the observed desensitization of 5-HT_{1A} autoreceptors is due to a reduction of its expression (Fabre *et al*, 2000), the concentration of 5-HT_{1A} autoreceptors in the dorsal raphe in our 1473G/G mice is unaltered.

Although we cannot exclude the involvement of other neurotransmitter systems contributing to the observed phenotypes in 1473G/G mice, these observations and our data suggest that a potentially adaptive desensitization or downregulation of 5-HT_{1A} autoreceptors during development appears to be a common denominator for an anxiogenic phenotype in mice in which serotonergic neurotransmission has been modulated during development.

Efficient Treatment of Anxiety by Chronic Escitalopram

An interesting new aspect shown by our study is the virtually selective anxiolytic treatment effect of escitalopram in our anxious 1473G/G mice. Chronic exposure to the SSRI escitalopram attenuates the anxiety phenotype in 1473G/G mice to levels similar to less anxious 1473C/C mice, thus mirroring the selective effect of chronic SSRI treatment in anxiety patients. Recently, increasing evidence has accumulated that the major mechanism of SSRIs in relieving anxiety- and depression-related symptoms is not simply increasing extracellular 5-HT concentrations via reuptake inhibition, but that chronic treatment with drugs like escitalopram, citalopram, or fluoxetine causes a complex modulation of the entire serotonergic neurotransmission, in which the desensitization of 5-HT_{1A} autoreceptors is one of the major changes observed (Blier and de Montigny, 1994; El Mansari *et al*, 2005; Johnson *et al*, 2007; Le Poul *et al*, 2000). In 1473G/G mice, we could identify normal extracellular 5-HT levels and a functional desensitization of 5-HT_{1A} autoreceptors before escitalopram treatment.

Nonetheless, a strong anxiolytic treatment effect could be observed in our anxious 1473G/G mice. Although the exact mechanism of 5-HT_{1A} autoreceptors desensitization in 1473G/G mice remains to be elucidated in the future, these mice may be a new tool for *in vivo* interrogation of SSRI-induced molecular changes that lead to an amelioration of anxiety. These mice could also provide an animal model for preclinical testing of other anxiolytic drugs.

Human *TPH2* Polymorphisms

Human studies have revealed controversial results regarding the association of *TPH2* polymorphisms and major depression. Although earlier studies reported significant associations between several *TPH2* SNPs and both depression and suicidal behavior (Haghighi *et al*, 2008; Ke *et al*, 2006; Lopez de Lara *et al*, 2007; Van Den Bogaert *et al*, 2006; Zhou *et al*, 2005; Zill *et al*, 2004a, b) more recent studies failed to confirm these findings (De Luca *et al*, 2006; Lopez *et al*, 2007; Mann *et al*, 2008; Zill *et al*, 2007). Among functionally characterized *TPH2* polymorphisms only a few (including G1463A) result in a clear reduction in enzymatic activity, whereas other polymorphisms have only a minor effect on TPH2 activity (McKinney *et al*, 2009). This might explain why a clear association with depression was only seen in the case of an extremely rare G1463A *TPH2* polymorphism (Zhang *et al*, 2005) that led to a marked reduction of 5-HT synthesis and 5-HT brain levels in knock-in mutant mice with the corresponding G1463A *Tph2* SNP (Beaulieu *et al*, 2008).

In humans, the *TPH2* polymorphism –703G/T may be associated with harm avoidance (Gutknecht *et al*, 2007; Reuter *et al*, 2007), an 'anxiety' personality trait characterized by excessive anticipatory worry and fear of uncertainty. This 703G/T *TPH2* SNP was also shown to be associated with increased reactivity of the amygdala in an emotional face-processing task (Brown *et al*, 2005; Canli *et al*, 2005). However, recent studies (Juhasz *et al*, 2010; Middeldorp *et al*, 2010) could not confirm a correlation between anxiety symptom scores and several *TPH2* polymorphisms but reported on reduced risk-taking behavior in subjects with the most prevalent *TPH2* haplotype (Juhasz *et al*, 2010). In contrast to the conflicting human studies, our homozygous 1473G C57BL/6N mice displayed a defined anxiety phenotype in several conflict-based paradigms and in behavioral tasks investigating anxiety toward novelty. Several reasons may account for these discrepancies, the foremost being species-related, as human anxiety obviously cannot be comprehensively reconstructed in mouse models. Associations of *TPH2* polymorphisms with multiple facets of human anxiety, like anxiety personality traits (neuroticism/harm avoidance) or reduced risk-taking behavior may converge in the mouse analysis to a simpler anxiety phenotype.

It should be noted that in human *TPH2* SNP studies effects are complicated not only by the heterogeneous genetic background, but also by difficulties in controlling for environmental factors such as stress. In contrast in this study, we used a laboratory C57BL/6N mouse strain housed under controlled conditions, thus the only genetic difference is the C1473G *Tph2* polymorphism and other confounding genetic and environmental factors are excluded. Furthermore, many human *TPH2* SNPs assessed previously have not been characterized with respect to their functional consequences on TPH2 transcription or 5-HT synthesis rate and consequently 5-HT brain content and 5-HT neurotransmission. One interesting finding of our study is that despite a decreased 5-HT synthesis rate we nonetheless encountered a fully compensated 5-HT system with respect to tissue 5-HT content and 5-HT neurotransmission. This apparent picture of a fully functional serotonergic system may disguise compensatory changes as shown here for the 5-HT_{1A} autoreceptor. The functionally desensitized 5-HT_{1A} autoreceptor during development might be one reason for the anxiety phenotype observed in our 1473G/G *Tph2* SNP mice. In fact, this view is also supported by a study investigating the human 5-HT_{1A} receptor polymorphism C1019G, responsible for an alteration of 5-HT_{1A} autoreceptor concentrations in the DRN (Fakra *et al*, 2009). In this report, the authors show that the genetic variation, responsible for reduced 5-HT_{1A} receptor availability, is linked with higher amygdala reactivity, which itself is correlated with increased trait anxiety.

CONCLUSION

On a defined C57Bl6/N genetic background, we have demonstrated that a significant reduction of 5-HT synthesis in mice with a 1473G/G polymorphism in the *Tph2* gene leads to a functional desensitization of 5-HT_{1A} autoreceptors but does not evoke reductions in basal and stress-induced 5-HT release. As these mice do not display depressive-like behavior yet clearly show increased anxiety, developmental compensations, and not acute alterations in serotonergic neurotransmission seem likely to be responsible for the anxiety phenotype. This supports the view of anxiety as a developmental disorder (Leonardo and Hen, 2008). Although we cannot exclude secondary effects of reduced TPH2 activity on other neurotransmitter systems, the anxiolytic effect of the highly selective SSRI escitalopram on the 1473G/G mice supports a direct serotonergic effect. Although the exact molecular pathways by which TPH2 activity affects anxiety remains to be elucidated, the functional desensitization of 5-HT_{1A} autoreceptors appears to be promising common denominator for increased anxiety.

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DISCLOSURE

The authors declare no conflict of interest.

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