Growth retardation and altered autonomic control in mice lacking brain serotonin

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Serotonin synthesis in mammals is initiated by 2 distinct tryptophan hydroxylases (TPH), TPH1 and TPH2. By genetically ablating TPH2, we created mice (*Tph2*$^{-/-}$) that lack serotonin in the central nervous system. Surprisingly, these mice can be born and survive until adulthood. However, depletion of serotonin signaling in the brain leads to growth retardation and 50% lethality in the first 4 weeks of postnatal life. Telemetric monitoring revealed more extended daytime sleep, suppressed respiration, altered body temperature control, and decreased blood pressure (BP) and heart rate (HR) during nighttime in *Tph2*$^{-/-}$ mice. Moreover, *Tph2*$^{-/-}$ females, despite being fertile and producing milk, exhibit impaired maternal care leading to poor survival of their pups. These data confirm that the majority of central serotonin is generated by TPH2. TPH2-derived serotonin is involved in the regulation of behavior and autonomic pathways but is not essential for adult life.

growth retardation | maternal care | respiration | serotonin | sleep

Serotonin (5-hydroxytryptamine, 5-HT) is an extracellular signaling molecule with a multitude of functions in the central nervous system (CNS) and in the periphery. 5-HT effects are conveyed by at least 13 receptors classified in 7 families, 5-HT1 to 5-HT7. Serotonin synthesis from tryptophan is initiated by the enzyme tryptophan hydroxylase (TPH) generating 5-hydroxytryptophan followed by aromatic amino acid decarboxylase (AADC), which produces 5-HT. We have recently discovered that 2 TPH isoenzymes exist in all vertebrates, TPH1 and TPH2, encoded by 2 distinct genes (1, 2). *Tph1* is mainly expressed in the gut, generating serotonin that is distributed into the whole body by thrombocytes, and in the pineal gland, where the resulting 5-HT is metabolized to melatonin. The *Tph1*-deficient mice generated by us (2) and others (3, 4) revealed that 95% of peripheral 5-HT is produced by TPH1. They also revealed that 5-HT in platelets and other peripheral cells is involved in such diverse processes as thrombosis (5), liver regeneration (6), hepatitis (7), colon cancer (8), mammary gland plasticity (9), pulmonary hypertension (10), and bone formation (11). TPH2, on the other hand, is responsible for the synthesis of serotonin in the raphé nuclei of the brainstem, from where all central serotonergic projections originate (12). Accordingly, polymorphisms and functional mutations in the human and mouse genes for this enzyme have been linked to neurological and behavioral abnormalities (13–16).

In this study, we generated mice lacking TPH2 by gene targeting and analyzed the physiological consequences resulting from a lack of brain serotonin.

Results and Discussion

Generation and Basic Characteristics of *Tph2*-Deficient Mice. *Tph2*-deficient (*Tph2*$^{-/-}$) mice were generated by deleting the coding sequence in exons 1 and 2 (supporting information (*SI*) Fig. S1 *A* and *B*). In the resulting *Tph2*$^{-/-}$ mice, no *Tph2* mRNA could be found by RT-PCR (Fig. S1*C*) and in situ hybridization in the brain (Fig. 1*A*). Immunohistochemistry did not detect serotonin in sections of the raphé nuclei (Fig. 1*A*). By high-performance liquid chromatography (HPLC) quantification we measured <1–4% of residual levels of the monoamine compared to control animals and no detectable amounts of its metabolite 5-HIAA in the whole brain, in raphé nuclei, and in other brain areas such a striatum, hippocampus, cortex, and hypothalamus of *Tph2*$^{-/-}$ mice while the concentration of the precursor tryptophan was unchanged (Fig. 1*B*, Table 1, Table S1). TPH1 may be responsible for this minute residual 5-HT generation, because it is expressed in the brain at a level of about 4% compared to TPH2 (17). However, TPH1 is obviously

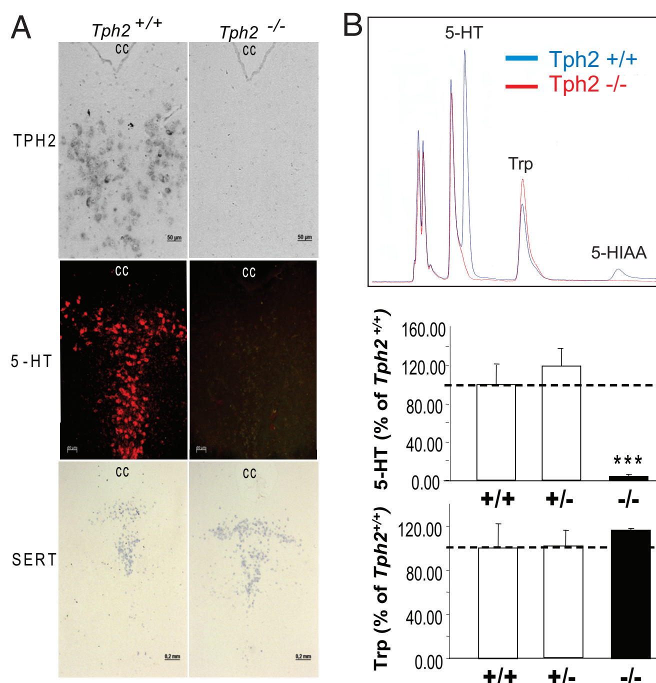

Fig. 1. Serotonin system in the brain of *Tph2*-deficient mice. (*A*) Detection of serotonin (*Middle* panel) by immunofluorescence and of *Tph2* and serotonin transporter (SLC6A4, SERT) transcripts by in situ hybridization (*Upper* and *Lower* panels, respectively) in the dorsal raphé (DR) of *Tph2*$^{-/-}$ mice. CC, central canal. (*B*) Detection of serotonin (5-HT), its degradation product 5-hydroxyindoleacetic acid (5-HIAA), and the serotonin precursor tryptophan (Trp) in the DR by HPLC (representative HPLC-chromatogram, *Upper* panel and its quantification, *Middle* and *Lower* panels). ***, $P < 0.001$ *Tph2*$^{-/-}$ vs. *Tph2*$^{+/+}$ and *Tph2*$^{+/-}$, Student's *t* test.

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Table 1. Monoamines and their metabolites, GABA, and glutamate levels in the brains of *Tph2*-deficient mice

	5-HT (pg)	5-HIAA (pg)	NA (pg)	Glutamate (pmol)	GABA (pmol)	DA (pg)	DOPAC (pg)	HVA (pg)
Control	710.1 ± 56.9	147.4 ± 13.2	419.6 ± 17.2	10.2 ± 0.5	2.93 ± 0.09	1116.4 ± 32.8	119.6 ± 4.2	147.3 ± 12.3
$Tph2^{-/-}$	28.4 ± 3.6***	ND	395.7 ± 23.9	10.1 ± 0.6	2.80 ± 0.04	1139.4 ± 27.6	121.1 ± 12.7	156.4 ± 10.5

Serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), norephinephrine (NA), Glutamate, γ-aminobutyric acid (GABA), dopamine (DA), dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) levels were measured in whole brain lysates per milligram of wet tissue of $Tph2^{-/-}$ (n = 4) and control (n = 6) mice (mixed background). Data are presented as mean ± SEM; ND, not detectable. ***, $P < 0.001$ vs. control group, 1-way ANOVA, followed by Tukey's post hoc test.

not able to compensate for the loss of TPH2, confirming that TPH2 is the only relevant enzyme for 5-HT synthesis in the CNS. There was no difference compared to control mice in the brain 5-HT concentrations of heterozygous mice ($Tph2^{+/-}$) (Fig. 1*B*, Table S1). Only 5-HIAA levels were slightly but significantly reduced in some brain regions (Table S1). These data show that 50% of a *Tph2* gene dose is enough to maintain normal brain serotonin levels, partially the result of a decreased metabolism of the monoamine. Furthermore, there was no difference in serotonin levels in any other organ or in the blood of $Tph2^{-/-}$ mice, confirming that peripheral serotonin is not depending on TPH2 (Table S2). The bowel is an exception because expression of both isoforms has been described there, TPH1 in enterochromaffin cells (2) and TPH2 in enteric neurons (18). Because TPH2-derived serotonin is only a minor portion of total intestinal serotonin, the 5-HT concentration in the duodenum remains unchanged in $Tph2^{-/-}$ mice (Table S2).

Serotonergic neurons were still present at their normal location and pattern in the raphé nuclei of $Tph2^{-/-}$ mice as detected by in situ hybridization for serotonin transporter (SLC6A4) mRNA (Fig. 1*A*). Consistent with the findings in other $Tph2^{-/-}$ mice (4, 19) and *Pet-1*-deficient mice, which lack most serotonergic neurons (20), we did not detect major alterations in brain structures using magnetic resonance imaging (Movies S1 and S2) but detailed morphological studies are still pending. Thus, neither the development of serotonergic neurons nor whole brain morphogenesis depends on TPH2-derived serotonin. However, we cannot exclude that during embryogenesis maternal or TPH1-derived circulating 5-HT reaches the brain because of the lack of a blood–brain barrier at early stages of development.

We next studied the effects of the near complete lack of serotonin on other neurotransmitters such as dopamine, γ-aminobutyric acid, norepinephrine, and glutamate in the brain of adult $Tph2^{-/-}$ mice. To our surprise the level of no other transmitter was altered in comparison to control animals (Table 1). Moreover, gene expression profiling in FVB/N-F4 *Tph2*-deficient mice using Affymetrix Mouse genome arrays also did not reveal any marked change (>1.8-fold) in mRNA abundances in the whole brains of $Tph2^{-/-}$ animals (data not shown). Thus, we could not detect any compensatory mechanisms in the CNS of mice drastically deficient in serotonin. Nevertheless, these studies need to be repeated with defined brain structures to detect possible local alterations in gene expression or neurotransmitter levels.

Survival and Growth. $Tph2^{-/-}$ mice were born at normal Mendelian ratios and at the day of birth were undistinguishable from control littermates. However, already 3 days later $Tph2^{-/-}$ mice were visibly smaller, had softer skin, and appeared weaker in comparison to control littermates and this difference was aggravated in the following 3 weeks (Fig. 2 *A*, *B*, and *C*). Moreover, $Tph2^{-/-}$ mice on a mixed genetic background showed considerable lethality (27%) in the first 4 weeks of life. Backcross to FVB/N background improved the survival rate (88.6%), whereas after backcross to the C57BL/6 strain the lethality got more pronounced and about half of the $Tph2^{-/-}$ pups were lost (Fig. 2*D*, 52.6% survival). Because of the growth retardation it was not possible to wean $Tph2^{-/-}$ mice at 3 weeks of age but only 2 weeks later. After weaning they started a catch-up growth and reached near normal size at 4 months of age (Fig. 2*C*), when also no increased mortality could be observed anymore at least until 1.5 years of age on the mixed genetic background.

We do not yet know the reasons for the lack of weight gain in early postnatal life. Newborn $Tph2^{-/-}$ mice drink milk and show normal digestion. All trials to quantify food intake or to force feed failed, because any handling of the pups worsened the phenotype.

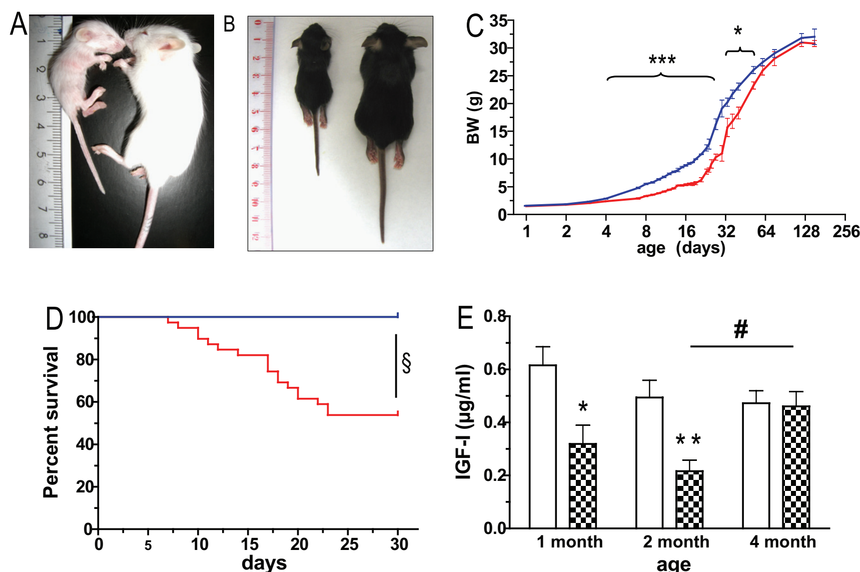

Fig. 2. Growth retardation and postnatal lethality in *Tph2*-deficient mice. Representative photographs of 15-day-old FVB/N-*Tph2*-deficient (*A*) and 21-day-old C57BL/6-*Tph2*-deficient (*B*) mice. On both panels: *Left*, $Tph2^{-/-}$ mouse; *Right*, $Tph2^{+/+}$ mouse from the same litter. Body weight (BW) development (mixed genetic background) (*C*) and survival (C57BL/6 genetic background) (*D*) of *Tph2*-deficient animals. Red line, $Tph2^{-/-}$; blue line, control mice. (*E*) IGF1 concentration in the serum of the *Tph2*-deficient animals (FVB/N-F4 genetic background) at different ages. Filled bars, $Tph2^{-/-}$; open bars, control mice. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ (Student's *t* test); and §, $P < 0.0001$ (Logrank test) $Tph2^{-/-}$ vs. age-matched control mice; #, $P < 0.05$ (Student's *t* test) 4-month-old vs. 2-month-old $Tph2^{-/-}$ mice.

Table 2. Maternal neglect in *Tph2*-deficient mice

Mother genotype	Number of dams	Litter size	Number of pups born	Number of pups survived until day 5	% survival	Number of dead litters on day 5
Control	*n*=23	10.13 ± 0.45	233	228	97.9%	0/23
$Tph2^{-/-}$	*n*=29	9.28 ± 0.48	269	149	55.4%	11/29

$Tph2^{--}$ and control females (mixed background) were mated with NMRI (wild type) males and separated into single cages when visibly pregnant. The number of pups was counted on the day of birth (day 0) and at day 5. Litter size is given as average ± SEM.

Pups raised in litters with mixed genotypes showed the same phenotype as pups raised in pure $Tph2^{-/-}$ litters excluding competition with control littermates as cause for the effect. The growth retardation also did not depend on the genotype of the mother because it appeared in pups born and raised by $Tph2^{-/-}$ and $Tph2^{+/-}$ dams. $Tph2^{-/-}$ pups vocalize equally as controls when separated from their mothers excluding a deficit in this behavior as cause of the growth retardation (Fig. S2). Serum levels of insulin-like growth factor I (IGF1) were markedly lower in $Tph2^{-/-}$ mice until 2 months of age but afterward reached the levels of control animals, when the animals had attained normal size (Fig. 2*E*). Although these reduced IGF1 concentrations are probably causing the postnatal growth retardation in $Tph2^{-/-}$ mice, they could easily be secondary to undernutrition (21). Thus, further studies are required to clarify whether behavioral or sensory deficits causing malnutrition or a primary impairment of the growth hormone/IGF1 axis by the lack of stimulatory serotonin actions (22) lead to the impaired thriving of these mice.

Fertility and Maternal Care. The number of pups per litter was normal even when both parents lacked *Tph2* (6.0 ± 1.5 in $Tph2^{-/-}$ vs. 5.9 ± 0.4 in controls; mixed genetic background). Thus, neither male nor female fertility was affected by the lack of brain serotonin. However, a high percentage of the dams did not take care of their pups. Of 10 litters born by $Tph2^{-/-}$ mothers bred with $Tph2^{+/-}$ fathers (FVB/N-F4 genetic background), only 4 survived for more than several days, compared to 8 litters of 9 born from couples of $Tph2^{+/-}$ mothers and $Tph2^{-/-}$ fathers. The same phenotype was observed when wild-type fathers were used for breeding and, thus, did not depend on the genotype of the offspring (Table 2). At the day of delivery (day 0) $Tph2^{-/-}$ mothers showed normal milk production, were feeding pups (the stomach of newborns was visibly filled with milk a few hours after delivery), organizing and keeping a nest. However, in the following days pups of $Tph2^{-/-}$ were neglected and even often cannibalized by the mother in an aggressive manner. Consequently, most of the pups of $Tph2^{-/-}$ mothers were dead on day 2 or 3 after birth. The same results were obtained when we performed cross-fostering experiments. $Tph2^{-/-}$ dams ate 50% of the pups (30% of whole litters) born by $Tph2^{+/+}$ mothers, while $Tph2^{+/+}$ mothers only cannibalized 5.6% of the pups (zero whole litter) from $Tph2^{-/-}$ mothers. This alteration in maternal instincts in $Tph2^{-/-}$ mothers was confirmed by the pups retrieval test: on day 1, 8 of 9 $Tph2^{-/-}$ dams were not able to collect their scattered pups within 30 min, whereas it took on average 3.9 ± 0.7 min for the control dams ($n = 6$) to achieve this (Movies S3 and S4). However, there was no significant difference between females of each genotype in the time it took to find a hidden cookie (42.3 ± 15.5 sec in $Tph2^{-/-}$ vs. 29.7 ± 9.3 sec in control mothers), indicating that maternal neglect is not caused by disturbed olfaction in $Tph2^{-/-}$ mice. These data confirm a recent study showing that mice lacking most serotonergic neurons exhibit a drastic impairment in maternal care (23). It has been shown that maternal neglect can go along with aggressiveness in mice (24) and, indeed, we observed a more pronounced aggressive behavior of female and male *Tph2*-deficient mice compared to controls. Even females housed with $Tph2^{-/-}$ females were often wounded by fighting that never happens in control animals of the same genetic background. These observations are consistent with the hypothesis that increased aggression is associated with states of low serotonergic system activity (25).

Body Temperature. Because central serotonin is known to be an important modulator of autonomic functions (26), we evaluated blood pressure (BP), heart rate (HR), respiration rate (RR), and the control of sleep and body temperature by telemetry. Basic body temperature was normal in $Tph2^{-/-}$ mice (36.60 ± 0.04 °C vs. 36.61 ± 0.22 °C in controls). However, after a challenge by a cold (4 °C) environment, the observed drop in body temperature was more pronounced in serotonin-deficient mice than in control animals (Fig. 3*A*). At the same time, $Tph2^{-/-}$ mice exhibited hyperactivity in the first hour at 4 °C probably compensating by skeletal muscle movement for a stronger loss in body temperature (Fig. 3*A*). When after 1 hour the animals reduced their locomotion to the levels of control animals, their temperature started to fall more rapidly than in the controls. This is in accordance with data recently obtained in mice lacking most of the serotonergic neurons (27). In this study, the authors show that shivering and nonshivering thermogenesis is impaired by the perturbation of the brain serotonin system, confirming an important function of central 5-HT in thermoregulation.

Sleep. Telemetric recordings revealed marked disturbances in sleep of $Tph2^{-/-}$ mice. During the daytime, these animals sleep more frequently (Fig. 3*B*, Fig. S3*A*) and for longer periods of time than their control littermates (3.96 ± 0.78 30-min inactivity periods from 7 a.m. to 7 p.m. in $Tph2^{-/-}$ vs. 1.04 ± 0.29 in control mice, $P < 0.01$). In the night, sleeping periods were rarer in both strains and did not significantly differ in length and frequency. There is a long-lasting debate on the role of serotonin in the regulation of sleep and wakefulness (28). Both sleep promotion and induction of arousal by 5-HT have been reported, mostly depending on the origin of the serotonergic neurons analyzed. Our animal model strongly supports sleep promotion and a suppression of arousal as the net effect of a lack of serotonin in the CNS.

Cardiovascular Parameters. The same animals were also analyzed for the circadian variation of cardiovascular parameters. Thereby, significant decreases in blood pressure and heart rate could be detected mainly in the late afternoon and in the night in $Tph2^{-/-}$ mice (Fig. 3 *C* and *D*, Fig. S3 *B* and *C*). In the CNS, serotonin is of major importance for cardiovascular control (29). Considerable evidence supports a role of 5-HT1A receptors in the brainstem, in particular in centers involved in sympathetic regulation, which get strong serotonergic innervation from the dorsal group of raphé nuclei, B1 to B4. When these 5-HT1A receptors are activated, a fall in blood pressure is observed accompanied by a decrease in sympathetic nerve activity. Concurrently, central administration of 5-HT1A agonists potentiates the vagal outflow to the heart (30). However, when these agonists are applied to forebrain nuclei, sympathoexcitation and hypertension is induced. These areas get their serotonergic input from the more rostral raphé nuclei, B5 to B9. To further complicate the issue, 5-HT2, 5-HT3, and 5-HT7 receptors have been shown to participate in central

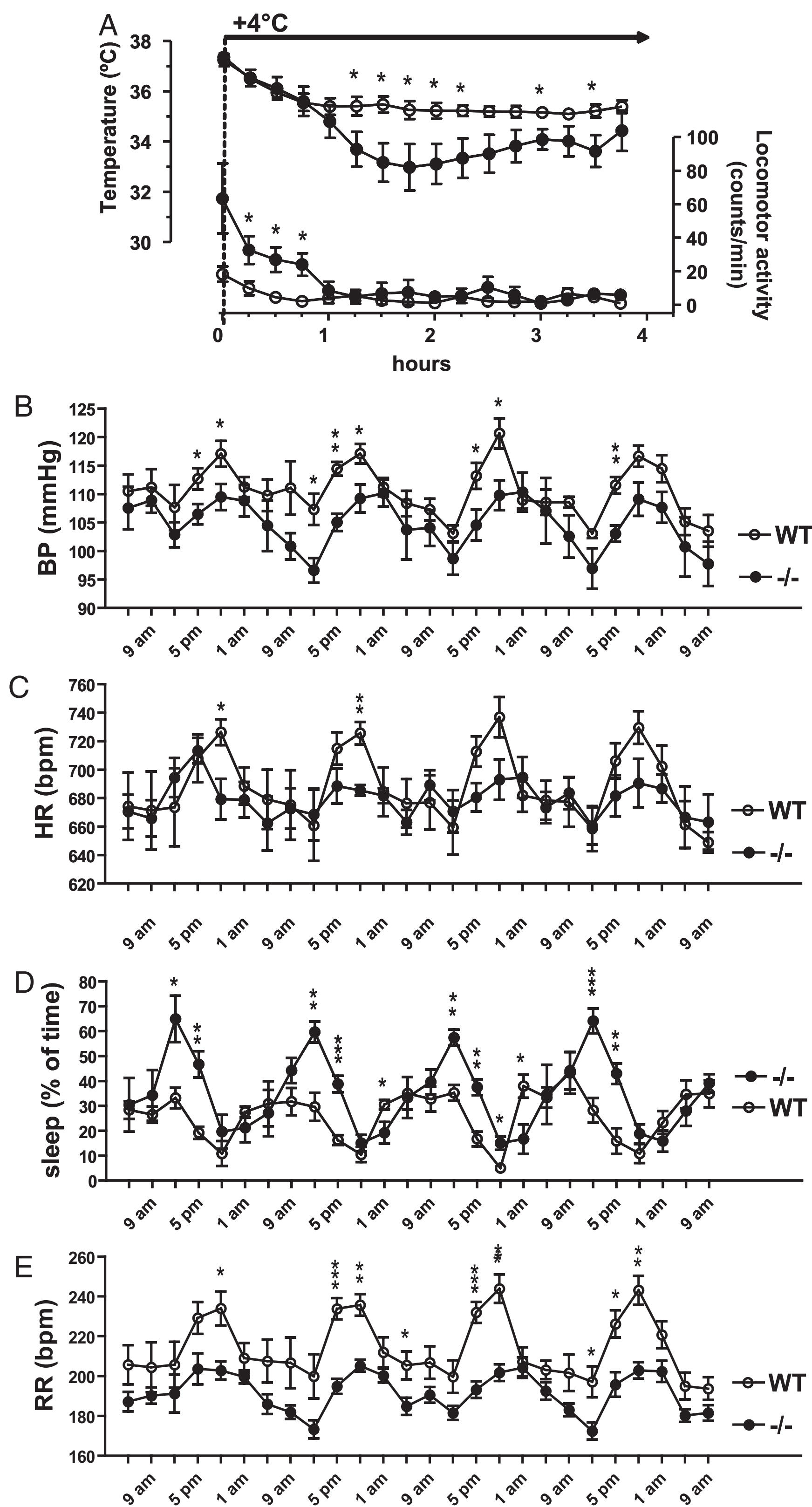

Fig. 3. Telemetric analysis of physiological parameters in *Tph2*-deficient mice. (*A*) Changes in the body temperature and locomotor activity after a challenge by a cold (4 °C) environment. Filled circles, $Tph2^{-/-}$ ($n = 7$); open circles, control mice ($n = 8$) (4-month-old female mice, FVB/N F4 genetic background). Circadian variations in sleep (*B*), mean arterial blood pressure (BP) (*C*), heart rate (HR) in beats per minute (bpm) (*D*), and respiratory rate (RR) in breaths per minute (Bpm) (*E*) in *Tph2*-deficient 3-month-old female mice (FVB/N F4 genetic background). Bold line indicates nighttime. Filled circles, $Tph2^{-/-}$ ($n = 5$), open circles, control mice ($n = 5$). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ (Student's *t* test) $Tph2^{-/-}$ vs. control mice at the same time point.

serotonergic regulation of the sympathetic and parasympathetic tone (29, 30). Despite that the role of single 5-HT receptors in specific brain areas remains to be clarified in future experiments, our data clearly show that a ubiquitous lack of serotonin decreases blood pressure and heart rate at least at nighttime probably by inhibition of the sympathetic and stimulation of the parasympathetic nervous system.

Respiration. Further analysis of the telemetric data revealed that respiration rate was significantly reduced in $Tph2^{-/-}$ mice (185.5 ±

9.7 min^{-1} in $Tph2^{-/-}$ vs. 231.5 ± 6.8 min^{-1} in WT, $P < 0.05$) but with normal circadian variations (Fig. 3*E*, Fig. S3*D*). These data were confirmed by whole body plethysmography (data not shown). Such dampening of respiration has been described in other pharmacologic (31) and genetic (27, 32) models of partial serotonin deficiency in the CNS. However, there were also contradictory studies postulating that serotonin inhibits breathing (reviewed in ref. 33). Our data strongly support the concept of a tonic excitatory input of serotonergic fibers on respiratory neurons.

Conclusions. In conclusion, the lack of serotonin in the brain of $Tph2^{-/-}$ mice confirms that TPH2 is the major enzyme responsible for serotonin synthesis in the brain and that circulating 5-HT cannot enter the brain after birth under normal conditions. The lack of central serotonin in these mice leads to impaired early postnatal growth and altered autonomic control of sleep, breathing, thermoregulation, heart rate, and blood pressure. Furthermore, it promotes aggressive behavior and maternal neglect. However strikingly, besides these relatively mild alterations, animals lacking most serotonin in the brain are viable, morphologically normal, and fertile. Thus, TPH2-derived serotonin is involved in the regulation of behavior and autonomic pathways but is not essential for adult life. Recently, mice lacking both TPH isoforms were generated and shown to be viable (ref. 4 and N.A., and M.B., unpublished work). Using these animals, it should be possible to clarify the functional importance of the serotonin system as a whole.

Materials and Methods

Generation of *Tph2*-Knockout Mice. A $Tph2^{+/-}$ embryonic stem (ES) cell line was created by homologous recombination of an ''expression-selection cassette'' described below into the *Tph2* locus of the ES cell line E14Tg2A (BayGenomics) leading to the deletion of a 5.2-kb-long region, which contains the coding part of exon 1 (Ex1) and Ex2, and, thus, disabling the transcription and translation of the entire *Tph2* gene (Fig. S1*A*).

First, 1- and 6-kb-long *Tph2*-homology arms were amplified by PCR from mouse genomic DNA. The pIRES-EGFP vector backbone (Clontech) was used to introduce the 5′ *Tph2* homology arm together with neoR and dsRed2 (dsRedN1 vector, Clontech) genes creating a Tph2-neoR-IVS-IRES-dsRed2-pA expression cassette. An additional SV40-EM7-Bsd cassette (kindly provided by F. Stewart, Technical University, Dresden, Germany) was inserted by homologous recombination in bacteria using Red/ET recombination kit (Genebridges) downstream of the dsRed2 to enable the selection in ES cells. The resulting expression-selection cassette was then flanked by the 3′ *Tph2* homology arm and used for homologous Red/ET recombineering with the *Tph2*-containing BAC RP23–226H2 to elongate the *Tph2*-homology arms. After the successful integration, Tph2-BAC was shaved according to the Red/ET protocol to a 24.6-kb-long plasmid with 14.7-kb 5′ and 3.8-kb 3′ homology regions. The targeting construct was then linearized with I-Sce I and electroporated into ES cells (Bio-Rad Gene Pulser, 800 mV, 3 μF) and clones were picked after 8 days of blasticidin selection. Correct genomic integration of the expression-selection cassette introduced an additional SpeI restriction site, which was used for restriction mapping and Southern blot analysis (Fig. S1*B*). The 298-bp Southern probe used to detect homologous recombination (Fig. S1*A*) was amplified by PCR with primers TPH2 South 5 (5′ CAG GAA GCG CTG GAT CTC C) and TPH2 South 3 (5′ CCA AGC ACG TTT ATG ACT CAG) and labeled with radioactive CTP using Prime-It RmT Random Primer labeling kit (Stratagene).

$Tph2^{+/-}$ ES cells were injected into C57BL/6 blastocysts and transferred into pseudopregnant females. After transmission of the knockout allele from chimera to F_1 generation, $Tph2^{-/-}$ mice were obtained from heterozygous breeding and the line was further maintained on the mixed background by breeding +/− with +/− animals. To obtain such mice on a pure genetic background, we bred F_1 (129/OlaHsd/C57BL/6 background) heterozygous *Tph2*-deficient animals to the inbred FVB/N and C57BL/6 mouse line (Charles River) for 7 and 6 generations, respectively.

Genotyping of animals was performed using PCR with primer TPH34 (5′AGC TGA GGC AGA CAG AAA GG), TPH54 (5′ CCA AAG AGC TAC TCG ACC TAC G), and Neo3 (5′ CTG CGC TGA CAG CCG GAA CAC). The absence of *Tph2*-transcripts in the brain of *Tph2*-deficient animals was confirmed by RT-PCR with primer pairs spanning Ex1–Ex6 (TPH2Ex1.5: 5′ GAT TCT GCT GTG CCA GAA GAT C; TPH2Ex6.3: 5′ GCA AGC ATG AGT CGG GTA GAG) and Ex10–Ex12 (TPH2 Ex10.5: 5′ CCA TCG GAG AAT TGA AGCA; TPH2 Ex12.3: 5′ GTC CTG CAC CAC ATT CTCA).

Animals. Mice were maintained in IVC cages (Tecniplast Deutschland) under standardized conditions with an artificial 12-h dark–light cycle, with free access to standard chow (0.25% sodium; SSNIFF Spezialitäten) and drinking water ad libitum. Local German authorities approved the studies with standards corresponding to those prescribed by the American Physiological Society.

Telemetry experiments, gene expression analysis, brain MRI, immunohistochemistry, HPLC, and behavior studies were performed in adult (12–24 weeks old) *Tph2*-deficient mice, using +/+ and +/− littermates as controls. The genetic background of the animals (mixed, FVB/N-F4, FVB/N-F7, and C57BL/6-F6) used in each experiment is indicated in the text and in the figure legends. However, unless otherwise stated, similar results were obtained with all analyzed backgrounds.

To collect organs for RT-PCR and gene expression analysis, animals were killed by cervical dislocation, and tissues were isolated and immediately snap frozen in liquid nitrogen. For the HPLC analysis, animals were first anesthetized by i.p. ketamine (100 mg/kg) and xylazine (10 mg/kg) injection and perfused with 1× PBS-heparin (5,000 IU/L) to wash out blood. To isolate brain areas, brains were rapidly removed, immediately frozen on dry ice, and stored at −80 °C until use. Various brain areas including frontal cortex, hippocampus, hypothalamus, and striatum, were dissected from the frozen brains on a cold plate (−10 °C).

For in situ hybridization and immunocytochemistry, animals were transcardially perfused first with 1× PBS-heparin and then with buffered 4% paraformaldehyde (PFA). Brains were removed and postfixed in the same buffered 4% PFA overnight at 4 °C.

Biochemical Analyses. For the evaluation of IGF1 levels, blood was taken periorbitally and 25 μL of serum were used for the IGF1 measurement using a commercially available EIA kit (DSL-10-29000, Diagnostic Systems Laboratories).

For the determination of monoamines and their metabolites, GABA, and glutamate, frozen tissues were homogenized in lysis buffer containing 10 μM ascorbic acid and 2.4% perchloric acid, centrifuged for 30 min at 20,000 *g*, and the supernatant was used for the HPLC measurement. The brain tissue levels of serotonin, 5-hydroxyindoleacetic acid, 3,4-dihydroxyphenylacetic acid and homovanillic acid, were analyzed as described previously using HPLC with electrochemical detection (HPLC-ECD) (34). Dopamine and noradrenaline were measured by HPLC-ECD technique with electrochemical detection after extraction to alumina, according to Felice et al. with minor modifications (35, 36). For determination of γ-aminobutyric acid (GABA) and glutamate tissue levels, amino acids were precolumn derivatized with *o*-phthalaldehyde/2-mercaptoethanol using a refrigerated autoinjector and then separated on a HPLC column (ProntoSil C18 ace-EPS, 50 mm × 3 mm i.d.) at a flow rate of 0.6 mL/min and a column temperature of 40 °C. The mobile phase was 50 mM sodium acetate pH 5.7 in a linear gradient from 5% to 21% acetonitrile. Derivatized amino acids were detected by their fluorescence at 450 nm after excitation at 330 nm (37).

Immunocytochemistry and in Situ Hybridization. PFA-fixed brains were incubated in 30% sucrose solution and cryosectioned at 20 μm. The sections were dried after mounting onto SuperFrost Plus (Menzel) slides and directly used for immunohistochemistry, as previously described (38) using rabbit polyclonal anti-5HT primary antibodies (1:1,000; Immunostar) and Cy3-conjugated anti-rabbit IgG secondary antibody (1:500; Jackson ImmunoResearch). Fluorescence images were collected using Axioplan2 imaging microscope and a Sensicam 12BIT camera (Zeiss).

For the in situ hybridization, PFA-fixed brains were embedded in paraffin and sectioned at 8 μm. The sections were deparaffinized, rehydrated, and treated with proteinase K (Roche). Hybridization was performed as previously described (39, 40) using digoxigenin-UTP (Qiagen)-labeled mouse *Tph2* and *Sert* antisense-RNA probes. Pictures were taken with Axioplan2 Imaging microscope/Axiophoto camera (Zeiss).

Telemetry. The telemetric techniques are described in detail elsewhere (41). Briefly, PhysioTel PA-C20 pressure transmitters (DSI) were implanted into the femoral artery and recordings of BP, HR, and locomotor activity were started 10 days after the surgery. RR was calculated from the telemetric blood pressure data using the RespiRATE module of the Dataquest A.R.T software (DSI).

The sleeping time was calculated from the locomotor activity data. An animal was considered ''sleeping'' when it was displaying 0 activity during at least 5 min. The percentage of sleeping over a period was calculated as relation between the number of ''5-min sleeping episodes'' and all 5-min intervals during this period. More than 30 min of immobility was considered an ''extended sleeping period.'' Data for the day/nighttime BP, HR, RR, and sleeping time were averaged from 4 or 5 consecutive days.

To perform the body temperature measurements, PhysioTel TA-F20 transmitters (DSI) were implanted into the peritoneal cavity of 4-month-old female mice (FVB/N-F4 genetic background) and 10 days after surgery temperatures were

recorded. After 2 days of baseline recordings at room temperature (22 °C), animals were subjected to a cold room (4 °C) for 4 h and then returned back to ambient temperature.

Behavioral Studies. For the evaluation of maternal care females of different genotypes were mated for 1 week with wild-type males of the NMRI mouse strain (which are considered to be good breeders). Several days before delivery, females were separated to single cages and were observed every morning for the presence of newborns. The day when pups were born was called day 0. The behavior of mothers and pups survival were recorded daily from day 0 until day 5. For the cross-fostering test, pups from *Tph2*$^{-/-}$ dams were transferred to control mothers and vice versa on day 1 after birth just after the lactation was started. Pups-retrieval test was performed in the home cage, covered with a transparent Plexiglas lid under normal light conditions. Mice were given a 30-min habituation time and thereafter the nest was destroyed and pups were scattered in the cage. Mothers were given 30 min for the construction of a new nest and huddling of the pups. Maternal behavior was monitored with a video camera and analyzed with a suitable software (Biobserve, version 2.2.0.91). ''Hidden cookie'' tests were conducted to check for gross malfunction of the main olfactory system in *Tph2*$^{-/-}$ females. After overnight food deprivation (≈24 h), the time to find a small piece of a food pellet, buried in the fresh sawdust, was recorded for each mouse.

Statistics. Results are expressed as mean ± SEM. Tests of significance (PRISM, GraphPad) were conducted by unpaired Student's *t* test and 1-way ANOVA, followed by Tukey's post hoc test. The survival of animals was analyzed using the Kaplan/Meier method followed by a Logrank test.

SI. Further details may be found in *SI Materials and Methods*.

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