Serotonin deficiency induced after brain maturation rescues consequences of early life adversity

B. Aboagye1,7, T. Weber4,5, H. L. Merdian6, D. Bartsch4, K. P. Lesch1,2,3,8 & J. Waider1,8

Brain serotonin (5-HT) system dysfunction is implicated in depressive disorders and acute depletion of 5-HT precursor tryptophan has frequently been used to model the influence of 5-HT deficiency on emotion regulation. Tamoxifen (TAM)-induced Cre/loxP-mediated inactivation of the tryptophan hydroxylase-2 gene (Tph2) was used to investigate the effects of provoked 5-HT deficiency in adult mice (Tph2 icKO) previously subjected to maternal separation (MS). The efficiency of Tph2 inactivation was validated by immunohistochemistry and HPLC. The impact of Tph2 icKO in interaction with MS stress (Tph2 icKO × MS) on physiological parameters, emotional behavior and expression of 5-HT system-related marker genes were assessed. Tph2 icKO mice displayed a significant reduction in 5-HT immunoreactive cells and 5-HT concentrations in the rostral raphe region within four weeks following TAM treatment. Tph2 icKO and MS differentially affected food and water intake, locomotor activity as well as panic-like escape behavior. Tph2 icKO prevented the adverse effects of MS stress and altered the expression of the genes previously linked to stress and emotionality. In conclusion, an experimental model was established to study the behavioral and neurobiological consequences of 5-HT deficiency in adulthood in interaction with early-life adversity potentially affecting brain development and the pathogenesis of depressive disorders.

Brain serotonin (5-HT) system dysfunction is implicated in disorders of emotion regulation, such as anxiety and depression, viewed as multifactorial conditions influenced by multiple gene-by-gene and gene-by-environment interactions. Previous research identified genetic variants regulating the expression of the gene encoding the rate-limiting enzyme of neuronal serotonin (5-HT) synthesis, tryptophan hydroxylase-2 (TPH2), which converts the essential amino acid tryptophan (TRP) into 5-OH-TRP, the direct precursor of 5-HT.

Depletion of TRP by dietary intervention (acute TRP depletion, ATD) has traditionally been used in assessing the influence of 5-HT deficiency on emotion dysregulation and the pathogenesis of depressive disorders. TRP-free diet produces an acute and profound reduction in brain TRP and consequently brain 5-HT synthesis. ATD decreases anxiety in patients with anorexia nervosa and also lowered mood in healthy participants and individuals with bulimia nervosa. However, the mechanisms of ATD and the impact of reduced brain 5-HT on behavior are still not well understood. Animal model studies provide evidence for alterations in brain 5-HT release following ATD, resulting in altered anxiety- and depression-related behavior.

Genetic manipulation in mice, which results in embryonic inactivation of Tph2 or other 5-HT neuron development-related genes (e.g. Pet1, Lmx1b), provided insight into the behavioral and physiological consequences of 5-HT deficiency in the brain. Several studies showed that neonates with a constitutive Tph2 inactivation present viability and growth-related problems compared to wildtype counterparts. While these mice retain normal 5-HT neuron morphology and physiology, and are indistinguishable from controls in adulthood, several behavioral phenotypes have been reported. These phenotypes arising from lifelong Tph2 inactivation may be the consequence of impaired functioning of other relevant genes, the expression of which is, at least
partly, dependent on 5-HT during brain maturation\(^{21,24}\) and may result in structural and functional differences of the networks involved in emotional processing of mice lacking Tph2\(^{25}\).

Here, we used Tamoxifen (TAM)-induced Cre/loxP-mediated inactivation of the Tph2 to investigate the effects of provoked 5-HT deficiency in the modulation of emotional responses and risk for anxiety disorders and depression in interaction with environmental adversity during brain maturation in adult mice (Tph2 icKO) previously subjected to maternal separation (MS).

### Material and methods

#### Animals.
In this study only male mice were used. They were housed in groups in a controlled environment (12/12 h light/dark cycle, 21 ± 0.5 °C room temperature, 50 ± 5% humidity) with food and water ad libitum. Mice were aclimatized to single housing conditions for ≥ 3 weeks prior to behavioral experiments. Behavioral tests were performed during the light phase between 10:00 and 15:00 with a recovery period of 7 days between different tests. All in vivo animal experiments were performed in accordance with the European Parliament and Council Directive (2010/63/EU) and ARRIVE guidelines. The study was approved by the institutional review board of the University of Würzburg and the Government of Lower Franconia (55.2-2531.01-57/12).

#### Induction of Tph2 inactivation.
For temporal and spatial control of Tph2 recombination, mice homozygous for Tph2 exon five flanked by loxP-sites (Tph2\(^2/0\)) and the palindromic recognition sites of Cre recombinase\(^{26}\) backcrossed onto a C57Bl/6N background were crossed with Tph2 null mutant mice (Tph2\(^{+/-}\)) to generate Tph2\(^{+/-}\)-hemizygous mice, which lack one Tph2 allele from the beginning of their life similar to heterozygous Tph2 knockout mice (Tph2\(^{+/+}\)). These mouse lines were crossed with C57Bl/6N.Tg\(^{phloxER2}\) transgenic mice, which express CreERT2 under the control of the murine Tph2 promoter exclusively in the raphe nuclei, to generate Tg\(^{phloxER2}\)/Tph2\(^{+/-}\) and Tg\(^{phloxER2}\)/Tph2\(^{+/-}\) mice respectively. Tg\(^{phloxER2}\)/Tph2\(^{+/-}\) or Tg\(^{phloxER2}\)/Tph2\(^{+/-}\) females were crossed with non-transgenic females in order to control the number of Tph2creERT2 transgenes (n = 1) in the animals. In order to initiate Cre/loxP-mediated recombination to generate an induced raphe nuclei specific Tph2 knockout (Tph2icKO), male Tg\(^{phloxER2}\)/Tph2\(^{+/-}\) or Tg\(^{phloxER2}\)/Tph2\(^{+/-}\) mice aged 10–12 weeks were injected twice a day for 5 consecutive days with 1 mg of Tamoxifen (TAM; Sigma Aldrich, St. Louis, USA)\(^{27}\) resulting in Tph2\(^{+/-}\)- mice or Tph2\(^{+/-}\)-. In this respect Tph2\(^{+/-}\)- mice model an Tph2icKO based on a hemizygous background, while Tph2\(^{+/-}\)- mice model an Tph2icKO based on a wildtype like genetic background during development. Tg\(^{thMrE}\)/Tph2\(^{+/-}\) mice injected with TAM (Tph2\(^{+/-}\)) were used as controls. Vehicle injected mice of same genotype were used as controls in the maternal separation experiments (Tph2 CON).

#### Immunohistochemistry.
The efficacy of TAM-induced time-specific Tph2 inactivation in the brainstem raphe region was assessed by fluorescence immunohistochemistry in the 4th and 6th week after treatment. Thin sections (30 μm) of brain of mice (n = 7–9/group) were cut on cryostat and stored at ~ 80 °C. Frozen sections were dried for 15–20 min. An antigen retrieval was conducted as previously described\(^{26}\). Sections were cooled down to 40 °C and washed 3 × 5 min in Tris-buffered saline (TBS). Unspecific binding sites for the antibodies were blocked for 90 min at room temperature (RT) with blocking solution (5% normal goat serum, 0.25% Triton-X100 in TBS). Sections were incubated overnight with the primary antibody (1:400, goat-anti 5-HT; Immunostar; Hudson, USA) diluted in blocking solution (TBS-T) at 4 °C in a humid chamber. Following three 5-min washing steps in TBS, sections were incubated in the dark with the respective fluorescent secondary antibodies (1:400 Cy3) diluted in blocking solution, lasting 90 min. Sections were washed 3 × 5 min in TBS. For staining of the cell nuclei, sections were treated with 300 μm DAPI diluted 1:1000 in TBS for 5 min. The number of raphe neurons that were immunoreactive (ir) for 5-HT in dorsal (DRN: B6, B7) and median raphe nuclei (MRN: B5, B8, B9)\(^{26,29}\) based on cell nuclei surrounded by anti-Tph2 signal using Fiji software\(^{30}\) on four consecutive pictures of anterior raphe (DRN and MRN) corresponding to Bregma—4.95 mm to —4.47 mm spaced 180 μm\(^{31}\).

#### Neurochemistry.
For high performance liquid chromatography (HPLC), three brain regions (hippocampus, the dorsal raphe and the amygdaloid complex) were quickly dissected under a stereo microscope. For this, the brain (n = 4–7) was sliced with the aid of a metallic matrix, which allows sectioning at equal intervals on a cold plate. The identified regions were dissected out with a preparation spatula and kept frozen at ~ 80 °C until use. The brain homogenates were prepared and analyzed to standard protocols\(^{19,22}\).

#### Body weight and food intake.
Body weight of mice (n = 18–25) used for the baseline behavioral study was measured weekly for 7 weeks starting from the first week of injection. Body-weight measurements were conducted from 10 to 11 a.m. Two weeks after the last injection, mice were single-housed and their food and water-intake was measured weekly.

#### Behavioral assessment.
Mice were subjected to behavioral testing starting from 4 weeks after TAM injections. In the baseline study, one group of mice (n = 13–15/group) was first tested for anxiety-like behavior in the light–dark transition test (LDT), followed by an open-field test (OFT) to assess locomotor activity in a novel inseparable environment\(^{23}\). A second group of mice (n = 7–11/group) was used to assess anxiety- and depression-like behavior including an elevated-plus maze test (EPM), sucrose preference test and lastly Porsolt swim test (PST)\(^{24}\). Observations were recorded with VideoMot2 (TSE Systems, Bad Homburg, Germany) and later analyzed with EthoVision XT 11.5 (Noldus, Wageningen, The Netherlands). For details see supplementary methods.
Maternal separation. In the second study, pups of the Tg\textsuperscript{Tph2\textunderscore \textalphaRT2/\textalphaTph2}\textsuperscript{Gens}\textalpha\textsuperscript{gen} genotype were separated on the second postnatal day (P2) from their mothers and kept in fresh cages for 3 h daily (between 10.00 and 13.00), for 14 consecutive days (P2-16). Ambient temperature was ensured by infrared light, positioned 70 cm above the cage. Non-MS mice were not separated from the dams but were handled during routine cage changes. Mice were weaned at 25 days after birth and kept in groups of 2 to 5 mice per cage and injected with either TAM (Tph2\textsuperscript{Gens}) or vehicle (CON) (n = 8–10/group). After 4 weeks, mice were tested for anxiety-related behavior in the EPM, LBD and OF followed by SPT and FST for depression related behavior with 3 days inter-trial time, all tests were done as described in supplementary methods.

Quantitative real-time PCR. Quantification of relative gene expression was performed by quantitative real-time PCR (qRT-PCR). cDNA was generated as previously described\textsuperscript{31}. The reaction was run in triplicates using SYBR green dye according to manufacturer instructions. Reaction mixture comprised 6 µl (SYBR green + Primer (F + R)) mix and 4 µl cDNA making 10 µl reaction volume each. Mean efficiencies were calculated by LinReg\textsuperscript{35}. Relative expression data were calculated by \textit{qBase} + (Bioazetta, Zwijnaarde, Belgium), with the normalization factors obtained from geNorm (geNorm M < 0.5)\textsuperscript{36}. Reference genes: glyceraldehyde 3-phosphate dehydrogenase (GAPDH\textsubscript{3}), beta-2 microglobulin (B2m\textsubscript{2}), ubiquitin C (UBC\textsubscript{1}) and ribosomal protein lateral stack subunit P0 (RpLp0). Selected target genes were: tryptophan hydroxylase 2 (Tph2), 5-HT receptor 2a (Htr2a), 5-HT receptor 2a (Htr2a), monoamine oxidase A (Maoa), arginine vasopressin receptor 1a (Avpr1a).

Data analysis. Data obtained from this study was analyzed and displayed using GraphPad Prism version 6.07 for Windows (GraphPad Software, La Jolla California USA, \url{www.graphpad.com}). The total numbers of 5-HT ir cells counted in the anterior raphe of Tph2 icKO were compared with that of Tph2 CON mice using Kruskal–Wallis statistic (H), while Dunn's multiple comparison test was used to compare means between the groups of mice.

Behavioral outcomes on baseline anxiety- and depression-like behavior were analyzed by one-way ANOVA. The course of 5-HT depletion from week 2, 4 and 6 were analyzed by two-way ANOVA as well as MS and gene expression data, with Tukey's multiple comparison post hoc test used to compare means between groups of mice.

Results

Efficiency of induced Tph2 inactivation. The half-life of Tph2 is approximately 2.5 days\textsuperscript{37}. Thus, we first evaluated the time required after induced Tph2 recombination to reduce the amount of Tph2 synthesis to a level similar to constitutive Tph2 KO mice by immunohistochemistry (Fig. 1a). Four weeks after TAM treatment, effective recombination of Tph2 was indicated by a significant drop of 5-HT ir cells in the DRN (Fig. 1b) of Tph2 Δ\textsuperscript{fl} mice. Of note the number of 5-HT ir cells in DRN and MNR of Tph2Δfl mice was increased 5-HT concentrations (p = 0.0783) and 95.0% respectively compared to Tph2\textsuperscript{Δ/−} mice. The concentration of 5-HT in the DRN was decreased in Tph2 icKO after four weeks (p = 0.0006; Fig. 1b, right panel). The number of 5-HT ir cells in the raphe region of Tph2\textsuperscript{Δ/−} (p = 0.0107) and Tph2\textsuperscript{Δfl} (p = 0.0789) mice was reduced by 97.6% and 95.0% respectively compared to Tph2\textsuperscript{Δ+/+} mice. After six weeks following TAM injection, only a few scattered 5-HT ir cells were observed in the DRN (Fig. 1b) in Tph2\textsuperscript{Δ/−} and Tph2\textsuperscript{Δfl} in comparison to Tph2\textsuperscript{Δ+/+} mice (H) = 16.43; p = 0.0003; Fig. 1b, right panel). Tph2\textsuperscript{Δfl} (p = 0.0006) and Tph2\textsuperscript{Δ/−} (p = 0.0023) showed a 93.7% and 92.2% reduction of 5-HT ir cells compared to Tph2\textsuperscript{Δ+/+} mice. Similar to the DRN, the number of 5-HT ir cells in the MRN was decreased in Tph2 icKO after four weeks (H) = 16.65; p = 0.0002) and six weeks after treatment (H) = 7.758; p = 0.0062). Compared to Tph2\textsuperscript{Δ+/+} mice, a significantly reduced number of 5-HT ir cells in Tph2\textsuperscript{Δfl} (p = 0.00053) and Tph2\textsuperscript{Δ/−} (p = 0.0003) was detected by week four. Similar observations were recorded by week six in Tph2\textsuperscript{Δfl} (p = 0.0783) and Tph2\textsuperscript{Δ/−} (p = 0.016), which accounted for 92.7% and 82.1% reduction, respectively (Fig. 1c). Of note the number of 5-HT ir cells in DRN and MNR of Tph2\textsuperscript{Δ+/+} mice did not differ to vehicle treated Tph2\textsuperscript{Δ+/+} mice (Fig. S1).

Neurochemistry. In order to relate the absence of 5-HT ir cells to concentrations of monoamines in the raphe and target brain regions, we measured the concentrations of 5-HT and its metabolite, 5-hydroxyindolacetic acid (5-HIAA), as well as norepinephrine (NE) and dopamine (DA) in the raphe, hippocampus and amygdala at all three time points after TAM induction. Two-way ANOVA revealed a tendency towards significance in the raphe for genotype × time interaction (F\textsubscript{4,40} = 2.299; p = 0.0756). Indeed, from week 2 to 6 Tph2\textsuperscript{Δ+/+} pre-sented increased 5-HT concentrations (p = 0.0032) in the brainstem at a comparable level to wildtype (Tph2\textsuperscript{Δ/−}) controls\textsuperscript{38}, and showed significantly higher concentrations compared to Tph2\textsuperscript{Δfl} (p = 0.016) and Tph2\textsuperscript{Δ/−} mice (p = 0.0783) mice. The concentration of 5-HT in Tph2\textsuperscript{Δfl} and Tph2\textsuperscript{Δ/−} mice was relatively stable at all time points, remaining at low concentrations (67.0 ± 13.6 ng/ml), similar to those reported in constitutive Tph2\textsuperscript{Δ+/+} mice\textsuperscript{19} (Fig. 2a, left panel). Moreover, the concentrations of the 5-HT metabolite 5-HIAA remained consistently low in both genotypes of Tph2 icKO mice at all time points examined. However, two-way ANOVA revealed a significant genotype × time interaction (F\textsubscript{4,40} = 8.157; p < 0.0001; Fig. 2b, left panel), showing higher 5-HIAA in Tph2\textsuperscript{Δ+/+} compared to Tph2\textsuperscript{Δfl} and Tph2\textsuperscript{Δ/−} (both p < 0.0001) only two weeks after injections.

In the hippocampus a significant genotype × time interaction was detected (F\textsubscript{4,40} = 6.493; p = 0.0045; Fig. 2a, middle panel). Similar to the raphe, a rise in 5-HT concentrations in Tph2\textsuperscript{Δ+/+} mice from week 2 to 6 was observed. The amount of 5-HT detected in Tph2\textsuperscript{Δ+/+} was considerably higher than Tph2\textsuperscript{Δfl} (p = 0.017) and Tph2\textsuperscript{Δ/−} (p = 0.013) in week 6. However, in contrast to the raphe, Tph2\textsuperscript{Δfl} had higher concentrations of 5-HT at week 2, which declined until week 6, whereas in Tph2\textsuperscript{Δ/−} mice low 5-HT concentrations were detected at all time points. This provides evidence that recombination of two functional Tph2 alleles requires more time to effect changes in 5-HT concentrations in hippocampal projections. Furthermore, hippocampal 5-HIAA concentrations revealed a significant genotype × time interaction (F\textsubscript{4,40} = 4.776; p = 0.003; Fig. 2b, middle panel). In week
2, $Tph2^{Δ+}$ displayed significantly higher concentrations of 5-HIAA than $Tph2^{Δfl/Δfl}$ and $Tph2^{Δfl/−}$ (both $p<0.0001$) like in the brainstem, which was also detected in week 4 [$Tph2^{Δfl/Δfl}$ ($p=0.0767$); $Tph2^{Δfl/−}$ ($p=0.0258$)] and week 6 [$Tph2^{Δfl/Δfl}$ ($p=0.00683$); $Tph2^{Δfl/−}$ ($p=0.0285$)] also less pronounced.

In the amygdala a significant genotype × time interaction effect was observed ($F_{4,40}=9.316; p<0.0001$; Fig. 2a, right panel). Similar to the other brain regions, 5-HT concentrations in $Tph2^{Δ+}$ were significantly lower compared to $Tph2^{Δfl/Δfl}$ ($p=0.0023$) and $Tph2^{Δfl/−}$ ($p=0.0214$) at week two after induction and increased towards week 6 resulting in higher concentrations of 5-HT than $Tph2^{Δfl/Δfl}$ ($p=0.0004$) and $Tph2^{Δfl/−}$ ($p=0.0044$). However, no
significant changes were detected over time in Tph2 icKO mice, the concentration of 5-HT remained constantly high. Yet, the concentrations of 5-HIAA documented in the raphe, amygdala and hippocampus showed a significant genotype × time interaction effect (F(4,40) = 7.356; p = 0.0002; Fig. 2b right panel). In week 2, Tph2Δ+/+ displayed significantly higher concentrations of 5-HIAA compared to Tph2Δfl/fl and Tph2Δfl/− (all p < 0.0001). Whereas in week 4, no significant differences were observed, Tph2Δ+/+ showed higher concentrations of 5-HIAA than Tph2Δfl/fl and Tph2Δfl/− mice at week 6 (p = 0.0184 and p = 0.0114) in week 6.

For NE two-way ANOVA revealed significant genotype × time interaction effects in the raphe (F(4,40) = 6.327; p < 0.0001; Fig. 2c, left panel), hippocampus (F(4,40) = 9.862; p < 0.0001; Fig. 2c middle panel) and amygdala (F(4,40) = 62.804; p < 0.0001; Fig. 2c, right panel). Post-hoc analyses revealed that in all brain regions Tph2Δ+/+ had lower NE concentrations compared to Tph2Δfl/fl and Tph2Δfl/− (p = 0.0184) and Tph2Δfl/− (p = 0.0114) in week 6.

Only in the amygdala a significant genotype × time interaction was detected for the concentrations of DA (F(4,40) = 2.822; p = 0.038; Fig. 2d, right panel). Post-hoc testing showed lower DA in Tph2Δ+/+ compared with Tph2Δfl/fl (p = 0.0024) and Tph2Δfl/− (p = 0.0837) at week 2. At week 4 DA concentrations in Tph2Δ+/+ dropped significantly compared to week 2 (p = 0.0385). At week 6, no differences between genotypes were detected. Increased DA concentrations in Tph2Δ+/+ at week 6 approached significance compared to week 2 (p = 0.065), indicating a
balancing effect in \( Tph2^{\Delta/-} \) over time. In the raphe (Fig. 2d, left panel) and hippocampus (Fig. 2d, middle panel) DA concentrations remained constant beyond all groups.

**Locomotor hyperactivity but unaltered anxiety- and depression-like behavior in Tph2 icKO mice.** After demonstrating the time course of 5-HT reduction after \( Tph2 \) icKO induction, we investigated anxiety and depressive-like behavior 4–6 weeks after the injections (Fig. 3a). In the OF test, \( Tph2^{\Delta/-} \), \( Tph2^{\Delta/fl} \) and \( Tph2^{\Delta/fl} \) mice put up similar performances in the frequency of visits to the aversive center (\( F_{(2,39)} = 2.431, \))
mental groups showed a statistical tendency towards quantity of water consumed (F(2,61) = 3.059, p = 0.0455; Fig. 3). Indeed, Tph2Δfl mice traveled longer distances than Tph2+/− and Tph2Δfl mice (p = 0.004). Furthermore, a tendency in favor of Tph2Δfl was seen in jumping activity (F(2,129) = 2.944, p = 0.0644; Fig. 3). In the LTD, Tph2Δfl mice did not differ from Tph2+/− mice in all behavioral measures including entries and time spent in the lit compartment as well as total distance traveled (Fig. 3b–d). However, vertical rearing activities varied between groups (F(2,129) = 5.865, p = 0.0058). Here Tph2Δfl mice differed significantly (p = 0.0379; Fig. 3e). In the EPM, Tph2Δfl and Tph2Δfl mice showed similar results regarding the frequency of visits to the open arms (F(2,61) = 0.7034, p = 0.5048; Fig. 3f), time spent on open arms (F(2,61) = 0.5964, p = 0.5587; Fig. 3g) and total distance traveled (F(2,61) = 0.7257, p = 0.4943; Fig. 3h).

Behavioral despair and anhedonia were tested in the FST and sucrose preference test. Tph2Δfl and Tph2Δfl mice did not show any significant variation in latency to floating (F(2,24) = 0.2681, p = 0.7671; Fig. 3i) and duration of immobility (one-way ANOVA, F(2,24) = 0.0737, p = 0.9291; Fig. 3n). Of note, Tph2Δfl mice did not differ in behaviour to vehicle treated Tph2Δfl mice (Fig. S2). Tph2Δfl and Tph2Δfl mice consumed more fluid than Tph2+/− mice (F(2,22) = 5.858, p = 0.0449; Fig. 3o) with Tph2Δfl consuming significantly less fluid that Tph2Δfl (p = 0.0384) and a tendency in comparison with Tph2Δfl mice (p = 0.0992). In the SPT, differences in preference for sucrose were detected (F(2,24) = 5.972, p = 0.0085; Fig. 3p) such that Tph2Δfl (p = 0.0301) and Tph2Δfl (p = 0.0076) preferred sucrose more than Tph2Δfl.

Finally, we assessed whether reduced adult brain 5-HT synthesis affects body weight, food and water consumption in mice. There was no difference in body weight (F(2,64) = 0.3954, p = 0.6751; Fig. 3c). However, experimental groups showed a statistical tendency towards quantity of water consumed (F(2,64) = 3.059, p = 0.0542; Fig. S3b), with Tph2Δfl drinking more water. This is in line with Tph2Δfl consuming a significant higher quantity of food (F(2,64) = 18.98, p < 0.0001; Fig. S3a) than Tph2Δfl (p < 0.0001).

**Anxiety- and depression-like behavior in MS-exposed mice.** Several studies suggested that the functionality of 5-HT in adulthood may be primed by early-life adversity to render an individual susceptible to emotion-related disorders. Therefore, Tg[HphCreERT2/Tph2fl] mice, were subjected to MS stress from P2-P16, injected with TAM in adulthood and tested for anxiety- and depression-like behavior (Fig. 4a).

In the OD test, no MS×treatment interaction in the number of visits to the center (F(1,32) = 2.275; p = 0.1413; Fig. 4h) was detected. Considering the total time spent in the center, a significant MS×treatment interaction effect (F(1,32) = 6.562; p = 0.015; Fig. 4i) was observed. In the Non-MS group, Tph2Δfl mice showed a tendency towards less time in the center (p = 0.077) compared with Tph2 CON. Inter-group comparison revealed that Non-MS Tph2Δfl mice were more anxious than MS-exposed Tph2Δfl mice (p = 0.035). Furthermore, MS exposure potentially increased total distance travelled in the aversive center (F(1,32) = 6.562; p = 0.09; Fig. 4j).

In the LTD, mice were assessed based on their activities in the open compartment of the box. Two-way ANOVA revealed no significant MS×treatment interaction in measures of anxiety (F(1,32) = 0.9622; p = 0.334, Fig. 4b,c) and overall distance (F(1,32) = 0.6689; p = 0.4195) travelled. However, Non-MS mice covered longer distance than MS-exposed mice (F(1,32) = 12.49; p = 0.0013) and Tph2Δfl mice were more active compared to Tph2 CON mice (F(1,32) = 4.672; p = 0.0334).

In the EPM, no significant MS×treatment interaction effect of was observed in visits to open arms (F(1,31) = 0.1966; p = 0.6605; Fig. 4e), total time spent on open arms (F(1,31) = 0.002; p = 0.9644; Fig. 4f) and the overall distance covered (F(1,31) = 0.2576; p = 0.6153). However, MS-exposed mice (F(1,31) = 12.41; p = 0.0014) covered less distance than Non-MS mice, while Tph2Δfl mice (F(1,31) = 5.392; p = 0.027; Fig. 4g) covered longer distance than Tph2 CON mice, independent of MS exposure.

Evaluation of depression-like behavior in the PST in both MS- and Non-MS-exposed mice revealed no behavioral deficits (F(1,31) = 0.3670; p = 0.549; Fig. 4k,l).

Furthermore the SPT showed no significant effects on sucrose preference (F(1,31) = 0.3531; p = 0.5566; Fig. 4m). With respect to total fluid consumed, a significant main effect of treatment occurred (F(1,31) = 20.9; p < 0.0001; Fig. 4n). Tph2Δfl mice consumed more fluid than Tph2 CON mice independent of aversive early-life stress experience. These outcomes indicate that reduction in adult brain 5-HT concentrations may not predispose to lack of pleasure that characterizes anhedonia but rather increases energy metabolism similar to constitutive Tph2 KO mice.

**Gene expression.** The effect of MS-induced anxiety-related behavior on expression of genes, that are viewed as indicators of 5-HT system functionality in the raphe region, hippocampus and amygdala was also examined. Tph2 expression indicated no significant MS×treatment interaction in the raphe region (F(1,31) = 0.1104; p = 0.7419). However, a significant main effect of treatment (F(1,31) = 30.05; p = 0.0001) was apparent (Fig. 5a). Thus, expression of Tph2 was significantly reduced both in Non-MS and MS-exposed Tph2icKO mice which confirms the efficiency of conditional Tph2 inactivation.

The relative expression of Htr1a in the raphe showed a significant main effect of treatment (F(1,31) = 4.345; p = 0.0456; Fig. 5b) and a strong trend in the effect of MS (F(1,31) = 3.597; p = 0.0672; Fig. 5d). No change in hippocampus and amygdala was detected (Fig. S4c).

Relative expression of Htr2a in the raphe region indicated no significant MS×treatment interactions (F(1,31) = 0.3917; p = 0.536) but a significant main effect of MS (F(1,31) = 7.599; p = 0.0097) and a trend towards treatment (F(1,31) = 3.328; p = 0.0778; Fig. 5c). No alterations in hippocampus and amygdala were detected (Fig. S4b).

Assessment of Maoa expression responsible for the degradation of 5-HT in the raphe revealed a general effect of TAM treatment with significantly lower expression in Tph2 icKO than Tph2 CON mice (F(1,31) = 13.39;
Figure 4. Anxiety-like, depression-like and exploratory behavior in MS-exposed Tph2 icKO mice with homozygous genetic predisposition (Tph2Δfl/fl). (a) Timelines for behavior testing; (b)–(d) LDB: light box visits, time in light box, total distance; (e)–(g) EPM: open arms visits, time in open arms and total distance; (h)–(j) OFT: center visits, time in center, total distance; (k),(l) FST: latency to float and floating duration; (m),(n) SPT: total fluid consumed and sucrose preference. Data are shown as mean ± SEM. #0.05 < p < 0.1, **p < 0.01 and ***p < 0.001.
was reported to influence anxiety in humans and rodents34. In this study a significant MS × treatment Avpr1a interaction (F(1, 31) = 5.44; p = 0.0009; Fig. 5d). By contrast, a main effect of MS occurred in target brain areas, such as hippocampus (F(1, 31) = 4.399; p = 0.0442; Fig. S4c, upper panel) and amygdala (F(1, 30) = 3.068; p = 0.09; Fig. S4c, lower panel). Avpr1a was reported to influence anxiety in humans and rodents34. In this study a significant MS × treatment interaction (F(1, 31) = 5.44; p = 0.0263; Fig. 5e) was observed in Avpr1a expression in raphe. Post-hoc analysis revealed Avpr1a expression in MS exposed Tph2 CON mice was higher than MS-exposed Tph2 icKO (p = 0.017). No differences were detected in the Non-MS cohort in both hippocampus and amygdala (Fig. S4d).

**Discussion**

The first part of this study capitalized on the TAM induced Cre-mediated inactivation of Tph2 to significantly reduce 5-HT synthesis in adult mouse brain. A transgenic Tph2-CreERT2 mouse line and the induction protocol described by (Weber et al. 2011) was used to induce a Tph2 knockout in adulthood. Although few 5-HT positive cells in the raphe region remained, which may point to ineffective nuclear translocation of Tph2-CreERT2 probably due to reduced expression of Tph2 in certain 5-HT immunoreactive neurons, we achieved similar recombination efficiencies as reported for other induced Tph2 KO mice using CMV-CreERT2 transgenics as Cre driver line41 or using AAV-Cre viral injection into the rostral raphe nuclei42. Furthermore, measurement of 5-HT concentrations four weeks after TAM induction in the raphe, hippocampus and amygdala revealed efficiently reduced 5-HT concentrations in Tph2 icKO comparable to constitutive Tph2−/− null mutant mice19.

Efficient reduction of brain 5-HT and 5-HIAA within 4 weeks of TAM treatment has previously been reported43, which indicates that the TAM-mediated induction approach requires a relatively long time period before a significant deficiency in brain 5-HT concentration is established. However, the HPLC results showing low concentrations of 5-HT in Tph2Δ+/+ combined with highly increased 5HIAA levels at week 2 differed to previous results of non-injected Tph2Δ+/+ mice in previous studies19. The increased 5-HT turnover in wildtype controls due to the stressful TAM injection protocol may represent a short time adaptive mechanism of the 5-HT system probably similar to restraint stress models44. Although, 5-HT concentrations in Tph2Δ−/− and Tph2Δ+/− mice did not differ in comparison with Tph2Δ+/+ wildtype mice without any injection19. Nevertheless, it seems that it takes at least four weeks in Tph2Δ+/+ mice to recover from the injection stress indicated by increasing 5-HT concentrations in the DRN similar to that of Tph2Δ−/− null mutant mice19.

In line with the above explanation is an early increased concentration of NE in the brain coupled with expected reduction in 5-HT concentrations, which was probably caused by the injection procedure45. This shows that acute stress coupled with reduced 5-HT metabolism has an immediate influence on the NE and dopamine enzymatic activity, which may cross the blood brain barrier and be converted into 5-HT by AADC16,19.

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Figure 5. Expression of genes representing markers of 5-HT system function in the raphe region of MS-exposed Tph2 icKO mice with homozygous genetic predisposition. After behavioral testing maternally separated (MS) and normally reared non-MS Tph2Δfl/fl::Tph2CreERT2 (fl/fl) mice, which were either injected at 10–12 weeks of age with TAM (Δfl/fl) or vehicle (CON) were analysed for differential expression of (a) Tph2, (b) Htr1a, (c) Htr2a, (d) Maoa, (e) Avpr1a. Data are shown as mean ± SEM. #0.05 < p < 0.1, **p < 0.01 and ***p < 0.001.
Tph2 metabolic effect of 5-HT deficiency in investigation of maternal behaviour. MS exposure neither affected hedonic behavior in rats58, nor did it impact tions in 5-HT receptors in target regions of 5-HT neurons have previously been associated with altered anxiety with behavioral alterations, were largely done in rats62–64. Indeed, some C57BL/6 mouse strains appear to be result in anxiolytic and anti-depressive-like effects80,81. Thus, the potential anxiolytic effect observed in MS knockout as well as pharmacological blockade of Avpr1a and this effect was rescued by inducing a Tph2 deficiency. This shows that MS exposure and reduced brain 5-HT and this is present in the dorsal raphe, the mesencephalic central gray and the caudal linear raphe79. Additionally, an G × E interaction on anxiety and depression in mice50,51 and has been shown to impact anxiety-like behaviors in rodents via the 5-HT system52,53. Exposure of wildtype mice, on a mixed c57BL6/J–129S6/Sv background, to MS significantly decreased distance travelled and time spent in the center of the OFT, while MS had no effect in Tph2 KI mice44. Here, we could show that MS produced a similar effect in vehicle injected Tph2Δfl control mice and this effect was rescued by inducing a Tph2 deficiency. This shows that MS exposure and reduced brain 5-HT differentially influence anxiety-like behavior and may compensate each other.

Thus, this study complements numerous studies, which focused specifically on manipulation of Tph2 expression in adult mouse brain and its effect on emotion-related behavior45. Aberrant 5-HT neurotransmission in the adult brain either via pharmacological interventions54,55, or induced gene inactivation42,43 was unable to destabilize behavioral adaptive mechanisms that are established during early brain maturation. Notably, neither MS nor induced adult 5-HT deficiency altered preferences for sucrose solution indicating no differences in hedonic-like behaviors. Furthermore, no alterations in depression-like behavior in the PST were observed in this study. One explanation may be that maternal care on reunion with pups increased and this may have dissolved the MS effect on depression-like behaviors. Thus, it may well be that the MS protocol used was not robust enough to impact depression-like behavior in the mice57 and requires further studies including investigation of maternal behaviour. MS exposure neither affected hedonic behavior in rats58, nor did it impact anxiety- and depression-related behavior in C57BL/6 mice59. Even in TPH2 KI mice, which have reduced brain 5-HT concentrations throughout life, MS exposure did not alter anxiety- and depressive-like behaviors60. By contrast, some studies have associated MS exposure with abnormal behavior and stress induced alteration in neurotransmitter concentration61,62. Noteworthy, most rodent studies, which found an association between MS with behavioral alterations, were largely done in rats62–64. Indeed, some C57BL/6 mice strains appear to be resilient to neonatal MS stress57,59,65–70, which may explain the weak MS effects observed in this study.

However, Tph2 icKO mice consumed more fluid than Tph2 CON mice, which points towards an acute metabolic effect of 5-HT deficiency in Tph2 KO mice71. The observed increase in food, water and percentage sucrose consumption by Tph2Δfl−/− mice has also been reported in Tph2Δ/Δ−/− mice57,71, which reflects an increased energy need, rather than altered anhedonia as a symptom of depressive-like behavior. This supports the assertion that strong reductions in 5-HT metabolism in adulthood are implicated in the pathophysiology of eating disorders through various hormonal and receptor systems72–73, independent of 5-HT functions during development.

Gene expression in raphe, hippocampus and amygdala. Early-life stress does not alter the expression of Tph2 in mice49 and rats52. In contrast, an association between MS exposure and raphe region-specific reduction in Tph2 expression of C56BL/6 J mice has been reported39. Here, the relative expression of Tph2 in Tph2 icKO was significantly lower than Tph2 CON mice in raphe, while MS exposure alone did not alter Tph2 expression.

We detected no differences in expression of Htr1a, Htr2a and Avpr1a in target regions of MS-naïve mice. This confirms our earlier work, which also reporting no change in expression of Htr1a and Htr2a genes in non-stressed Tph2 conditional KO mice72. A slightly altered expression of Maoa in amygdala and hippocampus may point to specific compensatory mechanisms due to MS53. Furthermore, MS exposed mice showed altered expression of Htr1a and Htr2a in the raphe independent of Tph2 icKO, which is in line with previous MS studies57,71. Alterations in 5-HT receptors in target regions of 5-HT neurons have previously been associated with altered anxiety and exploratory behavior76. Here, this may explain the dampening effect of MS on total locomotor activity in OF and EPM.

However, a Tph2 icKO reduced expression of Maoa in the raphe region, pointing towards a direct effect of strong 5-HT depletion on MaoA dependent 5-HT turnover processes. Thus, effects mediated by adverse life experience and associated with altered Maoa expression44 may be prohibited in Tph2 icKO mice through a dysfunctional 5-HT system in adulthood. Interestingly, Avpr1a expression was only affected in the raphe after the mice were maternally separated. We did not find altered Avpr1a expression in the hippocampus, which was correlated after late adverse life experiences with reduced anxiety-like behavior46. Nevertheless, Avpr1a is present in the dorsal raphe, the mesencephalic central gray and the caudal linear raphe76. Additionally, an Avpr1a knockout as well as pharmacological blockade of Avpr1a function in rodents reduced aggression and resulted in anxiolytic and anti-depressive-like effects80,81. Thus, the potential anxiolytic effect observed in MS
Tph2 icKO mice may be directly attributable to altered Avpr1a expression. This highlights the interaction of arginine vasopressin-dependent signaling with the 5-HT system in the brainstem following MS as a potential therapeutic target for treating emotional dysregulation.

In conclusion, our findings establish a ‘double-hit’ experimental model to study the behavioral and neurobiological consequences of 5-HT deficiency in adulthood in interaction with early-life stress experience potentially affecting emotion regulation and the pathogenesis of depressive disorders.

Received: 24 May 2020; Accepted: 22 January 2021
Published online: 08 March 2021

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**Acknowledgements**
This study was supported by the German Research Foundation (DFG: CRC TRR58-A05 to KPL and WA 3446/2-1 to JW), the European Union’s Seventh Framework Program (FP7/2007-2013) under Grant No. 602805 (Agressotype), the Horizon 2020 Research and Innovation Program under Grant No. 728018 (Eat2beNICE), and the 5-100 Russian Academic Excellence Project (to KPL). BA was supported by a research grant of the German Academic Exchange Services (DAAD). This publication was supported by the Open Access Publication Fund of the University of Wuerzburg.

**Author contributions**
J.W., B.A. and K.P.L. designed and supervised the study. J.W. and B.A. performed and analyzed the experiments. T.W. and D.B. contributed mouse lines. J.W., B.A. and K.P.L. wrote the main part of the manuscript. All authors interpreted the results and reviewed the manuscript.

**Funding**
Open Access funding enabled and organized by Projekt DEAL.

**Competing interests**
The authors declare no competing interests.

**Additional information**
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-83592-4.

**Correspondence** and requests for materials should be addressed to K.P.L. or J.W.

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