Dissertation

BEHAVIORAL, NEURONAL, AND DEVELOPMENTAL CONSEQUENCES OF GENETICALLY DECREASED TRYPTOPHAN HYDROXYLASE 2 ACTIVITY

Mathematisch-Naturwissenschaftlichen Fakultät I

VALENTINA MOSIENKO

Präsident der Humboldt-Universität zu Berlin
Prof. Dr. Jan-Hendrik Olbertz
Dekan der Mathematisch-Naturwissenschaftlichen Fakultät I
Prof. Dr. Stefan Hecht

Gutachter/innen:

1. Prof. Dr. Thomas Sommer

2. Prof. Dr. Michael Bader

3. Prof. Dr. Heidrun Fink

eingereicht: 16.04.2013
Tag der Mündliche Prüfung: 17.09.13

Наука является основой всякого прогресса,
облегчающего жизнь человечества и уменьшающего его страдания.
Склодовская-Кюри М.

Science is the basis of every progress,
facilitating human living and reducing suffering
Marie Sklodowska-Curie

Dedicated to my loving parents

ABSTRACT

Serotonin (5-hydroxytryptamine, 5-HT) is a major neurotransmitter in the brain biosynthesis of which is initiated by tryptophan hydroxylase 2 (TPH2). Polymorphisms in the *TPH2* gene are suggested as risk factors associated with depression and anxiety in humans. Furthermore, the most frequently prescribed antidepressants and anxiolytics target the serotonergic system. However, the question whether a complete ablation or partial reduction in brain serotonin leads to the developmental, neurochemical, or psychological abnormalities remains unresolved.

In this study, I took advantage of mouse lines with various degree of decrease in TPH2 activity in order to dissect the impact of 5-HT loss on development, brain neurochemistry and behavior.

Using *Tph2*-deficient mice I showed that central serotonin is essential for normal postnatal, but not prenatal development. Growth retardation of *Tph2*$^{-/-}$ mice was not a result of a disruption of the hypothalamo-pituitary-adrenal axis, metabolic abnormalities, or impaired thermoregulation, but could result from reduced ultrasonic vocalization.

I tested *Tph2*$^{-/-}$ mice along with other newly generated mouse models with partial TPH2 reduction, and showed that 20% reduction in central serotonin is not enough to cause changes in anxiety- and depression-like behaviors most likely due to compensatory mechanisms including reduced serotonin metabolism and increased 5-HT$_{1A}$ receptor sensitivity.

However, complete loss of central serotonin leads to a depression-like phenotype, reduced anxiety-like behavior, and exaggerated aggression, but no differences in activity, olfaction, memory, and adult neurogenesis.

Fluoxetine treatment of *Tph2*$^{-/-}$ mice revealed serotonin-independent action of this antidepressant on anxiety- and depression-like behavior. Furthermore, fluoxetine drastically reduced the brain 5-HT content in mice with low TPH2 activity indicating that TPH2 activity may determine the efficiency of antidepressants targeting the serotonergic system.

Keywords: Tryptophan hydroxylase, Serotonin, Development, Behavior, Fluoxetine

ZUSAMMENFASSUNG

Serotonin (5-Hydroxytryptamin, 5-HT) ist ein wichtiger Neurotransmitter im Zentralnervensystem (ZNS). Seine Biosynthese erfolgt unter Beteiligung des Enzyms Tryptophanhydroxylase 2 (TPH2). Polymorphismen im *TPH2* Gen beim Menschen sind Risikofaktoren bei der Entstehung von Depressionen und Angstverhalten. Die gängigsten Antidepressiva und Anxiolytika wirken auf das Serotonin System. Unklar ist, ob das komplette oder teilweise Fehlen von Serotonin im Gehirn zu Entwicklungsstörungen und neurochemischen oder psychologischen Veränderungen führt.
In dieser Arbeit werden Mauslinien mit unterschiedlichen TPH2 Aktivitäten im ZNS verglichen und der Einfluss verringerter 5-HT Konzentrationen auf Entwicklung und Verhalten der Tiere untersucht. Zentrales Serotonin ist nur für die postnatale Entwicklung notwendig. Das verzögerte Wachstum von *Tph2*$^{-/-}$ Tieren ist nicht auf eine Störung der Hypothalamus-Hypophysen-Nebennieren-Achse oder auf metabolische Veränderungen zurückzuführen, sondern kann aus verringerter Vokalisation im Ultraschallbereich resultieren. *Tph2*$^{-/-}$ Mäuse wurden mit generierten Mausmodellen mit niedriger TPH2 Aktivität vergleichen. Die Ergebnisse zeigen, dass 20% weniger zentrales Serotonin nicht ausreichen, um Depression oder Angst-Verhalten herbeizuführen. Möglicherweise greifen kompensatorische Mechanismen wie ein verringerter Serotoninmetabolismus oder eine gesteigerte 5-HT1A-Rezeptorsensitivität. Der komplette Verlust von Serotonin im Gehirn führt zu einem starken depressiven und weniger ängstlich Verhalten, mit erhöhter Aggression - ohne Veränderung in Aktivität, Geruchsinn, Gedächnis und adulter Neurogenese. Fluoxetine Behandlung von *Tph2*-defizienten Mäusen zeigte einen Serotonin-unabhängigen Effekt dieses Antodepressivums auf Angst-Verhalten und Depression. Fluoxetine reduzieren den Serotoningehalt im Gehirn von Mäusen mit geringen *TPH2*-Aktivität, was zeigt, dass TPH-Aktivität die Effizienz von Serotonin beeinflussenAntidepressiva bestimmen.

Schlagwörter: Tryptophan hydroxylase, Serotonin, Entwicklung, Verhalten, Fluoxetine

Мы должны быть мужественней
и не прекращать своей деятельности от неудач.
Константин Циолоковкий

One should be more manly,
and despite multiple failures should not stop his activities
Konstantin Tsiolkovsky

ACKNOWLEDGEMENTS

First of all I very much like to thank Prof. Dr. Michael Bader and Dr. Natalia Alenina for their brilliant supervision of my PhD project. Without these two people this work would never come out. Michael gave me the possibility to work in his laboratory, but also to perform any kind of experiment. Both of them were diligent advisors in teaching me which questions to ask and how to think in order to answer them. Natasha was both friend and chief during the entire time of my PhD. She had always time for answering my questions, for helping me to solve technical problems arising during the experiments. I would like to thank her for her patience, kindliness, support, all the knowledge and skills she taught me, her never-ending enthusiasm, optimism and energy.

I would like to thank Prof. Dr. Heidrun Fink and Dr. Bettina Bert for supporting our collaborative studies and giving scientific ideas that arose during this collaboration.

Many thanks to the students I worked with during all the time of my PhD, and to all the members of the group, especially to Dr. Michail Todiras, Susann Matthes, Daniel Beis, and Dr. Friederike Klempin for their help in performing some of the experiments, for very valuable scientific discussions, and the extensive support throughout my PhD.

I would like to thank Dr. Herbert Schulz from the research group of Prof. Dr. Norbert Hübner for analysing numerous gene expression data.

I would like to thank Iris Apostel-Krause for her generous help with German bureaucracy, and for the warm atmosphere she is creating in the group.

I would like to thank Sylvia Sibilak for her help regarding the issues about the legal stay in Germany.

I would like to thank Susanna da Costa Goncalves, Thorsten Riepenhausen, Andrea Müller and Lisa Mallis for magnificent technical help in the lab, especially with valuable help in endless animal genotyping, in providing necessary conditions and safety for the experimental work in the laboratory. Special thanks go to Manfred Ströhmann and Sabine Grüger, who took care of the animal colonies on which

this work is based. I would like to thank as well Sabine Schmidt for processing of multiple samples for gene expression analysis.
I would like to thank the Deutsche Akademische Austausch Dienst (DAAD) for financial support during the first 4 years of my PhD. Special thanks go to the International Helmholtz Research School of 'Molecular Neurobiology' without their funding I would have not been able to go to some international conferences, and its coordinators, Dr. Jana Dröse, Dr. Annette Schledz, and Dr. Oksana Seumenicht for their support and endless patience.
I would like to thank my family that supported first of all my decision to make a PhD far away from them. Despite being not around they were and will always be my shelter and the source of positive emotions.
Last but not least, I would like to thank all my friends who were around to hear my (occasionally unreasoned) complains, who gave their advices and shared experiences, with whom I shared my fears and happy moments, and who would take me out to refresh and recharge all my mental energy.

CONTENTS

1 INTRODUCTION

1.1 SEROTONIN - HISTORY, METABOLISM, AND MECHANISMS OF ACTION

1.1.1 *Discovery of serotonin*

Serotonin (5-hydroxytryptamine, 5-HT) was discovered in the middle of the 20th century. Scientists from the Page group were the first to isolate and identify the vasoconstrictive substance from the serum which was released while the blood was clotting [152]. Due to the source, serum, and its action, tonic, this substance encountered the name 'serotonin' [152]. At the same time Erspamer was working on a muscle dilating substance, which he named 'enteramine' [184]. At the end, he found out that 'serotonin' and 'enteramine' are the same factors. A couple of years later, in 1953, Twarog and Page discovered serotonin in the central nervous system [199]. Further serotonin creatinine sulfate was synthesised, bursting the development of new analytical methods to study serotonin metabolism *in vivo* and *in vitro*. Between 1953 and 1970 the processes of serotonin synthesis and degradation were clarified [184]. Moreover, during this time many important drugs interacting with the serotonergic system were discovered. Among them are reserpine (further known as vesicular monoamine transporter (VMAT) inhibitor), monoamine oxidase (MAO) inhibitors (pargyline, phenelzine, tranylcypromine, isocarboxazid), lysergic acid diethylamine (LSD), aromatic amino acid decarboxylase (AADC) inhibitor (carbidopa), tryptophan hydroxylase (TPH) inhibitor (parachlorphenylalanine), and tricyclic antidepressants (imipramine, desipramine, amitriptyline, nortriptyline, protriptyline) [184].

1.1.2 *TPH1 and TPH2: serotonin synthesising enzymes*

The biosynthesis of serotonin is a two-step process. On the first step the essential amino acid L-tryptophan (Trp) is metabolized to 5-hydroxytryptophan (5-HTP) by the rate-limiting enzyme TPH Figure 1. Next, 5-HTP is decarboxylated to 5-HT by the AADC Figure 1. This enzyme is involved as well in the synthesis of dopamine from l-3,4-dihydroxyphenylalanine (L-DOPA) [91], tryptamine from tyrosine [102] or tryptophan [57], and its distribution and functions are not restricted just to the serotonergic system. Thus, the whole process of serotonin synthesis in the brain is limited by two factors: availability of its substrate, Trp, and activity of the TPH enzyme.

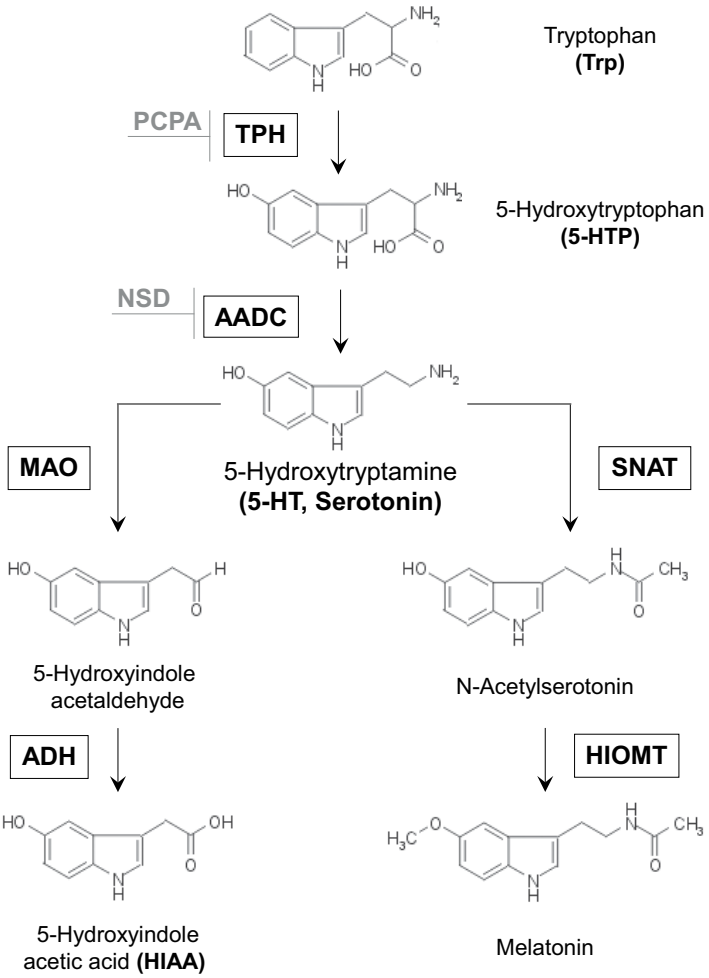

Figure 1: Serotonin synthesis and metabolism. TPH: tryptophan hydroxylase, AADC: aromatic amino acid decarboxylase, PCPA: parachlorophenylalanine (p-chlorophenylalanine), NSD: 3-hydroxybenzylhydrazine dihydrochloride, SNAT: serotonin N-acetyltransferase, HIOMT: hydroxy-indole-O-methyl transferase, MAO: monoamine oxidase, ADH - alcohol dehydrogenase, modified from [136].

TPH belongs to the superfamily of aromatic amino acid hydroxylases that also includes tyrosine hydroxylase (TH) and phenylalanine hydroxylase (PAH). These are iron (Fe^{2+})- and tetrahydrobiopterin (BH_4)-dependent monooxygenases with substantial structural similarities in their catalytic mechanism [65]. All aromatic amino acid hydroxylases are composed of 3 functional domains, a regulatory N-terminal domain, a catalytic domain and a C-terminal oligomerization domain [208, 65].
Since discovery of the serotonin synthesis pathway, it was supposed that in vertebrates only one form of TPH enzyme exists [105]. However, in 2003 the existence of a second isoform, *Tph2*, was unravelled by genetical ablation of the only known *Tph* gene [214, 213]. This spec-

tacular discovery gave new opportunities for studying the functions of two independent serotonin systems — central, driven by *Tph2*, and peripheral, driven by *Tph1*. Follow-up studies of mRNA and protein levels in rodents and human tissues confirmed TPH2 to be the central isoform, which is predominantly expressed in the neurons of Raphe nuclei in the brainstem [78, 155, 213], and in peripheral myenteric neurons in the gut [147, 43], but is absent in peripheral organs like lung, heart, kidney, or liver [173, 231]. On the other hand, TPH1 is mainly expressed in the enterochromaffin cells of the gut and also in the pineal gland, where it produces 5-HT serving as a precursor for melatonin synthesis [185, 213] Figure 1.

1.1.3 *Serotonin metabolism*

In the brain, there are two main serotonin-degradation pathways. In serotonergic neurons, serotonin can be degraded to 5-hydroxyindoleacetic acid (5-HIAA) by one of the forms of MAO — MAO-A Figure 1. MAO-A also metabolises norepinephrine [101]. Furthermore, another form of MAO, MAO-B, metabolises benzamidine [117]. Both enzymes are able to metabolize tyramine and dopamine [101]. Distribution of both isoforms in the brain reflects its substrate specificity [39]. In pituitary gland, retina and epiphysis serotonin serves as precursor in the synthesis of melatonin. First, it is degradated to N-acetylserotonin by 5-HT-N-acetyl-transferase (SNAT). Than the intermediate substrate get processed to melatonin by N-acetyl-5-HT-O-methyl-transferase (HIOMT) [41] Figure 1.

1.1.4 *Formation of serotonergic neurons*

Neurons producing serotonin are located in a restricted region of the brainstem. They are organized in 9 groups of cells that form two clusters according to their location — B1-B5 (corresponding to the raphe pallidus, magnus, obscurus, and pontis) and B6-B9 (corresponding to the dorsal and median raphe nuclei) [212, 121, 47]. The percentage of 5-HT to all neurons in the brain is only 0.0002%. However, extensive innervations of different brain regions and spinal cord allow to regulate a lot of essential physiological functions [121]. In mice, serotonergic neurons start to develop from embryonic day 10 to 12, whereas in primates it happens during the first month of gestation [118]. However, the full maturation of 5-HT neurons in rodents occurs only after birth [121].
Several molecular factors essential for the formation of the 5-HT neurons were identified. Among them *Lmx1b* and *Pet1* are two most essential transcription factors for the last stage of serotonergic neuron differentiation. *Lmx1b* is a LIM homeodomain-containing factor. The name of the family, LIM, was given by the initials of the three pro-

teins **L**inl-1, **I**sl-1, **M**ec-3, in which this homeodomain was first discovered. Its expression is not restricted to only serotonergic neurons, but was found as well in the subthalamic nucleus, substantia nigra, pons, and the dorsal part of the spinal cord [10]. In the mouse hindbrain, *Lmx1b* expression starts at E10.5 and persists during adult life [55, 188]. *Lmx1b* controls the expression of its downstream gene *Pet1*. The expression of the ETS (E26 transformation-specific) domain containing transcription factor *Pet1* (pheochromocytoma) was found primarily in neuronal tissue [70], starting at E12.75 in rat [88] and at E11 in mouse [162]. In the brain, *Pet1* expression is restricted to the raphe nuclei and specifically to the serotonergic neurons [88]. Moreover, a conserved cis-binding site for *Pet1* was found in several genes expressed exclusively in serotonergic neurons such as *Sert* and 5-HT_{1A} receptor [88].

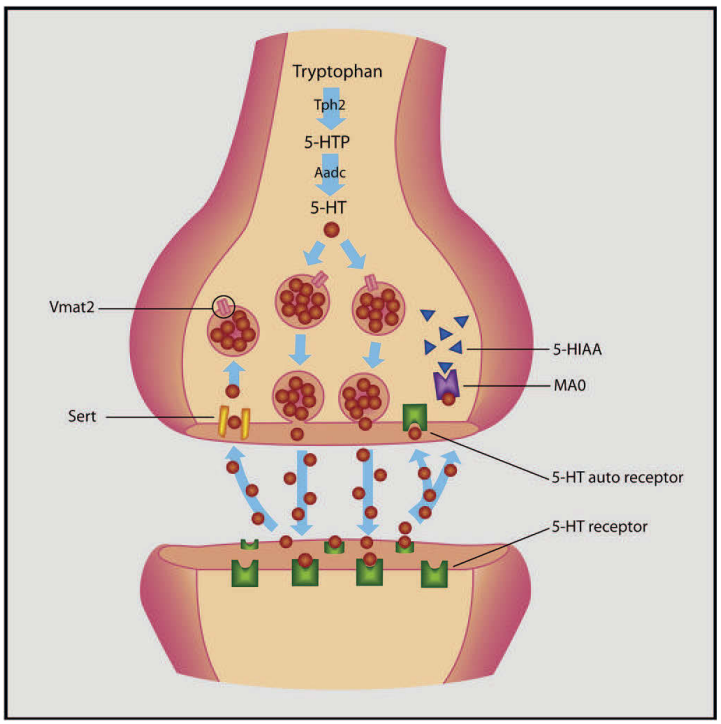

Figure 2: Serotonergic synaptic terminal. Abbreviations: 5-HT: serotonin, 5-HTP: 5-hydroxytryptophan, Tph2: tryptophan hydroxylase 2, AADC: aromatic amino acid decarboxylase, Sert: 5-HT transporter, MAO: monoamine oxidase, 5-HIAA: 5-hydroxyindoleacetic acid, Vmat2: vesicular monoamine transporter 2 [62].

1.1.5 *Serotonergic neurotransmission*

After synthesis VMAT-mediated serotonin packaging into vesicles occurs Figure 2. Similar to the serotonin-synthesising enzyme, VMAT exists in two forms: central — VMAT2, and peripheral — VMAT1 [58, 59]. VMAT2 is expressed in all monoaminergic neurons, thus its

expression is not restricted only to 5-HT neurons.
Once 5-HT is packed into the vesicles it is released into the synaptic cleft via exocytosis upon cell stimulation Figure 2. There it can implement its action on the postsynaptic cell via specific 5-HT receptors. By now, 16 different serotonin receptors have been described that are divided into 7 families by homology, signal transduction pathways, and sensitivity to pharmacological modulations [16, 129]. The most interesting receptor for this study is the 5-HT_{1A} receptor. It is expressed either as postsynaptic receptor in the hippocampus, cortical regions, septum, amygdala, and hypothalamus or as presynaptic receptor in dorsal and median raphe nuclei [114]. 5-HT_{1A} receptor is negatively coupled through G-proteins (α_i) to adenylate cyclase. Thus, due to the negative feedback mechanisms activation of presynaptic receptors causes a decrease in firing of the presynaptic cell, that in term causes a decrease of serotonin release into the synaptic cleft [2, 3, 24]. Stimulation of postsynaptic 5-HT_{1A} receptors causes decrease in firing rates of the neurons on which they are located [24, 189, 193].
Once serotonin completes its action it is reuptaken back into the presynaptic cell with help of the selective serotonin transporter (SERT) Figure 2. Besides the expression of *Sert* in 5-HT neurons, it was found as well in non-serotonergic cells in different structures of the telencephalon, diencephalon, and brain stem [19, 117]. Pharmacological inhibition or blockage of SERT leads to the prolongation of serotonin actions, and is largely used in the treatment of psychiatric disorders [207].

1.2 CENTRAL SEROTONIN AND *TPH2* IN HUMANS

1.2.1 *Mutations in TPH2 and its contribution to psychiatric states in humans*

It has been postulated that reduction in brain serotonin leads to increased depressive and aggressive behaviors. Discovery of a second, brain specific TPH isoform, TPH2, triggered genetic studies on polymorphisms in the *TPH2* gene to assess the implications of central serotonin in the development of neuropsychiatric disorders. Several single nucleotide polymorphisms (SNP) in the *TPH2* gene have been found to be associated with depression [82, 198, 203, 225, 229, 230], suicidal behavior [36, 82, 85, 84, 127, 169, 229, 232] and bipolar disorder [36, 85, 84, 122, 127, 169, 203, 232]. On the other hand a number of publications documented no association between *TPH2* SNPs and major depression or suicide [52, 71, 73, 98, 134, 142]. These inconsistent results may reflect the heterogeneous nature of neuropsychiatric diseases and populations on one side, and methodological differences, sample size and statistical power on the other.
In particular, the non-synonymous SNP (G1463A) published by Zhang

et al. (2005) seemed to be a promising step towards identifying a link between *TPH2* and depressive disorders [224]. The resulting missense mutation in the TPH2 protein (R441H) that they found in an elderly cohort of unipolar depressed patients showed an 80% decrease in 5-HT production in PC12 cells. The fact that the mutation is located within the part of the oligomerization domain that has previously been shown to be pivotal for TPH2 activity [195] together with the description of a corresponding severe pathogenic mutation in PAH (R408W) led to the expectation that the first loss of function mutation in human *TPH2* had been identified [223]. However, this polymorphism could not be found in any other study [22, 23, 53, 71, 74, 90, 158, 202, 228]. Thus, it is probably a rare mutation within a very unique cohort with unipolar depression. Interestingly, the same SNP in *TPH2* was later found as a result of RNA-editing in postmortem brain tissue of humans suffering from psychiatric disorders (drug abuse, schizophrenia, suicide) [75].
As the coding sequence of *TPH2* represents less than 2% of the gene, coding sequence variants are expected to be rather rare [171]. Therefore most of the so far known *TPH2* SNPs are located in introns and promoter regions. Although they are not likely to be of functional importance, they could affect gene expression on the transcriptional level e.g. via mRNA stability or splicing processes [25]. The T allele of the *TPH2* promotor polymorphism rs4570625 (-703G/T) has been shown to be involved in increased prefrontal brain activity [167], anxiety related personality disorders [79] and amygdala reactivity [29, 33]. Therefore it might have an impact on heightened stress responsivity and anxiety due to alterations in *TPH2* expression [191]. Another intronic *TPH2* SNP has been reported to reduce promoter activity by reduced binding of the transcription factor POU3F2 [122, 178]. Evidence for an inhibitory effect of the *TPH2* 5'-UTR on gene expression has been derived from studies on common polymorphisms and haplotypes in this region [35]. From the so far known *TPH2* missense mutations 4 have been reported in patients with clinical symptoms [137]. Nevertheless, there is still a great demand for functional and clinical data to define the role of *TPH2* polymorphisms in particular phenotypes of depressive disorders [81]. Furthermore, inconsistent results have been published for other types of affective disorders. Some studies showed *TPH2* polymorphisms to be related to schizophrenia, obsessive-compulsive disorder (OCD), attention-deficit hyperactivity disorder (ADHD), and autism or panic disorder [40, 106, 141, 182, 211], while others did not observe any association [51, 141]. Concordant response rates from several studies with relatives suggest that antidepressant treatment response is also an inheritable trait possibly influenced by *TPH2* polymorphisms [67, 150]. However, apart from some studies showing a significant link between a *TPH2* SNP haplotype and selective serotonin reuptake inhibitor (SSRI) response

[159, 198, 200] there are again others claiming no association between this trait and *TPH2* SNPs in various ethnic populations [71, 98, 158].

1.2.2 *Mouse as a model for human disorders*

Studies in genetically modified laboratory animals have an important impact on our understanding of psychiatric disorders and on testing new pharmacological tools that could be further used in treating these diseases in humans. Especially valuable in this respect is the mouse — a species in which human mutations can be easily mimicked by genetic modification of its genome. However, the usefulness and validity of mouse models in evaluation of human emotional behavior is always an issue. As there are differences in brain anatomy and capacity for processing complex psychological concepts between humans and mice, it is impossible to model certain aspects of disease symptoms in mice. For depression, for example, it is impossible to show behaviors such as low self-esteem, suicidal ideation or "fear of going crazy". On the other hand, among mammals the brains have a common structural organization, similar circuits connecting these structures, as well as conserved physiological and behavior responses. Therefore, to a certain extent mouse can be used as a model for understanding human behavior and disease, but it always has to be done with caution [45].
However, mouse models with complete gene inactivation are not the only source for studying the influence of certain genes on emotional behavior. In humans a more likely source of phenotypic difference is the accumulation of certain SNPs over generations. Due to extensive inbreeding laboratory mouse strains are good models to study the effect of SNPs on physiological processes and behavioral phenotypes.

1.3 HYPOSEROTONERGIC ANIMAL MODELS

1.3.1 *Tph2-deficient mice*

Since the discovery of *Tph2* mouse models genetically depleted of brain serotonin-synthesising enzyme were created by several laboratories [80, 78, 110, 125, 138, 196, 219]. In Table 1 the data obtained from various studies about the degree of central serotonin reduction, serotonin neuron formation and branching, and changes in other neurotransmitter systems in *Tph2*-deficient mice are summarized. Altogether, dramatic reduction in central 5-HT and no gross abnormalities in 5-HT neuron formation are the main characteristics of mouse models constitutively lacking *Tph2* [4, 80, 78, 110, 138, 176, 219]. However, reports about the state of other neurotransmitter systems in mice lacking *Tph2* are controversial, and indicate either no or mi-

Genetic model	Type/ deficiency	Back-ground	Brain 5-HT levels	5-HT+/Tph2+ neurons (IHC)	5-HT neuronal markers/ properties of 5-HT neurons	*Tph2* expression	Projection of 5-HT neurons	Other neurotransmitter systems/factors	Refe-rence
Tph2$^{-/-}$	constitutive, complete	C57Bl/6, 6th generation		complete absence of 5-HT in the raphe nuclei	normal SERT expression at some, dendrites and terminals of 5-HT neurons		no difference in SERT expression in different brain regions including hippocampus		[80]
Tph2$^{-/-}$	constitutive, complete	mixed	reduction by more than 92% in C, T, Ob, Hip, BS, Str and 68% in cerebellum					no difference in DA, DOPAC, HVA, NE, NM in C, T, Ob, Hip, BS, Str and cerebellum	[175]
Tph2$^{-/-}$	constitutive, complete	C57Bl/6, 6th generation	less than 1-4% is left in the brain	complete absence of 5-HT in the raphe nuclei	mSERT is present in the raphe	no detectable mTph2 expression		no detectable reduction in the whole brain content of NA, glutamate, GABA, DA, DOPAC or HVA	[4]
Tph2$^{-/-}$	constitutive, complete		near complete absence of serotonin in cerebral cortex, cerebellum, Hyp, BS			no detectable mTph2 expression in the brainstem		around 15% increased levels of NE in the whole brain	[218]
Tph2$^{-/-}$	constitutive, complete		complete absence of serotonin	complete absence of 5-HT or TPH2; no TPH2 protein is detected by western blot	unchanged protein levels of SERT and Lmx1b				[195]
Tph2$^{-/-}$	constitutive, complete		reduction by about 15%						[125]
Tph2$^{-/-}$	constitutive, complete	C57Bl/6 (97%)	1,1% in RR, 3,8% in Hip, 6% in FC and 3,7% in T	complete absence of TPH2 in the raphe nuclei	presence of SERT on the plasmatic soma membrane and projecting 5-HT fibers,in Hip; VMAT is present in raphe neurons; In the presence of 10 mM phenylephrine 5-HT neurons show regular firing rate			reduction in NE by 28.3% in RR, 38.6% in Hip, 43.3% in FC and 29.5% in T; DA level reduced by 71,9% in Hip and by 67,7% (relative to *Tph2*$^{-/-}$); reduction in NA neurons by 33.8% in arterior LC, but no difference in posterior LC; no difference in DA cell clusters in SN, VTA and A8	[78]
Tph2$^{Nes-cre}$	constitutive, in all neurons = cells expressing nestin	C57/BL6		some remaining TPH2-positive cells mainly in the dorsal part of rostral raphe region	no difference in the expression of 5-HT1a, 5-HT1b, 5-HT2a, 5-HT2c receptors, serotonin transporter (Sert) in F and PFC, Str, Hip, Hyp, A, rostral and caudal raphe region, and entorhinal cortex	80% and 90% mTph2 reduction in rostral and caudal raphe regions, respectively			[110]
Tph2$^{Pet1-cre}$	constitutive, in 5-HT neurons = cells expressing Pet1	C57/BL6 x 129/SvEMS x FVB/N		complete absence of 5-HT in the raphe nuclei		90% and 95% mTPH2 reduction in caudal and rostral raphe regions, respectively			[110]
Tph2$^{Pet1-icre}$	inducible, in 5-HT neurons = cells expressing Pet1	C57/BL6 x 129/SvEMS x FVB/N		some remaining TPH2-positive cells mainly in the dorsal part of rostral raphe region depending on the amount of tamoxifen injection and on the age of the animals		80% mTph2 reduction in rostral and caudal raphe region			[110]
Tph2::eGFP-/-	constitutive, complete	C57Bl/6, 9th generation	less than 5% in hind- and forebrain	complete absence of TPH2 in the raphe nuclei		no detectable mTph2 expression	normal innervation of primary somatosensory, S1, parietal cortex; reduced innervation in SChN (more than 50%); increased innervation in the NA and hippocampus (more than 50%)	increased mBDNF by 27% in Hip, but unchanged in cortex and PVN.	[138]

Table 1: Central 5-HT levels, serotonergic neurons formation and maturation, and other neurotransmitter systems in *Tph2*-deficient mice
RR: rostral raphe; Hip: hippocampus; FC: frontal cortex; T: thalamus; C: cortex; Ob: olfactory bulb; BS: brain stem; Str: striatum; Hyp: hypothalamus; A: amygdala; PVN: paraventricular nucleus; SN: substantia nigra; DA: dopamine; NE: norepinephrine; DOPAC: 3,4 - dihydroxyphenylacetic acid; HVA: homovanillic acid; NA: noradrenaline; NM: normetanephrine; BDNF: brain-derived neurotrophic factor

nor difference in the levels of other neurotransmitters in the brain [4, 80, 78, 138, 176, 219].

1.3.2 *Mouse models with altered formation of serotonergic neurons*

Besides mice lacking the serotonin synthesising enzyme, TPH2, there are other mouse models with reduced central serotonin levels due to disrupted formation of 5-HT neurons.

Lmx1b-KNOCKOUT MOUSE LINE *Lmx1b*-knockout mice die in utero [55]. *Lmx1b* is responsible for the development of not only serotonergic neurons but also of other neurons in the CNS, and other tissues such as kidney and limb.

$Lmx1b^{Pet1-cre}$ MOUSE LINE Disruption of *Lmx1b* gene in only *Pet-1* expressing cells ($Lmx1b^{Pet1-cre}$) allowed to specifically disrupt the expression of *Lmx1b* in 5-HT neurons [227]. In the brains of adult $Lmx1b^{Pet1-cre}$ mice there are just residual amounts of serotonin. Moreover, there is almost a complete loss of 5-HT neurons in the raphe or their fibers at the target areas [227]. Interestingly, no changes in the amount of DA or NE in the brain of $Lmx1b^{Pet1-cre}$ mice were reported.

$Lmx1b^{Pet1-icre}$ MOUSE LINE Inducible deletion of *Lmx1b* in the *Pet1* expressing cells in the adulthood causes reduction of central 5-HT by 40% and a decrease in the amount of TPH2-positive neurons by 85% [188].

Pet1-KNOCKOUT MOUSE LINE Disruption of another factor important for central serotonin neuron formation, *Pet1*, causes an 80% reduction in the number of 5-HT neurons in the raphe and 85-90% decrease in central serotonin in the cortex, hippocampus, and caudate without significant changes in DA levels [89].

$Pet1^{Pet-icre}$ MOUSE LINE Inducible disruption of Pet1 during 6-8 weeks of adult life ($Pet1^{Pet-icre}$) results in reduction of Pet1 expression by 70% in rostral raphe nuclei [124]. This leads to 50% reduction in *Tph2* expression and a drop of central serotonin levels to 25%. Surprisingly, *Pet1* disruption induced in adulthood did not cause any changes in serotonin content or formation of 5-HT neurons in B1-B3 raphe nuclei. In contrast, after tamoxifen treatment *Pet-1* mRNA was decreased by more than 70% in B5-B9 nuclei [124].

1.3.3 *Mouse models with impaired serotonin storage, release and reuptake*

Mice with disrupted 5-HT storage, release, or reuptake represent another type of hyposerotonergic models.

Vmat2-KNOCKOUT MOUSE LINE Mouse lacking *VMAT2* are not able to store also other monoamines apart from serotonin, and therefore, die within a few days after birth [6, 66, 215].

$Vmat2^{Sert-cre}$ MOUSE LINE. To overcome the lack of specificity *Vmat2* was ablated in SERT-expressing cells ($Vmat2^{Sert-cre}$) [146]. In this mouse model a dramatic decrease by 92-96% (depending on the region) of serotonin was observed in the cortex, striatum, hippocampus, and brainstem. DA levels in the striatum, and NA levels in the hippocampus and cortex were unchanged. However, in the hippocampus of $Vmat2^{Sert-cre}$ mice there was a 17% decrease in NA. There was no detectable 5-HT immunoreactivity in the adult raphe, while the amount of TPH2-positive cells was unchanged [146]. Furthermore, mice lacking *VMAT* in serotonergic neurons show normal fiber formation in cerebral cortex and hippocampus [146].

$Vmat2^{Pet1-cre}$ MOUSE LINE As both *Sert* and *VMAT2* are transiently expressed in a number of glutamatergic neurons and peripheral organs during development [116], it is possible that some aspects of the phenotype observed in $VMAT2^{Sert-cre}$ mice could be due to the deletion of *VMAT2* in these cells during development. To generate a more specific model mice with the raphe-specific inactivation of the *VMAT2* gene were created by using the ePet1-cre mouse line, where Cre is expressed exclusively in raphe neurons in the brain. The brain serotonin level in $VMAT2^{Pet1-cre}$ mice was uniformly decreased by 75% in the cortex, hippocampus, striatum, and brainstem in prenatal E18 embryos, postnatal pups as well as in adults. No changes in DA levels were found.

Sert-OVEREXPRESSING MOUSE LINE Another mouse model with reduced central serotonin levels is a *SERT*-overexpressing mouse [100]. This mouse shows a 2-fold increase in the amount of *SERT* mRNA, and 3-fold increase in the amount of SERT binding sites in the brain. Furthermore, the level of central serotonin decreased from 15 to 30% depending on the region. The extracellular 5-HT levels are reduced by 50-60% [100].
Interestingly, brain serotonin levels in mice lacking *SERT* are dramatically decreased. Thus, one might consider it as one of the hyposerotonergic mouse model. However, microdialysis studies revealed elevated extracellular 5-HT levels in the brain [135].

5-HT$_{1A}$ RECEPTOR RAPHE-OVEREXPRESSING MOUSE LINE 5-HT$_{1A}$ receptor expression in raphe nuclei is more than 10 times elevated in 5-HT$_{1A}$ receptor raphe-overexpressing mice (5-HT1A^{RO}) [11]. These mice have a 25% decrease in 5-HIAA/5-HT ratio in the whole brain lysate when compared to controls [11]. However, in the original and follow up studies extracellular 5-HT levels in different brain regions were not studied in these mice [11, 12, 34].

1.3.4 *Mouse models with polymorphisms in Tph2 gene*

It is known that certain mouse strains markedly differ in manifestation of different behavior traits. Zhang et al. first surmised that the difference in depression-like behavior between 129X1/SvJ and BALB/cJ mice may be caused by altered TPH2 activity [224]. Indeed, sequencing analysis revealed a (C1473G) single-nucleotide polymorphism in the coding region of *Tph2* between these two strains resulting in the substitution of a highly conserved proline (Pro) residue with an arginine (Arg) at position 447. Consequently, it led to lowered enzyme activity *in vitro* [172, 224]. Moreover, 5-HT content in the brain was reduced by approximately 50% in the mouse strains carrying only the 1473G allele in comparison to mice homozygous for the 1473C allele, which was also confirmed in an F2 intercross [172, 224].
However attempts to create congenic lines that differ only in the mTPH21473 polymorphic allele revealed controversial results. Osipova et al. showed that transfer of the 1473G allele into the C57Bl/6J genome over 3 generations (B6-1473G (G/G)) significantly decreased TPH2 activity in the brain [151]. Another study instead revealed that breeding the 1473G allele from DBA/2 mice over eight generations to the C57Bl/6 background did not result in difference in the 5-HT content in brain regions [194]. Recently in a third study it was shown that backcrossing 1473G polymorphism to C57BlB/6 background over 10 generations results in unchanged serotonin content either in the forebrain, midbrain, or in the whole brain [20]. Moreover, extracellular levels of 5-HT in dorsal and medial raphe nuclei were not different between C57Bl/6 mice carrying either 1473C or 1473G alleles [20].
At last, Beaulieu et al. generated knockin mice expressing a mutant form of *Tph2*. This mutation was engineered equivalent to the human R441H mutation found to be associated with major unipolar depression [17]. TPH2 activity in R439H TPH2 knockin mice was reduced by 80% in the striatum, cortex and hippocampus, while the expression of *SERT* was unchanged [17]. Extracellular 5-HT levels in frontal cortex and hippocampus were decreased by more than half in these *Tph2* knockin mice [99].

2

AIMS OF THE STUDY

Serotonin (5-hydroxytryptamine, 5-HT) was given a close attention since its discovery due to its involvement in the pathology of diverse psychiatric disorders and drug action. However, lack of suitable pharmacological or genetic tools to specifically influence the central but not the peripheral serotonin system did not allow drawing final conclusions about the processes that are directly influenced by serotonin in the brain.
In this study I took advantage of newly generated mouse models with complete or partial reduction in activity of TPH2, the enzyme responsible for central serotonin synthesis, to investigate the impact of this neurotransmitter on development, brain neurochemistry, and beha9vior.

Using these animal models I aimed at clarifying the following specific questions:

1. What is the consequence of life-long brain serotonin depletion on mouse prenatal and postnatal development?

In order to answer this question I assessed pathways involved in growth retardation of *Tph2*-deficient pups. In particular, functionality of the hypothalamo-pituitary-adrenal axis, lipid metabolism, thermoregulation, and the behavioral response to isolation were studied during the first postnatal weeks.

2. Does partial inactivation or complete depletion of TPH2 change central 5-HT levels and behavior?

To determine the role of brain serotonin in behavior first mice lacking *Tph2* were tested for alterations in general activity, olfaction, memory formation, adult neurogenesis, aggressive, anxiety-, and depression-like behaviors. To compare complete absence with partial inactivation of TPH2 anxiety- and depression-like behavior in mice with decreased TPH2 activity were evaluated. To test for potential compensatory mechanisms I checked 5-HT_{1A} receptor function and serotonin metabolism in the brains of hyposerotonergic animal models. In addition, the ability of selective serotonin transporter inhibitor, fluoxetine, to exert its actions on anxiety- and depression-like behaviors as well as brain neurochemistry in the partial or complete absence of serotonin was assessed.

3

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 *Reagents*

Name	Company, city
Chemicals	
5-HIAA	Sigma, Steinheim
5-HT	Sigma, Steinheim
5-HTP	Sigma, Steinheim
Acetic acid	Roth, Karlsruhe
Agarose	Lonza, Switzerland
Ascorbic acid	Sigma, Steinheim
Bromophenolblue	Sigma, Steinheim
DAPI	Sigma, Steinheim
DEPC	Serva, Heidelberg
DNaseI	Boehringer Mannheim, Mannheim
dNTPs	Amersham Bioscience, NJ, USA
Donkey serum	Sigma, Steinheim
DTT	Sigma, Steinheim
EDTA	Sigma, Steinheim
Ethanol	Roth, Karlsruhe
Ethidiumbromid	Sigma, Steinheim
First strand buffer	Invitrogen, Darmstadt
Glucose	Sigma, Steinheim
Glycerol	Roth, Karlsruhe
HCl	Roth, Karlsruhe
KCl	Roth, Karlsruhe
MgCl2	Roth, Karlsruhe
M-MLV	Invitrogen, Darmstadt
NaCl	Roth, Karlsruhe
NaOH	Roth, Karlsruhe
PCA	Sigma, Steinheim
PFA	Fischar, Germany
Proteinase K	Invitrogen, Darmstadt
Random Hexamer Primer	Boehringer, Mannheim
Rnase A	Promega, Madison, WI
RNasin	Promega, Madison, WI
SDS	Serva, Heidelberg
Sucrose	Roth, Karlsruhe
Taq polymerase	Invitrogen, Darmstadt
TissueTek	Sakura, Netherlands
Tris	Roth, Karlsruhe
Triton X-100	Sigma, Steinheim
Trizol	Invitrogen, Darmstadt
Tween 100	Sigma, Steinheim

Reagents used for behavior tests	
Chocolate cookie	Bisquiva GmbH, Germany
Powder milk	Marken-discount AG&Co.AG, Germany
Vanillin	Sigma, Steinheim
Diets	
High fat diet (45% fat)	Ssniff, Soest
Normal chow (0.25%sodium)	Ssniff, Soest
Reagents used for animal treatment	
5-HTP	Sigma, Aldrich
8-OH DPAT	Tocris-bioscience, Wiesbaden-Nordenstadt
BrdU	Sigma, Aldrich
fluoxetine	Ratiopharm, Ulm
hIGF-1	Cellsciences, Canton MA
NSD-1015	Sigma, Aldrich
tryptophan	Roth, Karlsruhe
WAY 100635	Tocris-bioscience, Wiesbaden-Nordenstadt

Table 2: Chemicals, reagents, enzymes and animal diets used in the study

3.1.2 *Kits and Markers*

Name	Company, location
100 bp Marker	Biolabs, USA
ABC Kit	Vector laboratories, USA
Affymetrix 1. ST	Affymetrix, USA
IGF-1 ELISA	Diagnostic System Laboratories, USA
Leptin ELISA	R&D System, UK
RNeasy Mini Kit	Qiagen, Hilden
RT2 qPCR primer assay for Tph2	Sabioscience, USA
WT expression and labelling KIT	Qiagen, Hilden
Φ174 DNA/*BsuRI* (*HaeIII*) Marker, 9	Fermentas, Burlington, CDN

Table 3: Kits and markers used in the study

3.1.3 *Antibodies*

Antibody	Dilution	Company, location
Primary		
Growth hormone, rabbit	1:1000	Millipore, Germany
BrdU, rat	1:500	Biozol, Germany
Secondary		
anti-rabbit AlexaFluor488	1:1000	Invitrogen, Germany
anti-rat biotin-SP	1:500	Jackson ImmunoResearch, USA

Table 4: Primary and secondary antibodies used in the study

3.1.4 *Equipment and expandable material*

Name	Company, location
8 channel pipette	Biohit, Rosbach v.d Höhe
Accu Check	Roche, Manheim
AlphaImager (UV)	Alpha Innotech, Germany
Automatic Pipette Witoped XP	Witeg Labortechnik GmbH, Wertheim
Camera	Panasonic, Japan
Centrifuge 5415C	Eppendorf, Hamburg
Centrifuge Sorvall RC 5C	Heraeus, Hanau
Cryostat	Leica, Germany
Falcon tubes TPPR	Trasadingen, Switzerland
FastPrep	MP Biomedicals, France
Fine balance	Kern&Sohn GmbH, Germany
Fluorescent microscope	Keyence Corp., USA
Incubator	Binder, USA
InfraMot	TSE systems, Bad Homburg
Microwave 8020	Privileg, Fürth
Nanodrop	Thermo Scientific, USA
PCR tubes	Biozym Scientific GmbH, Oldendorf
pH Meter	pH Level 1 WTW, Weilheim
Pipettes Gilson	Disposable pipettes CellstarR 1, 2, 5, 10, 25ml
Power supply for the gel chamber	Appligene, France
Real time PCR machine	Biosystems, USA
SasLabPro	Avisoft BioAcustics, Germany
Superfrost Plus slides	Menzel Gläser, Braunschweig
Telemetry transmitters	Data Science International (DSI), USA
Thermometer	Thermo Fischer Scientific, USA
Thermomixer 5437	Eppendorf, Hamburg
USV microphone	Avisoft BioAcustics, Germany
Viewer^2	Biobserve, Bonn
Vortex: VibroFix Janke & Kunkel	IKA, Germany
Water bath	GFL, Burgwedel

Table 5: Equipment and expandable materials used in the study

3.1.5 *Primers*

Primers used for qPCR

gene of interest	primer name	primer sequence
SERT	mSERT_fw_1	GGACAAAGAGGACTGCCAAG
	mSERT_rev_1	GGCATAGCCAATGACAGACA
TBP	TBP_fw	CCCTATCACTCCTGCCACACC
	TBP_rev	CGAAGTGCAATGGTCTTTAGGTC

Primers used for animal genotyping

region of interest	primer name	primer sequence	annealing temperature	agarose gel, %
Tph2 deletion	TPH34	5'AGC TGA GGC AGA CAG AAA GG	55^0C	2
	TPH54	5' CCA AAG AGC TAC TCG ACC TAC G		
	Neo3	5' CTG CGC TGA CAG CCG GAA CAC		
Tph2 1473G allele	control for	5' TTT GAC CCA AAG ACG ACC TGC TTG CA	65^0C	2
	control rev	5' TGC ATG CTT ACT AGC CAA CCA TGA CAC A		
	G allele	5' CAG AAT TTC AAT GCT CTG CGT GTG GG		
Tph2 1473C allele	control for	5' TTT GAC CCA AAG ACG ACC TGC TTG CA	65^0C	2
	control rev	5' TGC ATG CTT ACT AGC CAA CCA TGA CAC A		
	C allele	5' CAG AAT TTC AAT GCT CTG CGT GTG GC		

Table 6: Primers used in the study

3.2 METHODS

3.2.1 *Molecular biology methods*

DNA ISOLATION FROM TAIL/EAR BIOPSIES For genotyping of experimental animals DNA was isolated from tail or ear biopsies. The tissue was incubated overnight at 55°C in 100 µl of the ear buffer containing 1 mg/ml Proteinase K. Next the tissues were incubated 10 min at 95°C in order to inactivate Proteinase K. Thereafter 750 µl of TE/RNase buffer were added to the sample in order to digest RNA. After 15 min incubation at the room temperature (RT), 2 µl of genomic DNA was used as a template for the PCR reaction.

DNA ISOLATION FROM HAIR FOLLICLES For some mice genotyping needed to be performed more than once. To minimize the stress of repeated ear or tail biopsies genotyping of animals was performed from the hair follicles. A small bundle of fur together with the follicles was collected. Hair roots were cut off and incubated 55°C overnight in 60 µl of hair buffer with 3 mg/ml proteinase K (prepared first as stock 20 mg/ml in Tris-HCl, pH=7.5). Thereafter the same steps as for DNA isolation from ear/tail biopsies were preformed.

Ear buffer
100 mM Tris pH 8.5
5 mM EDTA
200 mM NaCl
0.2% SDS

TE/RNase-buffer
20 µg/ml RNase A in 1x TE buffer

TE buffer
10 mM Tris-HCl pH 8.0
1 mM EDTA

Hair buffer
10 mM Tris pH 8.3
50 mM KCL
0.5% Tween

RNA ISOLATION RNA from the whole brain was extracted with Trizol reagent. 100 mg of the organs were diluted in 1000 ml of Trizol and homogenized with Fast Prep. Further steps were performed according to manufacture protocol. RNA from the hypothalamus was isolated with the RNeasy Kit according to the manufacture protocol. Independent from the method of isolation residual genomic DNA was removed by DNase I treatment.

DETERMINATION OF NUCLEIC ACID CONCENTRATION DNA and RNA concentrations were determined by optical density (OD) 260 compared to OD280 in a spectrophotometer (Nanodrop). When OD 260/280 > 1.7, the protein concentration can be neglected and the concentration of nucleic acids was determined by the following ratio:
DNA OD260 = 1 = 50 µl/ml for double stranded DNA
RNA OD260 = 1 = 40 µl/ml
When OD260/OD280 < 1.7 the protein concentration of the solution is too high and requires additional purification steps (phenol-chlorophorm extraction or purification on a column).

STORAGE OF NUCLEIC ACIDS For long-term storage DNA was diluted in DNase-free water or TE buffer and kept at -20°C, RNA was diluted in DEPC or RNase-free water from QIAGEN, and kept at -80°C. Primer stocks were diluted in DNase- and RNase-free water, and kept at -20°C. Before performing PCR the stock solutions were diluted to working concentration in ddH_2O and kept at -20°C.

DEPC water
0.1% DEPC in ddH_2O
overnight mixing at 37°, than autoclaved

SEPARATION OF NUCLEIC ACIDS ON AGAROSE GEL DNA molecules were separated by length using 1-3% (w/v) agarose gels, containing 0.5 µg/ml ethidium bromide. DNA was mixed with 0.1V of 10 x Loading buffer, applied on agarose gel chambers and electrophorised at 1-8 V/cm in 1x TAE buffer. DNA was visualized under 300 nm UV light. The size and the approximate concentration of the DNA

bands was determined by comparison with standardized molecular weight markers (ϕX174 DNA/BsuRI (HaeIII) and 100bp ladder).

TAE buffer
40 mM Tris-Acetate pH 7.4
1 mM EDTA

10 x DNA Loading buffer
40% Sucrose
0.02% Bromphenolblue
in TE buffer

REVERSE TRANSCRIPTION (RT) The synthesis of cDNA from total RNA was done using M-MLV first strand synthesis Kit. 1 µg of total RNA was mixed with 1 µl of Random Hexamer primers, 1 µl 10 mM dNTPs and 0.5 µl RNasin in 12.5 µl volume. RNA was denaturated for 10 min at 60°C and quickly chilled to 4°C. Then 7.5 µl of the following mix was added:

5x first strand buffer 4 µl
0.1 M DTT 2 µl
RNasin (40 u/ml) 0.5 µl
M-MLV (200 u/µl) 1 µl

The reaction was incubated for 40 min at 37°C. Than M-MLV was inactivated at 80°C for 10 min, afterwards the mix was kept on ice until 2 µl were used for PCR reaction.

MOUSE GENOTYPING For the genotyping the following PCR reaction was set using TaqPolymerase:

genomic DNA 2µl
10x PCR buffer 5 µl
50 mM $MgCl_2$ 2.5 µl
10 mM dNTPs 1 µl
10 mM primer 1 1 µl
10 mM primer 2 1 µl
10 mM primer 3 1 µl
Taq polymerase (5u/ml) 0.5 µl
ddH_20 36 µl

The reaction was kept on ice before transferred to preheated PCR machine. The program used for genotyping:

Denaturation 5 min 92°C
Denaturation 45 sec 92°C

Annealing 30 sec at further specified °C for certain primer pairs
Elongation 30 sec 72°C
Repeat 34x
Final elongation 10 min 72°C
Forever 10°C

Genotyping of animals was performed using primer combinations specified in table Table 7. Information about the primer sequences, annealing temperatures, and percentage of agarose gels used for DNA separation are summarised in table Table 6.

PCR	Bands/primers	Band length, bp
Tph2	**WT band** TPH54 TPH234	568
	KO band TPH54 Neo3	401
G/G	**control allele** Control for Control rev	523
	G allele Control for G allele	307
C/C	**control allele** Control for Control rev	523
	C allele Control for C allele	307

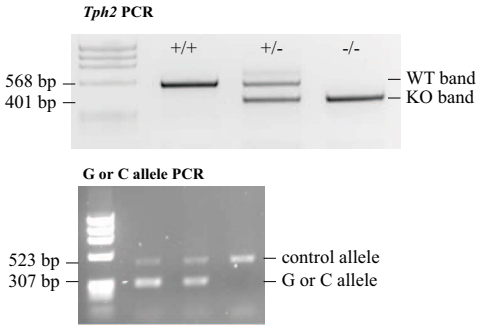

Table 7: Primers used for genotyping of mice

QUANTITATIVE REAL TIME PCR To quantify relative gene expression quantitative (real-time) polymerase chain reaction (qPCR) was performed. Real-time PCR was run in a technical triplicate using SYBR green reagent according to manufacturer protocol in a 384-well plate format. For the qPCR reaction 9 µl of 2 ng/µl cDNA was used. The expression of the *Tph2* gene was quantified using RT^2 qPCR primer assay, the expression of *SERT* — using primers listed in the Table 6. Expression of gene of interest was normalized to TATA-binding protein (TBP) mRNA expression. Oligonucleotides were custom made by Invitrogen. The method of Livak and Schmittgen [126] was applied to compare gene expression levels between groups, using the equation $2^{-\Delta\Delta CT}$.

MICROARRAY ANALYSIS For the analysis of gene expression pups at the day of birth were sacrificed by cervical dislocation. Brains were rapidly removed, hypothalamus was isolated, and then snap frozen. Isolated and cleaned from genomic DNA RNAs were fragmented and labeled ssDNAs with WT expression and Terminal labeling kits. The labeled RNA was hybridized against the mouse gene 1.0 ST Array. Differential expression of genes was calculated using Partek ANOVA statistic followed by FDR multiple testing corrections. Resulting p-values and FDR values indicated the probability of differential expression between the different conditions.

3.2.2 *Determination of plasma parameters*

MEASUREMENTS OF PLASMA HORMONE LEVELS IGF-1 and leptin levels were evaluated at postnatal day 35. Animals were anesthetized by an intraperitoneal (i.p.) injections of ketamine (100 mg/kg) and xylazine (10 mg/kg). Blood was taken transcardially. 25 µL of serum were used for IGF-1 measurement using a commercially available EIA kit. 50 µl of serum was used for the leptin measurement using a commercially available Leptin Kit.

GLUCOSE MEASUREMENTS Plasma glucose levels were measured with the glucometer according to manufacturer protocol. To obtain a drop of blood 1 mm of the mouse tail was cut with scissors.

3.2.3 *Staining*

GROWTH HORMONE IMMUNOHISTOCHEMISTRY For the analysis of pituitary glands in 2-day old *Tph2*$^{-/-}$ mice, the tissue was postfixated in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer for 4 hours followed by 2 hours 30% sucrose. After a short wash in PBS heads were embedded in Tissue-Tek O.C.T compound and cryosectioned in 14 µm coronal sections. Sections were dried for 2 hours at room temperature and then stored at -80°C. For immunohistochemistry, tissues were washed in PBS followed by PBST, and blocked with 10% normal donkey serum. Slides were incubated overnight at 4°C with polyclonal rabbit anti-growth hormone. Anti-rabbit IgG secondary antibody conjugated with Alexa-Fluor488 was applied the next day. Fluorescence images were collected using a fluorescence microscope.

<u>10 x PBS</u>
for 1L ddH_2O
80 g NaCl
2 g KCl
14.4 g Na_2HPO_2
2.4 g KH_2PO_2
pH to 7.4

<u>PBST</u>
0.1% Triton X-100 in 1xPBS

BRDU LABELLING AND QUANTIFICATION Mice were deeply anaesthetized with isoflurane and perfused transcardially with 0.9% sodium chloride followed by 4% PFA in 0.1 M phosphate buffer. Brains were removed from the skulls, postfixed in 4% PFA at 4°C over night, and

transferred into 30% sucrose. Sequential 40 µm coronal sections were cut on a microtome and cryoprotected in cryo protection solution (CPS). For BrdU staining, DNA was denatured in 2 N HCl for 20 minutes at 37°C. Sections were then rinsed in 0.1 M borate buffer and washed in Tris-buffered solution (TBS). Sections were stained free floating with antibodies diluted in TBS containing 3% normal donkey serum and 0.1% Triton X-100. Primary anti-BrdU antibodies were applied in the concentration 1:500. Immunohistochemistry followed the peroxidase method with biotinylated secondary antibodies, ABC Elite reagent and diaminobenzidine (DAB) as chromogen. For BrdU labeling, one-in-six series of sections of each brain were DAB stained, and immunoreactive cells were counted throughout the rostra-caudal extent of the dentate gyrus. Results were multiplied by six to obtain the total number of BrdU-positive cells per dentate gyrus.

0.1 M phosphate buffer
for 1L ddH_2O
13.73 g Na_2HPO_2
3.18 g NaH_2PO_2

0.1 M borate buffer
for 480 ml ddH_2O
3.08 g Boric Acid
5 N NaOH
pH 8.5

10x Tris buffer solution (TBS)
for 1710 ml ddH_2O
180 g NaCl
264.40 g Tris HCL
38.80 Tris base

Cryo protection solution (CPS)
250 ml 25% Ethylenglycol
500 ml 0.1 M PO4

OIL RED O STAINING Aliquots of fresh stool samples collected prior staining were homogenised after adding water in a volume of 10 µl/mg stool. After centrifugation (200g, 5 min) 5 µl of supernatant was applied to a glass slide, mixed with 5 µl of staining solution, and examined by light microscopy [83]. For preparing the stock of staining solution 0.3 g of Oil RedO was dissolved in 100 ml of isopropanole. It was stirred overnight, filtered and stored at 4°C. Prior to staining two parts of distilled water was added to three parts of the stock solution of Oil RedO [109].

3.2.4 *Animals*

GENERAL ANIMAL HANDLING All animal procedures were in accordance with the ethical principles and guidelines for care and use of laboratory animals adopted by German local authorities corresponding to the standards prescribed by the American Physiological Society. Mice were maintained in individually ventilated (IVC) cages, 34x19x13 cm at an ambient temperature of 22°C, under a standard light/dark cycle from 7 am to 7 pm, with free access to standard chow and drinking water ad libitum.

ANIMAL BREEDING *Tph2*-deficient mice were on two genetic backgrounds, C57Bl/6 and FVBN. To obtain *Tph2* gene-deleted mice on a pure genetic background, heterozygous *Tph2*-deficient animals on C57Bl/6 background (6th generation) [4] were bred for further four generations to C57Bl/6 mice (Charles River, Germany). $Tph2^{-/-}$ mice on a FVB/N background were obtained by backcrossing heterozygous *Tph2*-deficient animals with FVB/N mice (Charles River, Germany) for 12 generations backcross (F12).

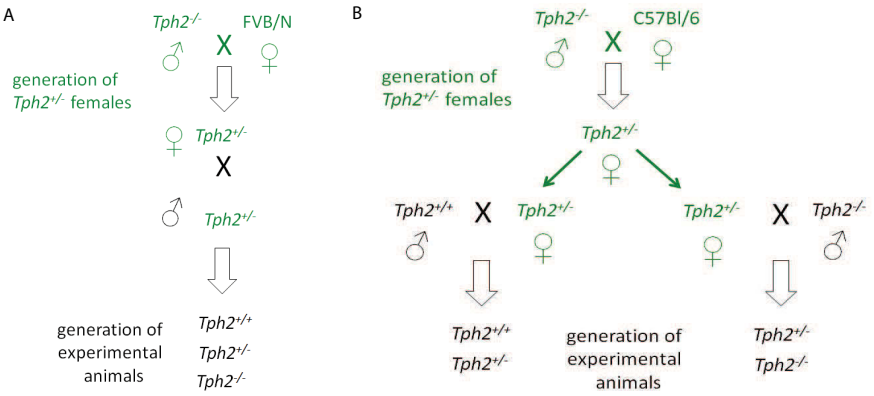

Figure 3: Breeding scheme used for obtaining $Tph2^{-/-}$, $Tph2^{+/-}$, and $Tph2^{+/+}$ animals for A). pups USV, experiments at 30°C, foot print test, and activity measurement at postnatal day 35 and for B). all the behavior, neurochemical and metabolic experiments

The pups USV, experiments at 30°C, foot print test, and activity measurement at postnatal day 35 were studied in mice on FVB/N background, derived from breeding $Tph2^{+/-}$ male mice with $Tph2^{+/-}$ female mice Figure 3(A). All the other experiments were performed in mice on C57Bl/6 background, obtained by breeding $Tph2^{+/-}$ female mice either with $Tph2^{-/-}$ or with $Tph2^{+/+}$ male mice Figure 3(B). The study of activity, muscle strength, anxiety- and depression-like behavior, aggression, memory, and brain neurochemistry was performed in 18-22 weeks old male mice. Neurogenesis was studied in female

Tph2$^{-/-}$ and *Tph2*$^{+/+}$ mice at the age of 32-37 weeks of age. The olfactory habituation-dishabituation test was performed in 18-22 weeks old male and female *Tph2*$^{-/-}$ and *Tph2*$^{+/-}$ (control) mice, and the hidden cookie test — in female *Tph2*$^{-/-}$ and *Tph2*$^{+/-}$ mice.

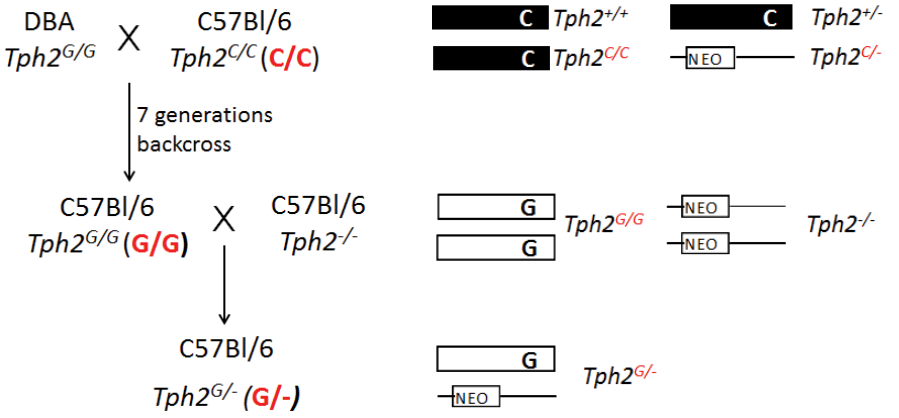

Figure 4: Breeding scheme used for obtaining experimental *Tph2*$^{C/C}$, *Tph2*$^{G/G}$, and *Tph2*$^{G/-}$ animals

To study the implication of partial reduction in central serotonin on behavior and brain neurochemistry 4 groups of mice were used. The first group was C57Bl/6 mice, homozygous for the 1473C allele further referred to as *Tph2*$^{C/C}$. The second group contained congenic C57Bl/6 animals homozygous for the 1473G allele (*Tph2*$^{G/G}$) generated by transferring the 1473G allele from DBA/2 mice via breeding over seven generations to the C57Bl/6 background [194] Figure 4. In the third group were mice heterozygous for the *Tph2*-null allele on C57Bl/6 background (10th generation backcross)[4], further called *Tph2*$^{C/-}$. The last group of animals, *Tph2*$^{G/-}$, was obtained by breeding *Tph2*$^{G/G}$ (1473G on C57Bl/6 background) with *Tph2*-deficient mice (*Tph2*$^{-/-}$ on C57Bl/6 background) Figure 4. Mice of the above mentioned genotypes were used at the age of 20-26 weeks.

3.2.5 *Behavior assessment*

For all behavior experiments mice were habituated to the experimental room for at least one week, or if that was not necessary at least an hour before testing. During this time repeated handling of animals was done by the same experimenter who performed the tests. Mouse behavior was video recorded for subsequent offline analyses by the experimenter. For analysis of EPM data Biobserve software was used. In all experiments the observer was blind to the genotype. The testing arenas if not specified were cleaned between trials or different animals with water, and dried with the paper towels.

ACTIVITY MEASUREMENTS Locomotor activity was recorded in the home cage by InfraMot system. The system is working through sensing the body-heat image, infrared radiation. The sensor was placed on a top of a home cage over 5 days. The last 3 days of measurements were analysed.

OPEN FIELD A large arena (50x50 cm) under low illumination (30 Lux) was used as an open field to measure locomotor activity. Each mouse was placed into the arena facing the middle of the wall and its activity was measured during 10 min. The total distance travelled, time spent in the center and near the walls were calculated.

FOOT PRINT TEST To evaluate the gate of mice the foot prints of mouse hind paws were obtained by dipping them into the dye. The mice were placed on a narrow (5 cm) gangway 45 cm long. The floor of the gangway was covered with white paper. No pretraining was used. Each individual mouse was given 3 trials. After all the trials the mouse was placed for 1 min in an empty cage filled with about 1 cm of warm water in order to clean the paws from the ink. Thereafter the mouse was transferred into its home cage. As a result, for each mouse 10-15 clearly defined, single prints of foot steps were obtained Figure 5. After drying the steps were analysed. The stride length (distance between the hind paw of the same side) and stride width (distance between hind paws of different side) were calculated.

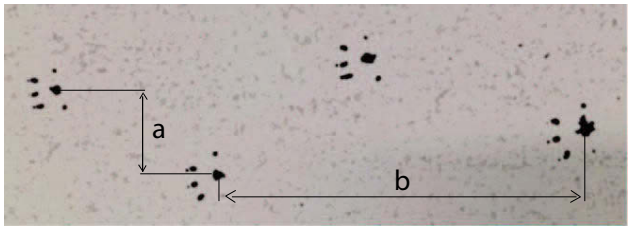

Figure 5: Representative picture of foot prints. a – stride width, b – stride length

RECORDING AND ANALYSIS OF ULTRASONIC VOCALIZATION Ultrasonic vocalisation was measured in *Tph2*$^{-/-}$ and *Tph2*$^{+/+}$ pups at postnatal days 3 (Pnd3), 6 (Pnd6) and 9 (Pnd9). Pups were one by one carefully isolated from their mother and nest, and transferred to a new small glass chamber (10x8x7 cm; open surface) with the 2 cm of sawdust. This chamber was in a sound-attenuated box made from Styrofoam. Ultrasonic vocalizations were recorded during 10 min using a condenser ultrasound microphone placed in the roof of the box, 10 cm above the floor Figure 6. The microphone was sensitive to frequencies of 15-180 kHz with a flat frequency response (±6 dB) between 25-140 kHz. Prior to each test, the glass chamber was cleaned using

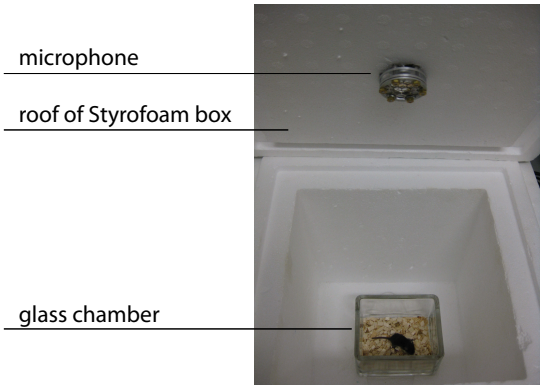

Figure 6: Representative picture of the testing chamber for recording pup ultrasonic vocalisations.

70% ethanol solution, followed by water, and dried with paper towels. The microphone was connected via an Avisoft UltraSoundGate 116Hbm device to a personal computer, where acoustic data were displayed in real time by an Avisoft Recorder and were recorded with a sampling rate of 250,000 Hz in 16 bit format. For acoustical analysis, recordings were transferred to SASLabPro, and a fast Fourier transformation was conducted (512 FFT length, 100% frame, Hamming window and 75% time window overlap). The spectrograms were produced at 488 Hz of frequency resolution and 0.512 ms of time resolution. Call detection was provided by an automatic threshold-based algorithm (amplitude threshold: -40 dB) and a hold-time mechanism (hold time: 10 ms). Since no USVs were detected below 30 kHz, a high-pass filter of 30 kHz was used to reduce background noise outside the relevant frequency band to 0 dB. The accuracy of call detection by the software was verified manually by an experienced user. When necessary, missed calls were marked by hand to be included in the automatic parameter analysis, and noise detected as a call was excluded from the analysis. Total number of USV was calculated for the entire session.

GRIP TEST According to the manufacturer's instruction, mice were hold by the tail and moved down until it grabbed the bar. Then the mouse was moved against the direction of the force gauge until the animal released the grid. The maximal grip strength value of animal was noted. An average of four readings was obtained at each occasion.

HIDDEN COOKIE TEST This test is based on the assumption that mouse driven by hunger will intensively look for hidden food in a secure environment orienting itself mostly by olfaction. It can give fast answer whether the mouse in general anosmic or not. Before testing mice were trained for a week by receiving small chocolate cookie that

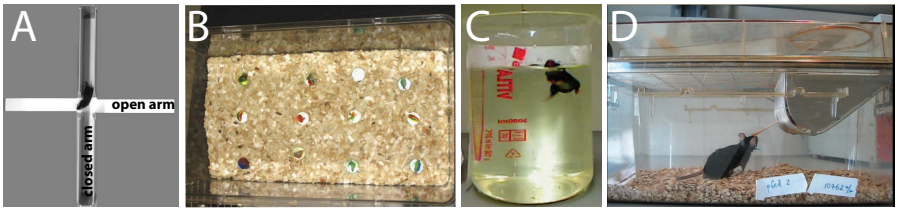

Figure 7: Representative pictures of behavior tests for assesing: **Anxiety-like behavior:** A) elevated plus maze and B). Marble burying test; **Depression-like behavior:** C). Forced swim test; **Olfaction:** D) habituation-dishabituation test.

was used afterwards for the test. 24 hr before testing the food was removed, and the cages were changed in order to prevent the feeding from food residues left in the bedding. The body weight and blood glucose levels were controlled before and after food deprivation. On the test day a mouse was placed in the arena (cage 42x25x18 cm, illuminated 10Lux) with the cookie hidden in a 5 cm of saw dust. Latency to locate the buried food was considered as an index of olfactory ability. Directly afterwards the mouse was placed in a new cage with the preweighed food. 30 min thereafter the amount of food eaten and blood glucose levels were measured.

ELEVATED PLUS MAZE This test is based on the inborn aversion of rodents to open, bright illuminated spaces [157]. The maze consisted of two open arms (30x5 cm, 300 lux illumination) and two closed arms (30x5 cm, 10 lux illumination) that were enclosed by a sidewall on all edges (height 15 cm) Figure 7(A). Mice were placed in the center of the maze (central platform) facing the closed arm. Total arm entries, percentage of entries into the open arm [(open arm entries/total arm entries)*100] and time spent in open arms [(open arms/total session duration)*100] were quantified. Arm entry was only defined when the center of the body of the animal was on an arm by at least three cm to distinguish true entries from stretched postures into the arms.

MARBLE BURYING TEST Marble burying is a common test for validating anxiolytic effect of drugs [148]. The test was conducted in a new cage (equally in size to the home cage) containing evenly spaced fifteen clear glass marbles (20 mm diameter) visible above of 5 cm of sawdust Figure 7(B). During the test mice had access to food and water, and the test cage was covered with a metal grid. After 30 min the test was terminated by removing the mouse and counting the number of buried marbles. A marble was scored as buried if more than two-thirds of it was covered with sawdust.

FORCED SWIM TEST This test, as originally described by Porsolt [164], assesses the tendency to give up attempting to escape from an

unpleasant environment, whereby fewer attempts are interpreted as behavioral despair. Here we used a plastic beaker (17.5 cm diameter, 24 cm high), filled with water (24°-26°C) to a height of 18 cm Figure 7(C). The time mice spent floating on the water (immobility time, sec) during 6 min as well as latency (sec) to the first immobility episode were manually scored. A mouse was judged to be immobile when it ceased struggling and remained floating motionless in water, making only those movements necessary to keep its head above the water. Swimming was defined as vigorous movements with forepaws breaking the surface of the water.

HABITUATION-DISHABITUATION TEST This olfaction test allows assessing the ability to distinguish smells between each other, and the ability to get used to one smell. 30 min before testing the mouse was put into fresh test cage (sized as home cage) with a closed plastic lid. Each test cage was used only once. During next 10 min a dry and clean cotton stick was introduced into the cage through the hole for water bottle in order to decrease unspecific sniffing not connected with the odor. Next four odors were tested along with water as control: vanilla, peppermint, female and male urine Figure 7(D). Each smell was introduced three times for 2 min with a 1 min inter-trial interval. In order to be able to test social odors (female and male odors) only test mice that were familiar with the opposite gender were used. Vanilla was dissolved in water. Peppermint was prepared freshly as a water extract from peppermint leaves. Female and male urine was collected at the day of testing from mice with which the tested mice never had contact. The time mice spent sniffing different odors was measured.

NOVELTY SUPPRESSED FEEDING This test is based on a provoked conflict between the fear of mice to enter bright illuminated spaces and food seeking induced by hunger [21, 123]. Animals were food deprived 23 hr prior testing. On the test day mice were first placed into a new cage for 30 min. Then they were introduced into a new brightly illuminated test environment (cage 42x25x18 cm, 350 lux illumination in the center of the arena) where a single food pellet was centrally placed. After the first feeding event animals were returned to their home cages where they could eat pre-weighed food over a period of 5 min. Latency to the first eating episode (time between mouse introduction to arena with food pellet in the middle and the first feeding event) was used as an index of induced anxiety-like behavior. The amount of food consumed in the home cage provided a measure of appetitive drive.

RESIDENT INTRUDER TEST The resident intruder test is based on the territory defensive behavior against unfamiliar intruding cons-

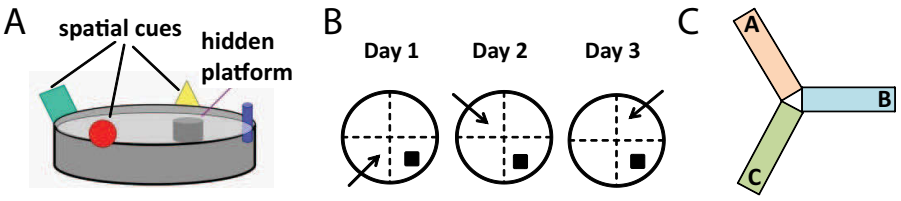

Figure 8: Schematic presentation of memory tests. A) and B) **Morris water maze**: filled with water circular maze (1.2 m diameter) with 4 cues of different shape and color on the side of it. During the three testing days the platform stayed at one position, and during each trial (from 1 to 6) of the same day mouse was placed in the same position. C) **Y-maze:** each arm is randomly assigned the letter A, B, or C.

pecifics [113]. Each single-housed resident male was confronted in its home cage by a group-housed (five mice per cage) intruder male FVB/N mouse for 10 min. Each intruder mouse was used only once to avoid submissive/dominance effects after first interaction. Behavioral interactions during each confrontation were recorded and subsequently scored by an observer. Latency to the first attack, total amount of attacks and cumulative duration of attacks were analyzed.

MORRIS WATER MAZE One of the tasks to assess special memory is the morris water maze (MWM) [209]. Mice were trained to find a hidden platform in a circular maze (1.2 m diameter) using cues situated around the pool. The maze was placed alone in a room with four cues on its walls Figure 8(A). Water was made opaque with milk powder and kept at the temperatures 20-22°C. Each mouse was given 6 trials a day during 3 consecutive days with an inter-trial interval of 1hr. The position of the transparent square platform (8cmx8cm) hidden 0.5cm under the water during the testing days was not changed Figure 8(B). Each day the mouse was released from one of the sides of the pool and was given 120 sec to find the platform. To introduce mouse into the pool it was placed into an opaque plastic beaker with the lid attached to a stick. It helped to prevent visual orientation during the release of the mouse. Next the cup was moved to the wall of the pool and turned so that the mouse glided into the water facing the wall. Irrespective at the trial performance mice were guided to the platform and allowed to remain there for at least 15 sec. The mouse was removed from the platform with the help of a wire mesh attached to a stick onto which it would readily climb. Between trials mice were kept in single cages under infrared light and were allowed to dry and warm up.

SPONTANEOUS ALTERNATIONS IN Y-MAZE Spontaneous alternations in the Y-maze are extensively used as a measure of working memory [61]. The Y-maze was slightly illuminated (10Lux). Each arm

of the symmetrical Y-maze was 30 cm long, 30 cm high, 3,5 cm broad at the bottom and 15 cm at the top. Each arm was randomly assigned the letter A, B, or C Figure 8(C). The mouse was placed in the middle of the maze facing arm B and was given 10 min to freely explore the maze. The amount and consequence of each arm entry was recorded by the observer. Percent alternation was calculated as the number of nonrepeating triads (for example, ABC, CBA, CAB, and etc.) divided by the maximal possible alternations (total number of arm entries minus 2) and multiplied by 100.

3.2.6 *Telemetric measurement*

PhysioTel PA-C20 pressure transmitters were implanted into the femoral artery of 3 months old female mice and recordings of blood pressure (BP), heart rate (HR) and locomotor activity were started 10 days after the surgery. Data for the day/night-time activity and sleeping time were averaged from 6 am to 6 pm and vice versa of 96 hours recording time. The sleeping time was calculated from the locomotor activity data. An animal was considered as sleeping when it was displaying no activity during at least 5 minutes. More than 30 minutes immobility was considered as an extended sleeping period. The locomotor activity of animals was averaged for the non-sleeping time periods during both day- and night-time.

3.2.7 *Neurochemical assessment*

To prepare brains for high-performance liquid chromatography (HPLC) analysis, animals were anesthetized by intraperitoneal (i.p.) injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Animals were transcardially perfused with PBS containing 300 U/ml heparin to remove the blood, containing peripheral serotonin. Brains were removed, weighed, and snap frozen on dry ice. For the determination of serotonin and its metabolites, frozen tissues were homogenised in lysis buffer containing 10 µM ascorbic acid and 1.8% perchloric acid (PCA), centrifuged for 30 min at 20 000 g, 4°C, and the supernatant was used for HPLC measurement. Tissue levels of 5-HTP, 5-HT, and its metabolite 5-HIAA were analysed using high sensitive HPLC with fluorometric detection [220]. Sample separation took place at 20°C on a C18 reversed-phase column using a 10 mM potassium phosphate buffer, pH 5.0, containing 5% methanol with a flow rate of 2 ml/min. Fluorescence of 5-HTP and 5-HT was excited at 295 nm and measured at 345 nm. For the evaluation of serotonin synthesis *in vivo* animals were injected i.p. with 100 mg/kg of the AADC inhibitor 3-hydroxybenzylhydrazine dihydrochloride (NSD-1015) one hour before brain dissection. Tryptophan was injected i.p. in a dose of 100 mg/kg alone or in a combination with NSD-1015 one hour prior brain

removal. Amounts of 5-HT, 5-HTP, and 5-HIAA were normalized to the wet tissue weight for statistical analysis. Calculation of substance levels was based on external standard values.

3.2.8 *Animal treatment*

FLUOXETINE TREATMENT Behavior changes followed fluoxetine treatment were assessed after oral treatment with the drug in a dose of 20 mg/kg/day. Fluoxetine was prepared fresh every third day by first dissolving it in a small amount of tap water, then filtering and adding the required amount of drinking water. 2 weeks after treatment mice were either sacrificed for further analysis of brain neurochemistry, or respective behavior test was performed.
To investigate the role of fluoxetine on proliferation and survival of precursor cells mice were divided to two groups. The first group was injected i.p. once a day with fluoxetine in a dose 10 mg/kg (dissolved in saline). The second group served as a control group, and received the saline injection once a day. Both groups received the drug or saline in a volume of 10 µl/g body weight during 21 day.

BRDU TREATMENT To analyse proliferation, animals received three i. p. injections of bromodeoxyuridine (BrdU) in a dose of 50 mg/kg (dissolved in saline) 6 hours apart at the day 22 after saline or fluoxetine treatment, and were sacrificed 24 hours after the first injection.
Another group of mice was tested for survival of proliferating precursor cells 3 weeks after BrdU. In this case BrdU was injected three times at day 0 (one day before fluoxetine treatment was initiated) in a dose of 50 mg/kg (dissolved in saline) 6 hours apart, and animals were sacrificed the next day after the last fluoxetine injection (day 22).

IGF-1 TREATMENT Recombinant human IGF-1 was dissolved in saline first until the stock concentration 1 mg/ml. The aliquots were stored at -20°C. For the injections stock solution was dissolved with the saline 10 times until the final concentration 100 µg/ml. hIGF-1 was injected from P5 till P19 twice a day in a dose 1 mg/kg/day, subcutaneously, in an amount 10 µl/g BW. Body mass measurements were performed on the selected days (D5, 8, 11, 14, 16, 18 and 20).

5-HT$_{1A}$ AGONIST-INDUCED HYPOTHERMIA Saline, 1 mg/kg 8-OH DPAT or 0.1 mg/kg WAY100635 were injected i.p. in a volume of 10 µl/g. Mice were injected first with saline, then one week later with 8-OH DPAT, and another week later with a combination of WAY 100635 and 8-OH DPAT. Body temperature was measured using a thermometer probe inserted about 10 mm into the rectum. The probe was dipped in Vaseline before insertion and held in the rectum until a stable temperature was obtained (about 6 s). Prior to substance injec-

tions three baseline body temperature measurements were recorded every fifteen minutes (time points -30 min, -15 min and 0). After the initial baseline temperature reading at 0 min, mice were administered sterile saline or 8-OH-DPAT, and body temperature was recorded every 15 min during 90 minutes in total. A specific 5-HT_{1A} antagonist, WAY 100635, was injected at the time point -30 min, directly after the first baseline temperature measurement, followed by 8-OH DPAT injection at 0 min and temperature measurements every 15 min during the following 90 min.

HIGH FAT DIET To rescue growth retardation phenotype, the diet containing 45% fat was given starting at P15 for 11 days. At P35 animals were sacrificed for further analysis.

30°C EXPERIMENT Pregnant female mice at the day 14 after plug check were put in the chamber with the normal dark/light regime. In this chamber the temperature was maintained at 30°C. When the pups were born the litter was kept there until the weaning. The body weight was measured each second day.

4 RESULTS

4.1 GROWTH RETARDATION IN *Tph2*-DEFICIENT MICE

4.1.1 *Phenotype*

Tph2$^{-/-}$ mice on FVB/N background were born in normal Mendelian ratios and were undistinguishable from their *Tph2*$^{+/-}$ littermates at birth. Analysis of late embryonic stages (E14 and E18) did not reveal body or brain weight or size abnormalities Figure 9 (A,B). However, starting 3-4 days after birth *Tph2*$^{-/-}$ pups were smaller in size, with decreased adipose tissue content and dry skin. The growth retardation worsened up to 4-5 weeks of age Figure 9 (C). Interestingly, at postnatal day 7 (P7) *Tph2*$^{-/-}$ pups were almost twice lighter than the control littermates, but there was no detectable difference in brain weight. At P14 there was a small but significant difference in brain weights between *Tph2*-deficient and control pups Figure 9 (D). No abnormalities in feeding behavior in the pre-weaning period were detected — pups were noticed to have suckling activity as well as a size of milk pouches similar to control pups. Moreover, diminishing the competition for the milk by decreasing the litter size did not restore normal growth. Although being smaller, at the age of 3-4 weeks *Tph2*$^{-/-}$ mice showed hyperactivity and jumpiness. However, at P35 *Tph2*$^{-/-}$ pups were as active as their control littermates in the open field Figure 10 (A).

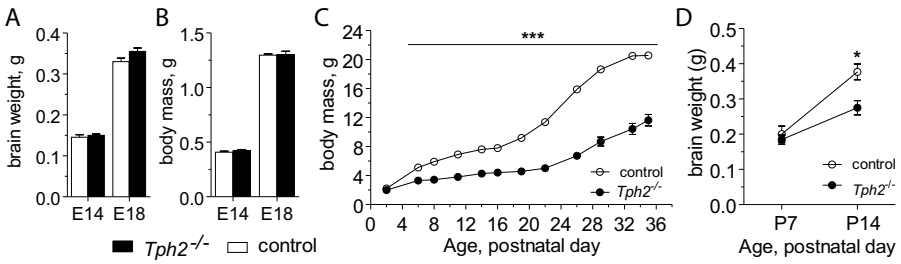

Figure 9: Growth retardation in *Tph2*$^{-/-}$ mice. A) Embryonic growth: no significant reduction in body mass of *Tph2*$^{-/-}$ embryos compared to control littermates was observed (E14 and E18 — embryonic day 14 and 18, respectively). B) Postnatal growth curves of control and *Tph2*$^{-/-}$ pups show a delay in body mass acquisition. The growth of the *Tph2*$^{-/-}$ mice becomes significantly different from that of control mice by P6 (***p<0.001, two way ANOVA). C) Brain weight gain over the first two postnatal weeks in *Tph2*$^{-/-}$ mice. Student's t-test, *P<0.05.

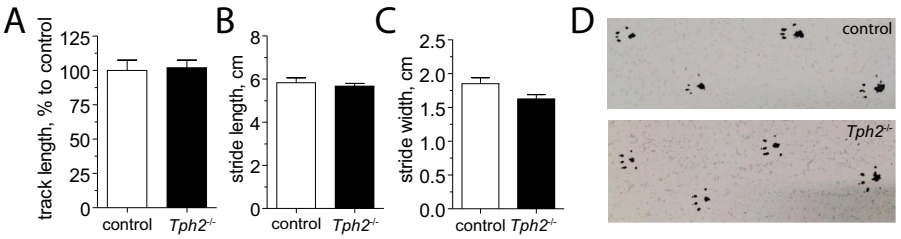

Figure 10: Activity and gait in *Tph2*$^{-/-}$ mice at postnatal day 35. A) *Tph2*$^{-/-}$ and control mice travel equal distance in the open field under low illumination during 10 min. B) Stride length, measured in cm as a distance between hindpaws of the same side. C) Stride width, measured in cm as a distance between hindpaws of different sides. D) Representative picture of steps obtained from control and *Tph2*$^{-/-}$ mice.

Furthermore, at this age *Tph2*-deficient mice have unchanged gait measured in the foot print test by the stride length and width Figure 10 (B,C,D).

4.1.2 *In search for explanation: hypothalamic-pituitary-adrenal (HPA) axis*

The HPA axis consists of hypothalamus, pituitary and adrenal glands, which are in tight connection with each other through complex feedback mechanisms. It is known that the HPA axis is involved in the regulation of metabolic processes (energy expenditure, feeding, and growth). The hypothalamus produces a number of releasing hormones to the pituitary gland that in turn stimulates growth. Hypothalamus receives 5-HT innervation of various density, which mainly originate from the B9 group of raphe nuclei.

GENE EXPRESSION IN HYPOTHALAMI OF NEWBORN *Tph2*-DEFICIENT MICE First the impact of central serotonin ablation on the gene expression profile of the hypothalamus was assessed. Only minor alterations in hypothalamic gene expression were detected in *Tph2*$^{-/-}$ mice at the day of birth in comparison to control pups. Analysis of differently expressed genes did not reveal any potential candidate gene which may be responsible for the observed growth retardation phenotype Table 8.

GROWTH HORMONE PRODUCTION BY PITUITARY GLAND IN *Tph2*-DEFICIENT PUPS The next component of the HPA axis that translates signals from hypothalamus is the pituitary gland. One of the important hormones of the pituitary gland is Growth Hormone (GH) that stimulates growth and regeneration. Analysis of GH by IHC re-

Gene Symbol	RefSeq	fold change *Tph2*$^{-/-}$ vs. WT	p-value
down-regulated gnes			
Ifi27l2a	NM_029803	-2,2	0,001
Atad4	NM_146026	-1,5	0,008
2410018E23Rik	XM_912668	-1,8	0,015
AY026312	NM_133359	-1,6	0,015
Slamf7	NM_144539	-1,6	0,015
Olfr430	NM_146718	-2,3	0,019
Apon	NM_133996	-1,8	0,020
Gzmc	NM_010371	-1,8	0,035
Semg1	NM_017390	-1,7	0,044
up-regulated gnes			
Exoc3l2	ENSMUST00000011407	1,6	0,004
Clca2	NM_030601	1,5	0,009
Zfp353	NM_153096	1,6	0,009
6720416L17Rik	ENSMUST00000100000	1,6	0,020
4932411G14Rik	NM_177711	2,2	0,023
Ms4a6b	NM_027209	1,5	0,029
Nkx2-6	NM_010920	1,6	0,030
1810037I17Rik	NM_024461	1,5	0,038
Crygb	NM_144761	1,5	0,043
Gtsf1l	NM_026630	1,9	0,045
Krt8	NM_031170	1,5	0,046

Table 8: Growth retardation in *Tph2*$^{-/-}$ mice: gene expression analysis of hypothalamus obtained from *Tph2*$^{-/-}$ and control pups at the day of birth (D0). Genes which expression was more than 1.5 fold and significantly ($p<0.05$) up- or down-regulated are shown.

vealed no differences in its production and distribution in *Tph2*$^{-/-}$ pups at D2 in comparison to control littermates Figure 11(A).

PLASMA LEPTIN AND IGF-1 LEVELS IN *Tph2*-DEFICIENT PUPS

Due to the short life-time and pulsatory excretion it is technically impossible to measure the level of GH in plasma. However, IGF-1 is excreted by the liver in response to GH stimulation, and its level can serve as a marker of GH secretion. Plasma IGF-1 levels were markedly decreased in growth-retarded *Tph2*$^{-/-}$ mice at p35 Table 9. Moreover, plasma leptin level was dramatically decreased in *Tph2*$^{-/-}$ mice in comparison to controls at this age Table 9.

	BW, g	IGF-1, ng/ml	Leptin, pg /ml
Control (n=5)	20,6±0,4	838,6±4,2	2930±240
Tph2$^{-/-}$ (n=3)	11,6±1,2*	212,4±4,5**	685±38**
FVB/N (n=6)	22,0±0,2	918,2±39,1	6463±839
FVB/N malnutrition (n=5)	18,6±0,4$^{\#\#\#}$	349,3±20,5$^{\#\#\#}$	2430±324$^{\#\#}$

Table 9: Metabolic parameters in 35-days old *Tph2*$^{-/-}$, control and malnourished mice. Values are expressed as mean ± SEM. *$p<0.05$, **$p<0.01$ *Tph2*$^{-/-}$ vs control. $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ FVB/N malnutrition vs FVB/N.

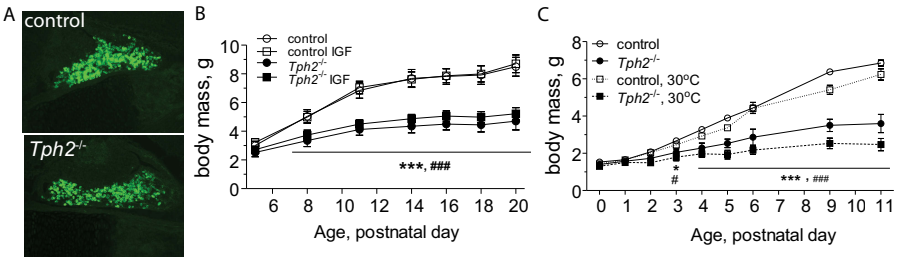

Figure 11: Growth retardation in *Tph2*$^{-/-}$ mice. A) Representative immunofluorescence images of growth hormone (GH) in the pituitary gland of 2-days old and control mice. B) Postnatal growth of *Tph2*$^{-/-}$ pups under the treatment of IGF-1 is not accelerated in comparison to untreated *Tph2*$^{-/-}$ pups (***p<0.001 vs. untreated control mice, $^{###}$p<0.001 vs. hIGF-1 treated control mice). C) Postnatal growth curves of pups brought up at 30°C degree do not differ from the litters grown up at room temperature (*p<0.05, ***p<0.001 vs. control mice raised at room temperature,$^{\#}$p<0.05, $^{###}$p<0.001 — at 30°C degree, two-way ANOVA).

GROWTH PHENOTYPE IN IGF-1 TREATED *Tph2*-DEFICIENT PUPS Taking into account the low levels of IGF-1 I hypothesized that dysfunction of IGF-1 excretion, induced by altered GH release, is the cause of delayed growth. To test this hypothesis pups were treated from P5 to P12 with IGF-1. No improvement in body weight gain was observed in treated *Tph2*$^{-/-}$ pups vs. untreated Figure 9(B). Similar to untreated mice, a significant difference in body weight between IGF-1 treated *Tph2*$^{-/-}$ pups and control mice was observed already at day 8 Figure 9(B). This difference persisted during the whole period of treatment, arguing that the decrease in plasma IGF-1 level is rather a secondary phenotype than the primary cause of growth retardation. Indeed, wild type FVB/N mice brought up by mothers having reduced milk availability developed significantly reduced body weight than the control pups of the same age, and showed decreased IGF-1 and leptin levels Table 9.

4.1.3 *In search for explanation: metabolic abnormalities*

GROWTH PHENOTYPE OF *Tph2*-DEFICIENT PUPS BROUGHT UP AT 30°C Previously it was shown that adult *Tph2*$^{-/-}$ mice exhibit alterations in body temperature control [4]. To test the hypothesis that a thermoregulation deficit is the primary cause of growth abnormalities during early postnatal life, pregnant mothers were placed at an ambient temperature of 30°C, and maintained at this condition for 2 weeks after the delivery. This had no effect on growth of *Tph2*$^{-/-}$ or control pups Figure 11(C).

FAT DIGESTION IN *Tph2*-DEFICIENT PUPS During the postnatal development in the pre-weaning period the main food source for pups is mother's milk. It contains mostly fat and few carbohydrates. When pups grow up, they start eating the normal chow which contains mostly carbohydrates. Thus, there is a switch in food composition between 2-3 weeks of age. Different metabolic processes are involved in digesting and getting the energy from fat or carbohydrates. *Tph2*$^{-/-}$ pups do not properly gain body weight in the pre-weaning period, but after weaning they grow even faster than their control littermates. Therefore, I hypothesized that *Tph2*-deficient pups despite being able to suck the milk, are not able to properly digest it. In this case keeping the mice on high fat diet (HFD) further after weaning may delay the catch up in body weight.

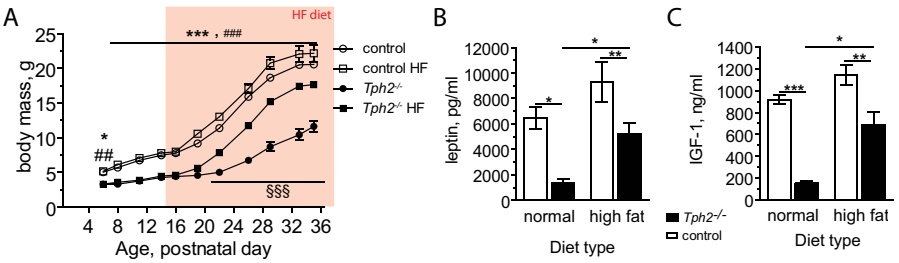

Figure 12: Growth retardation in *Tph2*$^{-/-}$ mice. A) Body weight of *Tph2*$^{-/-}$ mice fed with HFD at P15-P35 is slightly improved in comparison to *Tph2*$^{-/-}$ pups under ND (*p<0.05, ***p<0.001 *Tph2*$^{-/-}$ on ND vs. control mice on ND, $^{\#\#}$p<0.001, $^{\#\#\#}$p<0.001 *Tph2*$^{-/-}$ on HFD vs. control mice on HFD, §§§p<0.001 *Tph2*$^{-/-}$ mice on ND vs. *Tph2*$^{-/-}$ mice on HFD). B) Plasma leptin levels in *Tph2*$^{-/-}$ mice 2 weeks after HFD reached the level of control mice fed with ND. C) Plasma IGF-1 levels of *Tph2*$^{-/-}$ mice 2 weeks after HFD reached the level of control mice fed with ND (*p<0.05, **p<0.001, ***p<0.0001, two-way ANOVA). HFD — high fat diet, ND — normal diet

However, giving HFD starting from 2 weeks of age even increased the body weight gain of *Tph2*$^{-/-}$ pups Figure 12 (A). At P35 *Tph2*$^{-/-}$ pups on HFD had higher body weight, increased levels of plasma leptin and IGF-1 in comparison to *Tph2*$^{-/-}$ mice fed with ND Figure 12 (A,B,C). Moreover, after 10 days of HFD plasma leptin and IGF-1 levels in *Tph2*$^{-/-}$ were normalised to the levels of control littermates on a ND Figure 12 (B,C). However, *Tph2*-deficient pups fed with HFD were still lighter than their control littermates fed with the ND Figure 12 (A).

In order to test the hypothesis of improper fat digestion during early postnatal development in another experimental set up feces extracts were stained with the Oil Red. The analysis showed no major difference in the amount of fat found in *Tph2*$^{-/-}$ and control pups' feces extracts Figure 13.

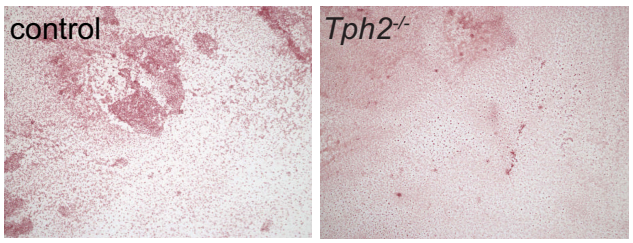

Figure 13: Representative Oil RedO staining of feces obtained from three weeks old control and *Tph2*$^{-/-}$ mice.

4.1.4 *In search for explanation: behavioral changes during early development*

ULTRASONIC VOCALIZATION OF *Tph2*-DEFICIENT PUPS AT THE PRE-WEANING PERIOD As mother's care is very important for the development during the pre-weaning period ability of *Tph2*$^{-/-}$ pups to communicate their needs for care at the early postnatal days was assessed. For this purpose ultrasonic vocalization of pups after their separation from mother and nest was evaluated. At both P3 and P6 *Tph2*$^{-/-}$ mice emited twice less USVs in comparison to control pups of the same age Figure 14. Furthermore, in contrast to *Tph2*$^{-/-}$ pups, control mice exhibited a U-shaped profile of the amount of emitted USV, with a clear peak at day 6 and reduction at day 9.

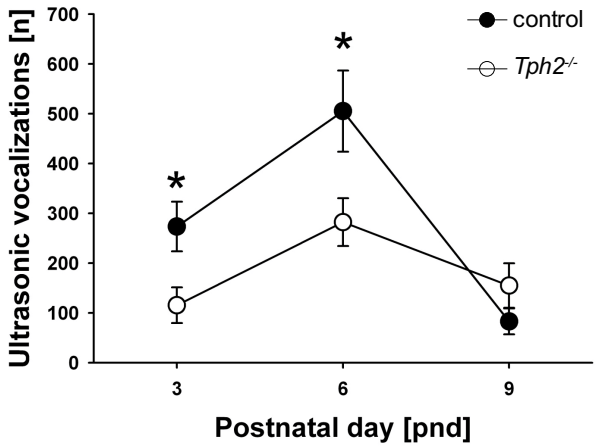

Figure 14: Amount of ultrasounds emitted by *Tph2*$^{-/-}$ mice at postnatal days 3, 6 is different from control pups, but undistinguishable at P9. (*p<0.05 vs. *Tph2*$^{+/+}$, two-way ANOVA).

4.2 BEHAVIORAL AND NEUROCHEMICAL ABNORMALITIES IN *Tph2*-DEFICIENT MICE

4.2.1 *Tph2 expression and serotonin levels*

First the amount of *Tph2* transcripts in the brains of $Tph2^{+/-}$ and $Tph2^{+/+}$ mice, containing one and two copies of the *Tph2* gene, respectively, was evaluated. Real-time PCR showed a 50% reduction in *Tph2* gene expression in the whole brain of $Tph2^{+/-}$ mice in comparison to $Tph2^{+/+}$ mice Figure 15(A).
Next the amount of 5-HTP, serotonin and 5-HIAA in the whole brain of $Tph2^{-/-}$, $Tph2^{+/-}$ and $Tph2^{+/+}$ mice was measured by HPLC. $Tph2^{-/-}$ mice contained less than 2% of control 5-HT levels and no detectable 5-HIAA in the brain Figure 15(B), Table 10. However, only around 10 % reduction in brain serotonin levels was observed in $Tph2^{+/-}$ in comparison to $Tph2^{+/+}$ mice, whereas the level of 5-HIAA was reduced nearly by half Figure 15(B), Table 10.
Further the 5-HT synthesis rate in $Tph2^{-/-}$, $Tph2^{+/-}$ and $Tph2^{+/+}$ mice was evaluated by blocking conversion of 5-HTP to 5-HT by NSD. An around 20% decrease in accumulation of 5-HTP was observed in $Tph2^{+/-}$ in comparison to $Tph2^{+/+}$ mice Figure 15(C), Table 10. As expected *Tph2*-deficient mice accumulated less than 2% of 5-HTP compared to $Tph2^{+/+}$ Figure 15(C), Table 10.

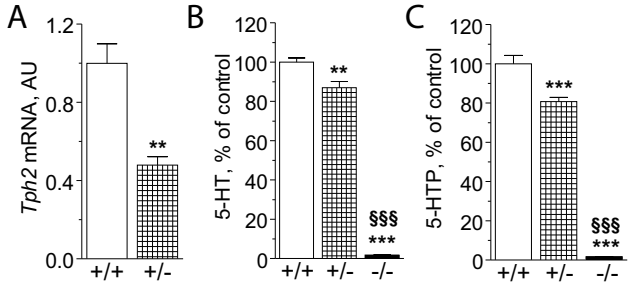

Figure 15: *Tph2* expression and serotonin synthesis in *Tph2*-deficient mice. A) Real-time PCR analysis of *Tph2* expression in the brain (AU - arbitrary units). B) 5-HT level in the whole brain (HPLC measurement). $Tph2^{+/+}$ 5-HT level is taken as 100%. C) 5-HTP level in the whole brain 1 hr after NSD administration (100 mg/kg, i.p.) (HPLC measurement). $Tph2^{+/+}$ 5-HTP level is taken as 100%. Data are shown as means ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. $Tph2^{+/+}$; $^{§§§}p < 0.01$ vs. $Tph2^{+/-}$, one-way ANOVA with Bonferroni correction.

4.2.2 *Activity and muscle strength*

Prior to behavior testing overall activity of freely moving *Tph2*-deficient mice in their home cages during 24 hours within couple of days

	5-HT (pg/mg)	5-HIAA (pg/mg)	5-HTP (pg/mg)	5-HTP / NSD (pg/mg)
Tph2$^{+/+}$	753±16.3	366.3±34.9	3.7±0.3	329.1±14.0
Tph2$^{+/-}$	655.6±24.1[2]	217.0±11.7[1]	2.0±0.2[1]	266.7±7.1[1]
Tph2$^{-/-}$	13.6±1.0[1,3]	0.0±0.0[1,3]	0.0±0.0[1,3]	5.69±0.27[1,3]

Table 10: Serotonin, 5-HIAA, and 5-HTP in the brains of *Tph2*-deficient mice. Serotonin (5-HT), 5-hydroxytryptophan (5-HTP), and 5-hydroxyindoleacetic acid (5-HIAA) levels were measured by HPLC in whole brain lysates. Serotonin synthesis rate was evaluated by the accumulation of 5-HTP in the brain during 1 hour after administration of the AADC inhibitor, NSD (5-HTP/NSD column). Values are normalised to mg of wet brain tissue. Data are presented as means ± SEM. [1]p < 0.001, [2]p < 0.01 vs. *Tph2*$^{+/+}$; [3]p < 0.001 vs. *Tph2*$^{+/-}$ (one way ANOVA with Bonferroni's correction).

was evaluated. First, activity was measured by telemetry that allows recording the position of the animal every 5 sec Figure 16(A). Then, the activity of mice was measured in the home cages with the InfraMot system Figure 16(B). Both experiments did not reveal any differences in the activity of *Tph2*$^{-/-}$ mice in comparison to control mice. However, analysis of sleeping episodes from the activity data obtained in telemetric measurements revealed that *Tph2*$^{-/-}$ mice slept more than control mice Figure 16(C,D).
Next, activity of *Tph2*-deficient mice in a unknown environment (open field, under low illumination conditions) was evaluated. *Tph2*$^{-/-}$ mice did not show any difference in locomotor activity in comparison to *Tph2*$^{+/-}$ and *Tph2*$^{+/+}$ mice Figure 19(A).
Furthermore, to assess another important parameter, muscle strength, the grip test was performed. The maximum power that the mice displayed during this test was not different between *Tph2*$^{-/-}$, *Tph2*$^{+/-}$, and *Tph2*$^{+/+}$ Figure 16(E).

4.2.3 *Olfaction*

Brain serotonin depletion has been shown to influence olfaction [161]. Therefore, olfaction in *Tph2*$^{-/-}$ mice was determined.
24 hr of food deprivation performed before the hidden cookie test caused equal decrease in body weight by 15% in *Tph2*$^{-/-}$ and control mice Figure 17(A). Moreover, fasted glucose levels were not different between the 2 groups Figure 17(B). On the test day *Tph2*-deficient mice needed the same 30 sec as controls to find and start eating a hidden cookie in the middle of the tested area Figure 17(C). Further-

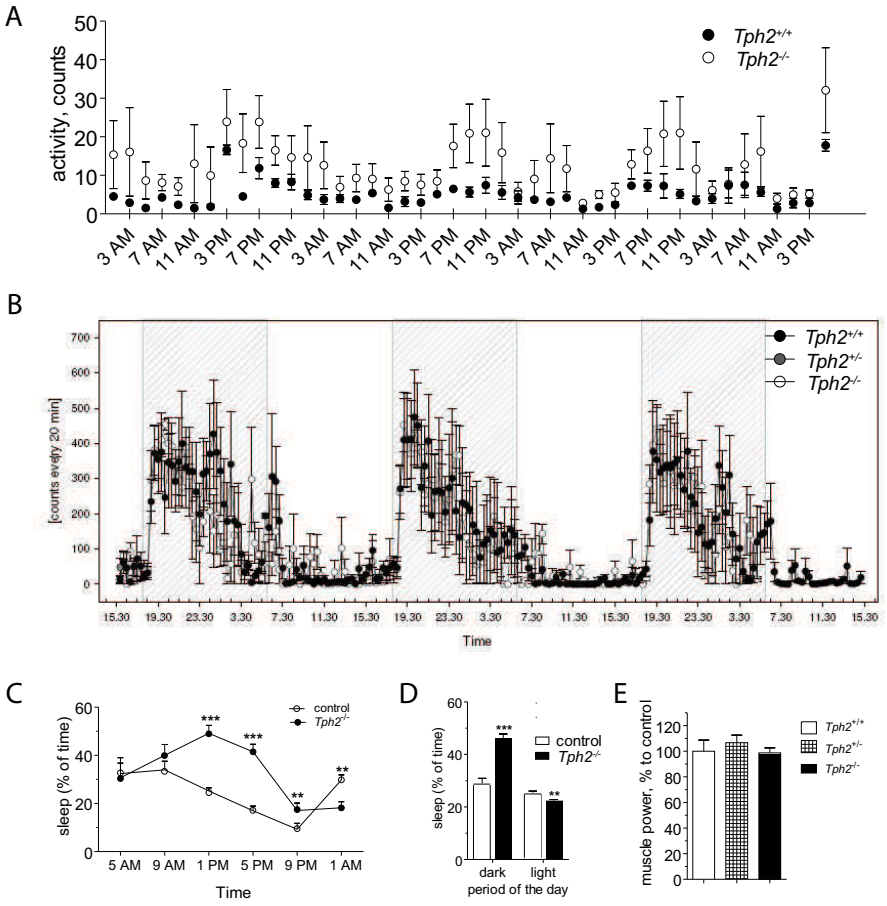

Figure 16: Activity, sleep, and muscle power in *Tph2*-deficient mice. A) Day-night activity of $Tph2^{+/+}$ and $Tph2^{-/-}$ mice over four days assessed by telemetric measurements. B) Locomotor activity of $Tph2^{+/+}$, $Tph2^{+/-}$, and $Tph2^{-/-}$ mice measured by InfraMot system in the home cage over three consecutive days. C) Sleep: % of time mice displayed no activity during 5 min period. Data are averaged over for 5 consecutive days. D) Sleep periods over dark and light phases. E) Grip test: muscle power, displayed as % of controls. Data are shown as means ± SEM. ***$p < 0.001$, **$p < 0.01$ vs. control, one-way ANOVA with Bonferroni correction.

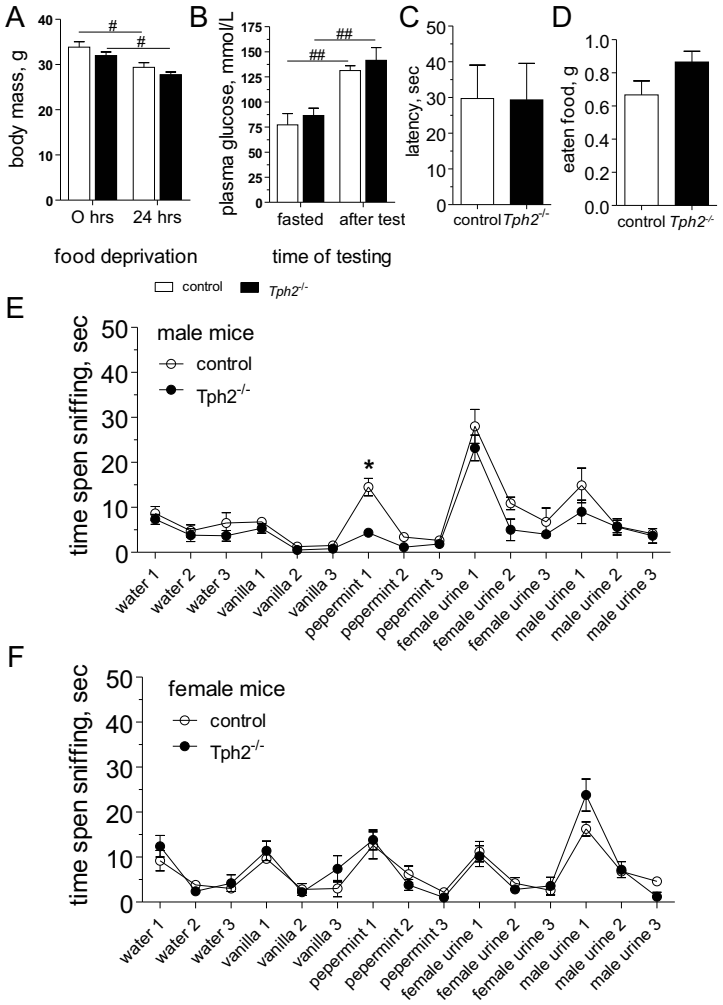

Figure 17: Olfaction in *Tph2*-deficient mice.

Hidden cookie test A) Body weight and B) Glucose levels before and 24 hrs after food removal. C) Latency to find the hidden cookie. D) Amount of eaten food after finding the hidden cookie during 30 min.

Habituation/dishabituation test: time male E) or female F) mice spent sniffing water (control), vanilla, peppermint, and the urine of female and male mouse.

*p < 0.05 vs. $Tph2^{+/+}$, #p<0.05, ##p<0.001, one-way ANOVA

more, the amount of eaten food and glucose levels after the feeding were the same in *Tph2*$^{-/-}$ and control mice Figure 17(B,D).
In another test for olfaction, the habituation-dishabituation test, *Tph2*-deficient mice could distinguish when the same odor was introduced a second or third time. *Tph2*$^{-/-}$ mice sniffed equal amounts of time in comparison to control mice at the cotton stick dipped into all odor solutions besides the peppermint Figure 17(E,F) when male *Tph2*$^{-/-}$ mice sniffed this odor less during the first introduced time. The longest sniffing time was observed for the urine of the opposite gender. When this odor was introduced second and third time the sniffing time decreased equally in *Tph2*$^{-/-}$ mice and control animals Figure 17(E).

4.2.4 *Aggressive behavior*

In the RI test, *Tph2*$^{-/-}$ mice attacked the intruder almost six times faster than *Tph2*$^{+/+}$ mice (p=0.0002) Figure 18(A). Furthermore, the number of attacks and the cumulative attack duration in the *Tph2*$^{-/-}$ versus *Tph2*$^{+/+}$ group were elevated sevenfold (p=0.0014 and p=0.01, respectively) Figure 18(B,C). A qualitative analysis of attacks revealed a striking difference between *Tph2*-deficient and *Tph2*$^{+/+}$ mice: within 5 min of the test all mutant animals displayed aggressive bouts, while only 22% of *Tph2*$^{+/+}$ mice showed such behavior.

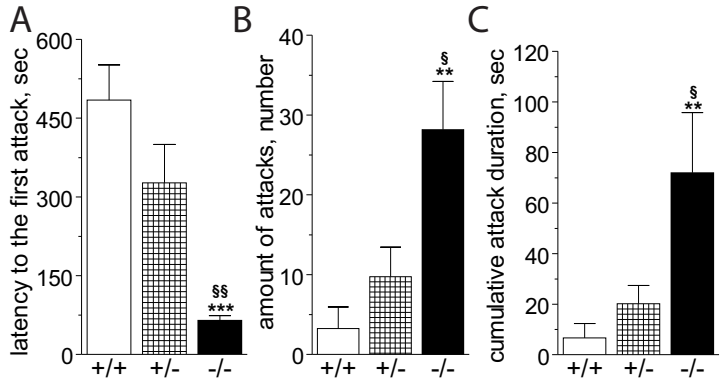

Figure 18: Aggressive behavior in *Tph2*-deficient mice. **Resident-intruder test**: A) Attack latency: time between the introduction of intruder and the first attack of the resident. B) Total amount of attacks by the resident. C) Cumulative attack duration during 10 min tested time. Data are shown as means ± SEM. **p<0.01, ***p<0.001 vs.*Tph2*$^{+/+}$, §p< 0.05, §§p<0.01 vs.*Tph2*$^{+/-}$, one-way ANOVA with Bonferroni correction.

Though *Tph2*$^{+/-}$ mice tended to show an intermediate state of aggressive behavior between *Tph2*$^{-/-}$ and *Tph2*$^{+/+}$ mice, neither the differences in the first attack latency nor the number of attacks were significantly different between *Tph2*$^{+/-}$ and *Tph2*$^{+/+}$ mice Figure 18(A,B)

4.2.5 *Anxiety-like behavior*

In EPM, *Tph2*$^{-/-}$ mice spent significantly more time in the open arms than *Tph2*$^{+/+}$ and *Tph2*$^{+/-}$ (p=0.0161 and p=0.0133, respectively) Figure 19(C). *Tph2*-deficient mice also exhibited twice the amount of open arm entries (p=0.0026 vs. *Tph2*$^{+/+}$, p=0.0054 vs. *Tph2*$^{+/-}$) Figure 19(D). However, total arm entries and total distance travelled were comparable between mice of all three genotypes Figure 19(B).

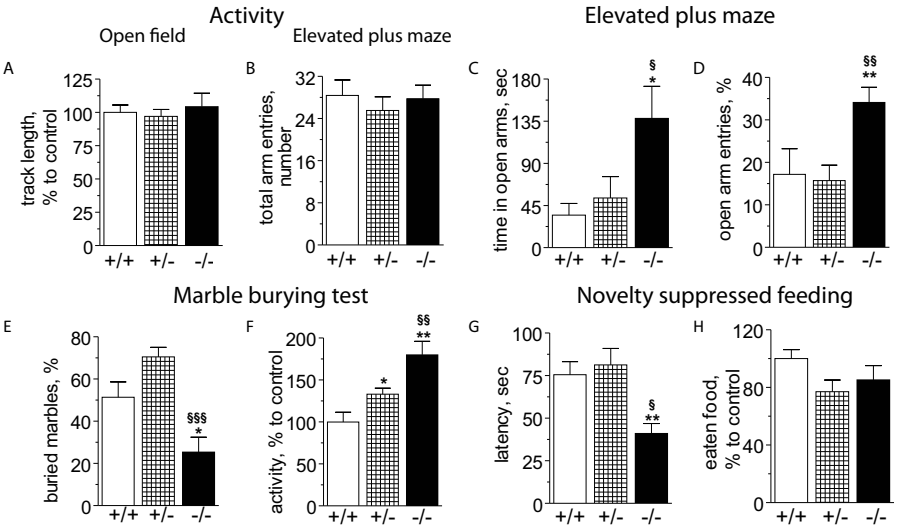

Figure 19: Locomotor activity and anxiety-like behavior in *Tph2*-deficient mice.
Open field: A) Total distance travelled.
Elevated plus maze: B) Activity: total number of entries to closed and open arms. C) Percentage of entries to open arms: entries to open arm/total arm entries. D) Total time spent in open arms.
Marble burying test: E) Percentage of buried marbles: buried/introduced amount of marbles. F) Locomotor activity during MBT.
Novelty supressed feeding: G) Latency to feed. H) Consumed amount of food during 5 min after reaching the food pellet.
Data are shown as means ± SEM. *p<0.05, **p<0.01 vs.*Tph2*$^{+/+}$, §p< 0.05, §§p< 0.01, §§§p<0.05 vs.*Tph2*$^{+/-}$, one-way ANOVA with Bonferroni correction.

Analysis of locomotion in the EPM over time showed that *Tph2*$^{-/-}$ mice extensively explored the brightest illuminated part of the open arms already during the first 5 min of testing, while *Tph2*$^{+/+}$ animals did not enter the distal parts of the open arms during the whole 10 min of the test. *Tph2*$^{+/-}$ mice did not show any significant difference compared to *Tph2*$^{+/+}$ mice neither in the total time spent in open arms nor in the open arm entries Figure 19(C,D).
The amount of marbles buried by *Tph2*$^{-/-}$ mice in the MBT was significantly lower than that of *Tph2*$^{+/+}$ and *Tph2*$^{+/-}$ animals (p=0.0199 and p<0.0001, respectively) Figure 19(E). Interestingly, the general ac-

tivity of *Tph2*-/- animals during this test was almost two fold higher than that of $Tph2^{+/+}$ (p=0.0046) Figure 19(F). There was no significant difference in the percentage of marbles buried by $Tph2^{+/-}$ mice compared to $Tph2^{+/+}$ mice Figure 19(E). However, $Tph2^{+/-}$ showed an intermediate activity, significantly different from both $Tph2^{-/-}$ and $Tph2^{+/+}$ mice (p=0.009 and p=0.023, respectively) Figure 19(F).
In the NSF task, $Tph2^{-/-}$ mice needed less time to reach and start eating the food pellet in the center of the area compared to $Tph2^{+/+}$ and $Tph2^{+/-}$ (p=0.002 and p=0.017, respectively) Figure 19(G). Food consumption, evaluated during the five minutes following the test did not differ between the genotypes Figure 19(H). $Tph2^{+/-}$ mice did not show a significant difference in the latency to reach and start eating the food in comparison to both, $Tph2^{+/+}$ and $Tph2^{-/-}$ Figure 19(G,H).

4.2.6 *Depresssion-like behavior*

In the FST, $Tph2^{+/-}$ mice did not show any significant difference in comparison to $Tph2^{+/+}$ in the total immobility time or the latency to the first immobility episode, whereas $Tph2^{-/-}$ mice demonstrated reduced struggling behavior Figure 20. They spent less time swimming until the first immobility episode (p=0.0001) Figure 20(A) and stayed longer immobile compared with $Tph2^{+/-}$ and $Tph2^{+/+}$ littermates (p=0.005, in comparison to both genotypes) Figure 20(B) . Moreover, $Tph2^{-/-}$ mice showed an increase in immobility time during each single 2 min episode compared with $Tph2^{+/+}$ mice Figure 20(B).

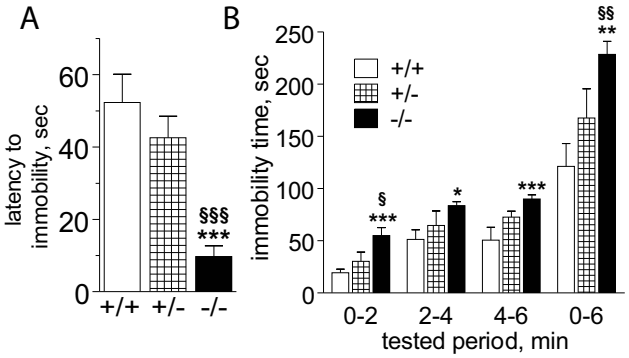

Figure 20: Depression-like behavior in *Tph2*-deficient mice. **Forced swim test**: A) Latency to the first immobility episode. B) Immobility time during 2 min intervals and the whole 6 min of tested period. Data are shown as means ± SEM. *p<0.05, ***p<0.001, **p<0.01 vs.$Tph2^{+/+}$, §p< 0.05, §§p<0.01, §§§p<0.001 vs.$Tph2^{+/-}$, one-way ANOVA with Bonferroni correction.

4.2.7 *Effect of fluoxetine in Tph2-deficient mice*

One of the first antidepressant, fluoxetine (FLX), has a capacity to influence anxiety- and depression-like behavior [136, 210]. Therefore, next effects of FLX treatment in mice lacking central serotonin were assessed. Baseline levels of *Sert* expression were not different between control and *Tph2*$^{-/-}$ mice Figure 21. Interestingly, after two weeks of oral treatment with FLX *Sert* expression was more than 3 times up-regulated in controls. However, the amount of *Sert* mRNA did not change in the brain of *Tph2*$^{-/-}$ mice after FLX challenge Figure 21.

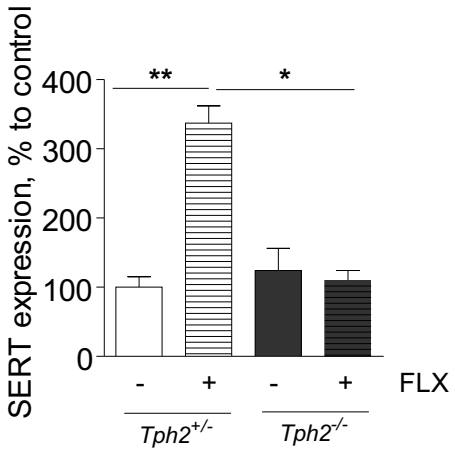

Figure 21: *Sert* expression in *Tph2*-deficient mice. Real-time PCR analysis of *Sert* expression in the whole brain. AU - arbitrary units, FLX - fluoxetine. *p < 0.05, **p < 0.01 two-way ANOVA with Bonferroni correction.

Effect of chronic FLX treatment was assessed first in the EPM. The low anxiety phenotype, observed in *Tph2*$^{-/-}$ mice Figure 19(C,D), was reversed by FLX treatment. After two weeks of FLX treatment *Tph2*$^{-/-}$ mice spent less time in the open arms than untreated *Tph2*-deficient mice Figure 22(A). Moreover, after FLX treatment *Tph2*$^{-/-}$ mice remained in the open arms as long as untreated control mice. FLX treatment did not have any effect on anxiety level in control mice as *Tph2*$^{+/+}$ mice spent the same amount of time in the open arms Figure 22(A).

Effect of chronic FLX treatment on depression-like behavior was assessed in the FST. As it was reported above, *Tph2*$^{-/-}$ mice have reduced latency to the first immobility episode, and increased immobility time Figure 20(A,B), Figure 22(B, C). Two weeks of FLX treatment in 2 different groups of male mice resulted in 35% decrease in the amount of time being immobile in *Tph2*$^{-/-}$ mice compared to untreated mice of the same genotype ($p<0.01$) Figure 22(C). In contrast, FLX treatment did not have any effect on the latency to the first immobility episode in *Tph2*-deficient mice Figure 22(B). In fact, FLX treatment did not have any influence on the immobility time in control mice

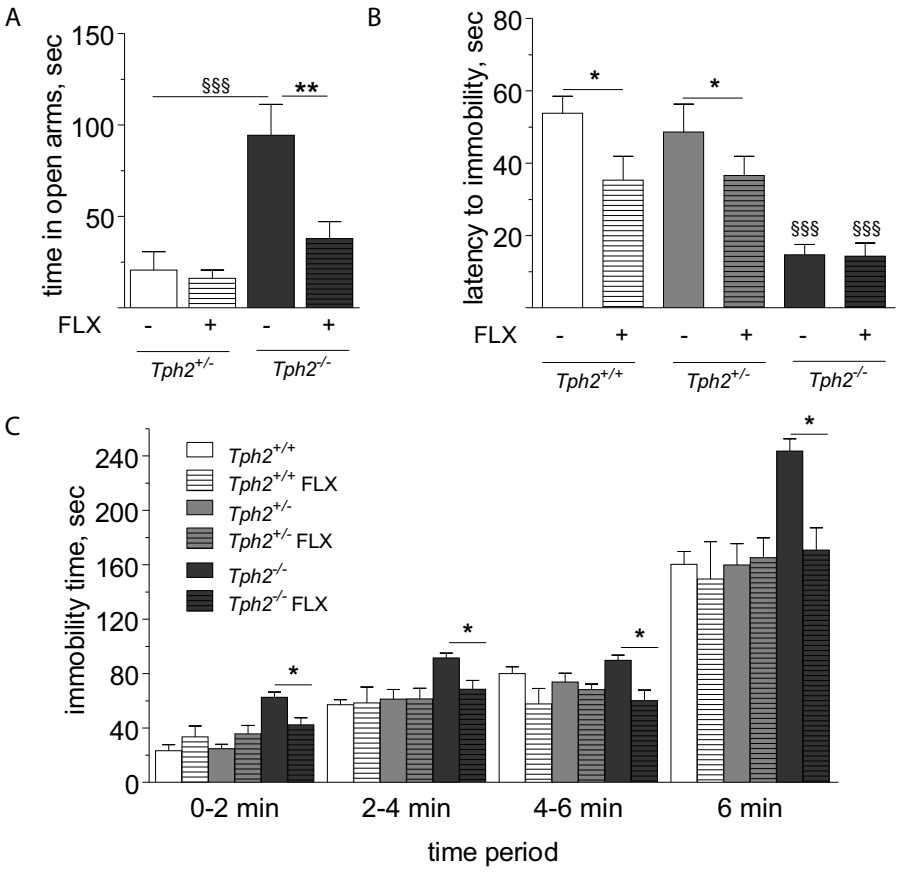

Figure 22: Anxiety- and depression-like behavior of *Tph2*-deficient mice after two weeks of oral fluoxetine treatment.
Elevated plus maze: A) Time spent in the open arms.
Forced swim test B) Latency to the first immobility episode. C) Immobility time during 2 min intervals and the whole 6 min of tested period.
*p<0.05, **p<0.05 vs. untreated mice of marked genotype, §§§p<0.001 vs. untreated control mice, one-way ANOVA with Bonferroni correction.

Figure 22(C), even though the first immobility episode tended to occur earlier than in untreated mice (p>0.05) Figure 22(B).

4.2.8 *Learning and memory*

Y-MAZE The percentage of spontaneous alternations was evaluated in a Y-maze, a test which is known to be sensitive to impairments in working memory. Both *Tph2*$^{-/-}$ and control mice completed the task with the same degree of alternation which reached almost 70% Figure 23(A). *Tph2*-deficient and control mice exhibit as well the same amount of total arm entries confirming similar activity in the arena.

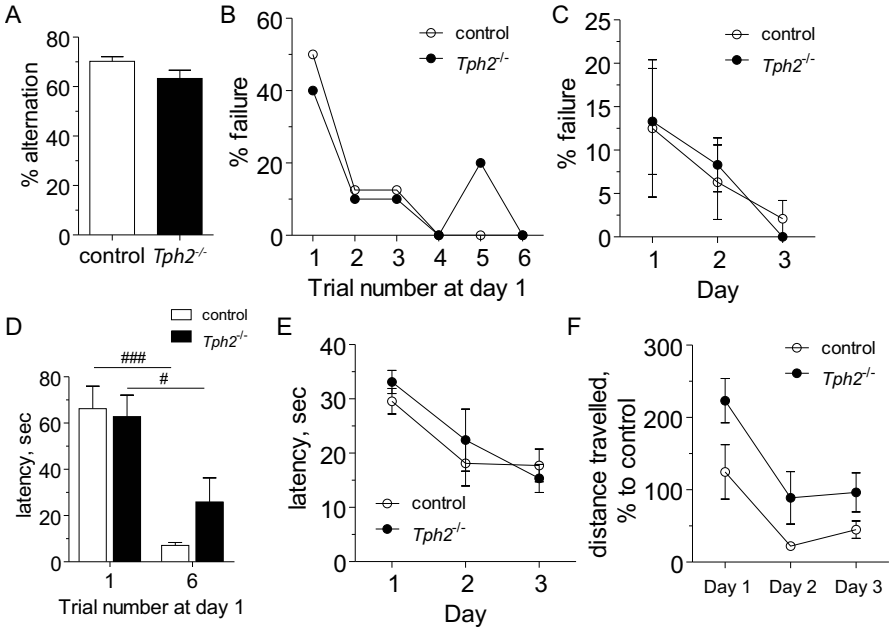

Figure 23: Memory in *Tph2*-deficient mice.

Y-maze: A) % alternations: number of nonrepeating triads divided by maximal possible alternations

Morris water maze: Percentage of failures to find the platform B) over trials 1-6 at day 1 of testing or C) at the days 1-6 calculated as average of failures made over trials 1-6. Latency to find the platform D) at day 1 of testing and E) at days 1 to 3 calculated as average distance needed to find the platform. F) Distance travelled at days 1-3 calculated as average from 6 trials. The averaged distance over trial 1-6 at day 1 control mice needed to reach the platform is taken as 100%.

$^{\#}p< 0.05$, $^{\#\#\#}p<0.001$ vs. mice of the same genotype of a marked condition, two-way ANOVA with Bonferroni correction.

MORRIS WATER MAZE Both *Tph2*$^{-/-}$ and control mice learned the navigation task very rapidly. On the first testing day the amount of failures to find the platform declined from trial 1 (40% for *Tph2*$^{-/-}$ and 50% for control mice) to trial 6 (all mice successfully reached the platform) Figure 23(B,C). Already on the first day of testing the amount of time needed to find the hidden platform decreased from trial 1 to trial 6 Figure 23(D). Cumulative statistics over 6 trials for each day showed no difference between the latency to find the hidden platform in *Tph2*$^{-/-}$ and control mice Figure 23(E). The distance travelled to the platform as well decreased from Day 1 to Day 3 for both *Tph2*$^{-/-}$ and control mice Figure 23(F) indicating that the path became more direct from the place where the mouse was placed to the platform.

4.2.9 *Neurogenesis*

Adult neurogenesis and memory formation are closely connected [104]. Thus, next neurogenesis in adult mice lacking central serotonin was assessed. First the amount of proliferating cells in adult *Tph2*$^{-/-}$ mice was quantified. The number of proliferating cells in *Tph2*-deficient mice 24 hours after the first bromodeoxyuridine (BrdU) injection did not differ compared to controls Figure 24(A,B).
Next the survival of neural precursor cells in the adult hippocampus following three weeks after BrdU injections was assessed. The number of surviving precursor cells were not different between *Tph2*$^{-/-}$ and control mice Figure 24(C,D).
Then the amount of proliferating and surviving cells in adult *Tph2*$^{-/-}$ mice after three weeks of FLX treatment was quantified. There was no difference in the amount of proliferating as well as surviving cells in *Tph2*$^{-/-}$ mice treated with saline or FLX Figure 24(B,D). Furtermore, the number of proliferating and surviving precursor cells were not different between *Tph2*$^{-/-}$ and control mice Figure 24(C,D).

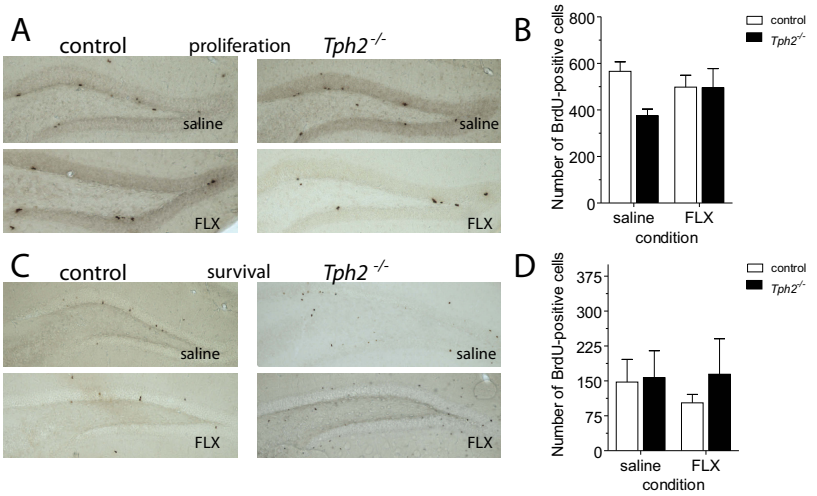

Figure 24: Neural proliferation in the adult hippocampus of *Tph2*-deficient mice at baseline and under fluoxetine treatment.
Cell proliferation: A) Representative images showing no effect of central serotonin deficiency as well as fluoxetine treatment on proliferating cells. B) Quantification of the number of BrdU-positive cells in the subgranular zone (SGZ) 24 h after the first of three i. p. injections of BrdU, after 3 weeks of either saline or fluoxetine (10 mg/kg/day) i.p. injection.
Cells survival: C) Representative images showing no difference in the neuronal cell survival in *Tph2*$^{-/-}$ mice at baseline and under fluoxetine (FLX) treatment in comparison to control mice. D) Quantification of surviving BrdU-positive cells in SGZ after 3 weeks of i. p. injections of BrdU and three weeks of either of saline or fluoxetine treatment (10 mg/kg/day).

4.3 EMOTIONAL AND NEUROCHEMICAL ABNORMALITIES IN MICE CARRYING *Tph2* HYPOMORPHIC AND NULL ALLELES

4.3.1 *Anxiety- and depression-like behavior*

First the effect of gradual reduction in *Tph2* activity on emotional behavior was investigated. To this end, I tested C57Bl/6 mice ($Tph2^{C/C}$), congenic C57Bl/6 mice homozygous for the hypomorphic *Tph2* 1473G allele ($Tph2^{G/G}$), heterozygous *Tph2*-deficient mice ($Tph2^{C/-}$), and mice carrying one *Tph2* 1473G and one null-allele ($Tph2^{G/-}$) in three paradigms for anxiety-like behavior and in two models of depression.
In the EPM, $Tph2^{C/C}$, $Tph2^{G/G}$, $Tph2^{C/-}$, and $Tph2^{G/-}$ mice spent equal time in the open arms of the maze Figure 25 (A, B). Moreover, on the maze animals of all genotypes showed the same activity measured as total arm entries Figure 25 (C).

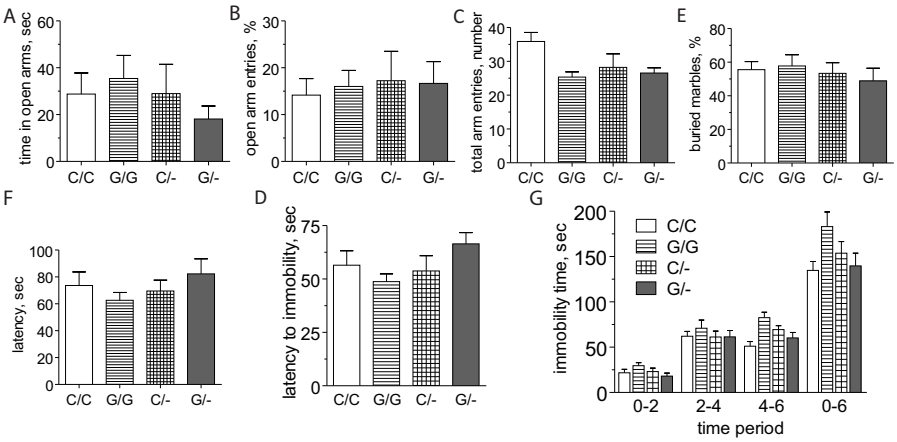

Figure 25: Anxiety- and depression-like behavior.
Elevated plus maze: A) Total time spent in open arms. B) Percentage of entries to open arms: entries to open arm/total arm entries. C) Activity: total number of entries to closed and open arms.
Marble burying test: D) Percentage of buried marbles.
Novelty supressed feeding: E) Latency to the first eating episode.
Forced swim test: F) Latency to the first immobility episode. G) Immobility time during 2 min intervals and 6 min of tested period .

In the MBT, the amount of buried marbles was equal for all tested groups Figure 25 (D).
In the third paradigm, NSF, $Tph2^{G/G}$, $Tph2^{C/-}$, and $Tph2^{G/-}$ mice were as quick as $Tph2^{C/C}$ mice to reach and start eating the food pellet in the middle of the tested arena Figure 25 (E). Food consumption, evaluated during the five minutes following the test did also not differ between the genotypes.

In the FST, $Tph2^{G/G}$, $Tph2^{C/-}$, and $Tph2^{G/-}$ mice did not show any significant difference in comparison to $Tph2^{C/C}$ animals in the latency to the first immobility episode Figure 25 (F). Moreover, mice of all genotypes stayed immobile for the same amount of time during each two min intervals and during the total test duration (6 min) Figure 25 (G).

4.3.2 *Serotonin content and metabolism*

Next the impact of the *Tph2*1473G polymorphism and the genetic deletion of one *Tph2* allele on serotonin levels in the brain was evaluated. $Tph2^{G/G}$ and $Tph2^{C/-}$ mice showed a 9 and 13% reduction in central 5-HT levels, respectively, relative to $Tph2^{C/C}$ mice, Figure 26(A), Table 11. Surprisingly, carriers of just one copy of the hypomorphic *Tph2*1473G allele, $Tph2^{G/-}$ mice, showed only a 19% reduction in serotonin content in comparison to $Tph2^{C/C}$ mice Figure 26(A), Table 11.

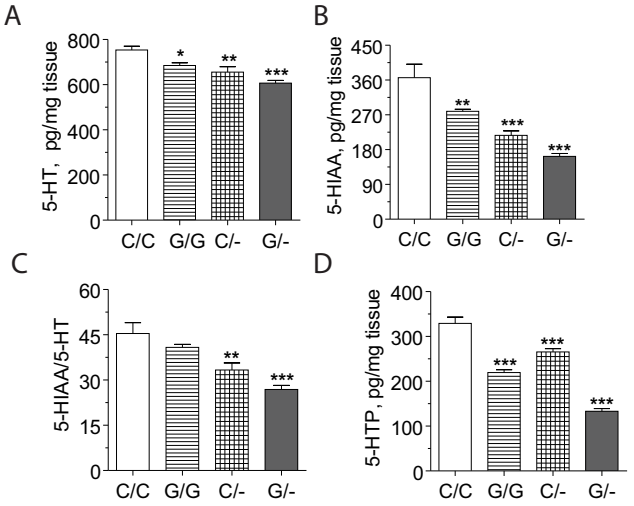

Figure 26: Brain serotonin content and synthesis rate (HPLC measurement). 5-HT levels (A), 5-HIAA levels (B), and 5-HT turnover (ratio between 5-HIAA and 5-HT) (C) in the whole brain lysates. D) 5-HT *in vivo* synthesis: 5-HTP levels in the whole brain 1 hr after NSD administration (100 mg/kg, i.p.). *p<0.05, **p<0.01, ***p<0.001 vs. $Tph2^{C/C}$, one way ANOVA with Bonferroni's correction

To understand the reasons for such a subtle reduction in brain 5-HT content in these mice next the 5-HT synthesis rate was analysed by blocking conversion of 5-HTP to 5-HT by NSD. The accumulation of 5-HTP was decreased by 33, 19, and 59% in $Tph2^{G/G}$, $Tph2^{C/-}$, and $Tph2^{G/-}$ mice, respectively, in comparison to $Tph2^{C/C}$ animals Figure 26(D), Table 11.

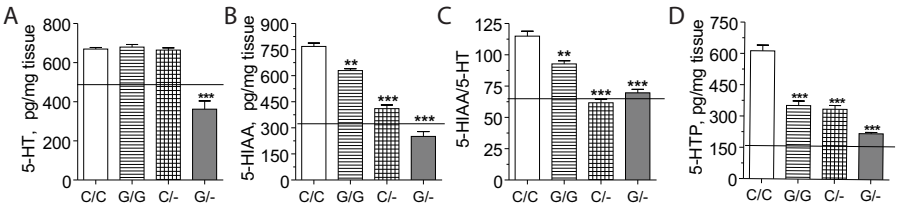

Figure 27: Brain serotonin content and synthesis rate 1hr after tryptophan treatment (100mg/kg, i.p., HPLC measurement). 5-HT levels (A), 5-HIAA levels (B), and 5-HT turnover (ratio between 5-HIAA and 5-HT) (C) in whole brain lysates. D) 5-HT *in vivo* synthesis : 5-HTP levels in the whole brain 1 hr after NSD administration (100 mg/kg, i.p.). *p<0.05, **p<0.01, ***p<0.001 vs. *Tph2*$^{C/C}$ (one way ANOVA with Bonferroni's correction). Horizontal lines reflect the levels measured in untreated *Tph2*$^{C/C}$ mice.

Despite considerable differences in the serotonin synthesis rate observed between the strains, they still did not reach the values predicted from the *in vitro* activity of *Tph2*1473G (50% of *Tph2*1473C) [151, 224] and inactivation of one *Tph2* allele, which results in a 50% reduction in *Tph2* transcript levels Figure 15(A).

	5-HT (% to *Tph2*$^{C/C}$)	**5-HT/ FLX** (% to untreated *Tph2*$^{C/C}$)	**5-HIAA** (% to *Tph2*$^{C/C}$)	**5-HIAA/5-HT** (% to *Tph2*$^{C/C}$)	**5-HTP/ NSD** (% to *Tph2*$^{C/C}$)
Tph2$^{C/C}$	100.0±2.6	74±2.9$^{###}$	100.0±9.5	100.0±8.76	100.0±4.3
Tph2$^{G/G}$	90.9±1.5*	78.7±0.7$^{##}$	76.2±1.4**	84.1±2.1*	66.7±2.3***
Tph2$^{C/-}$	87.0±3.2**	67.3±1.9$^{###}$	59.2±3.2***	68.1±4.8**	80.7±2.2***
Tph2$^{G/-}$	80.52±1.6***	42.7±1.8$^{###,***}$	44.4±2.1***	55.3±2.8***	40.5±1.9***

Table 11: Serotonin content and synthesis rate at baseline and after 2 weeks of oral fluoxetine treatment (20 mg/kg/d, HPLC measurement). Metabolite levels are presented as % of *Tph2*$^{C/C}$ levels. 5-HT levels after FLX treatment are presented as % to untreated (water dirnking) *Tph2*$^{C/C}$ mice. NSD was given i.p. in a dose of 100 mg/kg 1hr before brain removal. Data are presented as means ± SEM. *p<0.05, **p<0.01, ***p<0.001 vs. *Tph2*$^{C/C}$, $^{##}$p<0.01, $^{###}$p<0.001 vs. untreated mice of the same genotype (one way ANOVA with Bonferroni's correction). FLX – fluoxetine, NSD – 3-hydroxybenzylhydrazine dihydrochloride.

I hypothesized that this discrepancy may originate from a lack of the substrate tryptophan (Trp) [63] and therefore evaluated the impact of acute Trp administration on serotonin level and synthesis rate. After acute Trp treatment, the amount of accumulated 5-HTP reached 50%

in *Tph2*$^{G/G}$ and *Tph2*$^{C/-}$ animals and only 25% in *Tph2*$^{G/-}$ mice in comparison to Trp-treated *Tph2*$^{C/C}$ mice Figure 27(D), reflecting the 5-HT synthesis pattern expected from *Tph2* activity and expression.
However, the serotonin level after Trp injection was elevated to similar values in *Tph2*$^{C/C}$, *Tph2*$^{G/G}$, and *Tph2*$^{C/-}$ mice Figure 27(A). In contrast, *Tph2*$^{G/-}$ mice failed to increase their brain serotonin values following Trp administration, which remained below the level of untreated control mice Figure 27(A).
The reason for the almost indistinguishable serotonin levels in *Tph2*$^{C/C}$, *Tph2*$^{G/G}$, and *Tph2*$^{C/-}$ animals at both baseline and after Trp administration became evident after the evaluation of the serotonin degradation product, 5-HIAA. 5-HIAA was significantly decreased by 18, 47, and 67% in *Tph2*$^{G/G}$, *Tph2*$^{C/-}$, and *Tph2*$^{G/-}$ mice, respectively, in comparison to *Tph2*$^{C/C}$ animals at baseline Figure 26(B), Table 11 and by 24, 41 and 56%, respectively, in comparison to *Tph2*$^{C/C}$ mice after Trp administration Figure 27(B). As a result at both conditions — baseline and after Trp administration — there was a marked reduction in serotonin turnover (5-HIAA/5-HT) in all mutant mouse lines in comparison to the wild type *Tph2*$^{C/C}$ mice Figure 26(C), Figure 27(C), Table 11.
In the last series of experiments we evaluated the possible effects of chronic fluoxetine (FLX) treatment on serotonin metabolism in the brain of mice with reduced serotonin synthesis. Two weeks after oral FLX treatment the levels of central serotonin were reduced by around 20% in *Tph2*$^{C/C}$, *Tph2*$^{C/-}$, and *Tph2*$^{G/G}$ and by half in *Tph2*$^{G/-}$ mice in comparison to control groups with water drinking mice of the same genotypes Table 11.

4.3.3 *5-HT$_{1A}$ receptor function*

To evaluate the function of 5-HT$_{1A}$ autoreceptors in conditions of gradually reduced *Tph2* activity, we tested the body temperature response to the single injection of 5-HT$_{1A}$ receptor agonist, 8-OH DPAT. There was no difference between *Tph2*$^{C/C}$, *Tph2*$^{C/-}$, *Tph2*$^{G/G}$ and *Tph2*$^{G/-}$ mice at baseline temperature measured 30 and 15 min before treatment Figure 28(A) or in response to saline injection. However, 15 minutes after the injection of 8-OH DPAT there was a drastic drop of body temperature in *Tph2*$^{G/G}$ and *Tph2*$^{G/-}$ mice by 3.5°C and 4.5°C, respectively Figure 28(B), whereas only a slight decrease in body temperature was observed in *Tph2*$^{C/C}$ and *Tph2*$^{C/-}$ mice Figure 28(B). Furthermore, the hypothermic response was completely abolished in *Tph2*$^{-/-}$ mice Figure 28(B). The difference in body temperature in *Tph2*$^{G/G}$ mice persisted for one hour after 8-OH DPAT administration. Furthermore, the body temperature of *Tph2*$^{G/-}$ mice did not normalize even after 90 minutes had elapsed, whereas all other genotypes had returned to baseline level by this time point Figure 28(A). The

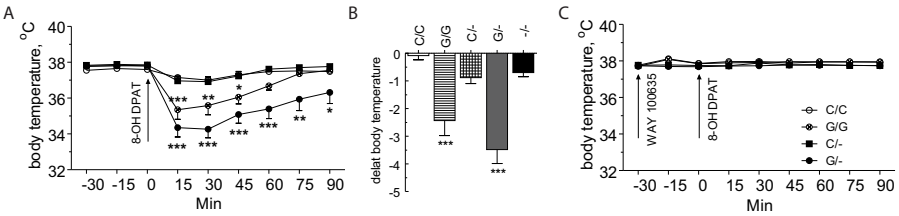

Figure 28: 5-HT$_{1A}$ receptor agonist-induced hypothermia. A) Temperature response to the 5-HT$_{1A}$ receptor agonist, 8-OH DPAT. B) Temperature drop 15 min after 8-OH-DPAT injection: delta body temperature between time point 0 and 15 min. C) Temperature response to the 5-HT$_{1A}$ receptor agonist after injection of the selective blocker of the 5-HT$_{1A}$ receptor, WAY 100635 (0.1 mg/kg i.p. at the time point -30 min). *p < 0.05, **p < 0.01, ***p < 0.001, vs. *Tph2*$^{C/C}$ under the same treatment. (ANOVA for repeated measurements with Bonferroni's correction).

hypothermic response was completely abolished by administration of WAY 100635, a selective 5-HT1A receptor antagonist, 30 min prior to 8-OH DPAT injection in all mouse strains Figure 28(C).

5 DISCUSSION

5.1 ROLE OF CENTRAL SEROTONIN IN POSTNATAL DEVELOPMENT

In the first part of my thesis postnatal growth but not embryonic development was shown to be dependent on brain serotonin.
Tph2$^{-/-}$ mice are depleted in brain serotonin by more than 95% [4], and exhibit growth retardation during the first weeks of postnatal life [4], Figure 9. Interestingly, another mouse model with impaired serotonin storage, thus as well depleted in brain serotonin, *Vmat2*$^{Sert-cre}$, exhibit almost identical growth alterations. These mice are characterized by normal weight at birth and most pronounced growth stunting during the first 3 postnatal weeks corresponding to the pre-weaning period. Furthermore, in the *Vmat2*$^{Sert-cre}$ mice, as in the *Tph2*$^{-/-}$ mouse pups, a normalization of weight gain occurred after weaning. Similarly, a growth retardation phenotype was observed in *Pet1*-knockout and *Lmx1b*$^{Pet1-cre}$ mice [92]. Furthermore, *VMAT2* ablation in *Pet1*-expressing cells in mice leads to 75% decrease in central serotonin [146], and results in a similar trend and temporal course of growth alterations, albeit to a lower degree [145]. It is possible that such moderate transient growth retardation may exist in other hyposerotonergic mice, such as the R439H *Tph2* knockin mice with 80% reduction in central 5-HT levels [17]. However, no data about growth of these mice have been reported.
In spite of life-long serotonin depletion, there is no visible alterations in body and brain embryonic development in *Tph2*$^{-/-}$ mice Figure 9. Instead, growth abnormalities became visible only during postnatal life. This is unlike observations in mice born from *Tph1*-deficient mothers, which were reported to display altered embryonic growth [42]. This suggests that the maternal source of 5-HT, rather than embryonic 5-HT, is a crucial determinant of embryonic growth. Alternatively, it is also possible that other embryonic sources of 5-HT in the body or the placenta could compensate for the lack of 5-HT production in the brain [205, 197]. Another explanation is that somatic growth relies on different trophic factors during embryonic and postnatal life, with embryonic growth being dependent on maternal and placental factors [181].
The growth phenotype in *Tph2*-deficient mice as well as in other hyposerotonergic models mentioned above, resembles the one caused by general malnutrition with dry skin, lack of adipose tissue, and reduction in organ size. Experiments performed with the rat pups that were underfed during the first two weeks of life show a similar

phenotype as *Tph2*$^{-/-}$ mice accompanied with increased lethality rate, growth retardation, and impaired temperature regulation in adulthood [143]. Moreover, in *Tph2*$^{-/-}$ pups this phenotype is accompanied by diminished plasma IGF-1 and leptin levels Table 9. However, despite their emaciated appearance *Tph2*$^{-/-}$ mouse pups had filled milk pouches and appeared to suckle normally, suggesting that they had no feeding abnormalities. A similar phenotype was observed in *Vmat2*$^{Sert-cre}$. This is unlike what has been previously observed in the constitutive *Vmat2*-deficient mouse, who had clear feeding abnormalities with empty milk pouches and weak suckling activity during early postnatal life [7, 66]. *Vmat2*-deficient mice showed massive depletion of catecholamines in addition to 5-HT depletion. The altered feeding behavior and the related growth deficiency is hardly corrected when normal 5-HT brain levels are reinstated [42], suggesting a causal role of the dopamine deficit in the altered feeding phenotype. Finally, measurements to reduce competition among pups had beneficial effects on survival, but no visible effects on growth, suggesting that altered access to the nipples was not a primary cause of growth alterations. This suggested that an intrinsic metabolic defect may occur in the *Tph2*$^{-/-}$ mice. Indeed, telemetric measurement indicated an impaired body temperature and altered respiratory control in *Tph2*-deficient mice [4], which may be an indirect cause of growth alteration. However, attempts to maintain the pups in normothermia had no visible effects on the growth phenotype Figure 11.

A remaining possibility to explain why decreased 5-HT causes the exclusive deficit in growth during the early postnatal period could involve the control of the somatotropic axis and growth hormone (GH) secretion. Although in the *Tph2*$^{-/-}$ mice GH synthesis in the pituitary gland was not altered Figure 11, it is possible that its secretion may be modified. Indeed, previous evidence indicated that 5-HT modulates GH release in two different ways. On the one hand it can directly activate the anterior pituitary gland [153]. On the other hand it can facilitate GH release indirectly via a hypothalamic control of GHRH and somatostatin (SST) secretion that control GH release [186]. GH serum levels are particularly elevated during the first postnatal weeks. 5-HT control of GH secretion appeared to be particularly marked during this period [192]. Thus, GH release is less dependent on central 5-HT after weaning which could explain growth normalization in *Tph2*$^{-/-}$ mice after weaning. SST, being activated by 5-HT also represses GHRH, resulting in a net inhibition of GH release. This inhibitory 5-HT-SST circuit may get more important during postnatal life and could explain the compensation after weaning, since then the lack of 5-HT would derepress the secretion of GH. However, there was no evidence for this hypothesis in hypothalamic gene expression profile of *Tph2*$^{-/-}$ mice Table 8.

Another possible cause of growth retardation in the postnatal period

is inability to digest mother's milk enriched with fat. However, giving chow enriched with fat starting at the second week after birth even improved the growth of *Tph2*-deficient pups Figure 12. Apart from the hypotheses of HPA-axis disruption or improper fat digestion as a cause of postnatal growth retardation in $Tph2^{-/-}$ pups, the nature of this phenotype could lie in some behavioral functions such as ultrasonic vocalisation of pups. Pups vocalisation attracts attention of the mother, thus notifying the mother that a pup needs some care. Loss of maternal care leads to starvation, decrease in core body temperature of pups, and thus may result in malnutrition and death [93, 111]. However, the reduced USV observed in $Tph2^{-/-}$ pups is not necessarily the primary cause of growth retardation. *Tph2*-deficient pups may vocalize less since they are already weaker at P3 Figure 14. A recent report about impaired sexual behavior in $Tph2^{-/-}$ mice revealed as well impaired vocalisation to a social stimulus in these mice in adulthood [125]. However, in this paper there was no data about pup vocalisation at the first days of their life. Impaired pups' USV was reported in two models targeting serotonin receptors — 5-HT_{1A} [216] and 5-HT_{1B} [30] receptor knockout mice. However, these mice are not reported to be growth retarded.

5.2 *Tph2*-deficiency and its psychological consequences

The implication of brain serotonin in animal behavior has been recognized already in the last century. Most of the studies were conducted using pharmacological or genetic inhibition of serotonin reuptake and 5-HT receptors. However, the role of serotonin per se in these studies was not completely clarified, because no suitable animal model was available yet.
In the second part of my thesis mice with reduced activity of serotonin-synthesising enzyme in the brain on a pure genetic background were used to evaluate the consequences of complete absence of this neurotransmitter in the CNS on behavior and brain neurochemistry.

Unchanged activity of *Tph2*-deficient mice in the home cage and in the new enviroment As any of the behavior tests used in this study could be influenced by changes in animal activity, first *Tph2*-deficient mice were examined for any alterations in locomotion. Neither activity in the open field (new environment), nor home cage activity measured by telemetry recording or InfraMot system were different between $Tph2^{-/-}$ mice and control animals Figure 16. These findings go in line with other studies in $Tph2^{-/-}$ mice [176, 210] as well as in other hyposerotonergic mouse models such as $Lmx1b^{Pet1-cre}$ [227], *Pet1*-knockout [89], and *Sert*-overexpressing mice [100]. In contrast, decreased locomotor activity was reported in mice with impaired *Vmat2* expression, *Vmat2* heterozygous and

Vmat2$^{Sert-cre}$ mice [69, 146]. However, observed changes in locomotion in these mouse models could arise from changes in other neurotransmitter systems.

NO CHANGES IN ODOR DISCRIMINATION IN *Tph2*-DEFICIENT MICE Impaired serotonin neurotransmission might impair olfactory sensation [161], thereby affecting the performance in behavior tests. Therefore, prior behavior testing, female and male mice were tested for the ability to detect and distinguish different odours. No major difference in the response to unsocial or social stimuli in two tests for olfaction was detected Figure 17.

EXAGGERATED AGGRESSION IN *Tph2*-DEFICIENT MICE Serotonin has been postulated to play a role in aggression [131, 201]. Low cerebrospinal 5-HIAA was correlated with elevated aggression in humans [37, 38, 190] and monkeys [221]. Furthermore, low-Trp diet resulted in increased aggressive behavior in humans [222], whereas tryptophan enriched diet initiated a reduction of physical aggression in subjects that had a history of elevated aggression [144]. Several genetic variations in serotonergic genes have been linked to impulsive aggression in humans [86, 177]. Moreover, a positive correlation between low serotonin release and increased aggression was confirmed by microdialysis in freely moving animals during the RI test [204, 64]. Inhibition of serotonin synthesis in rats led to increased aggressiveness, while enhancement of serotonin transmission suppressed aggressive behavior [130].
Using *Tph2*$^{-/-}$ mice I showed that central serotonin deficiency led to highly increased aggressive behavior in mice Figure 18. Interestingly, this phenotype was observed not only in males, but also in *Tph2*$^{-/-}$ females [4]. Thus, this model provides strong evidence for increased aggression as a consequence of complete serotonin deficiency in the CNS being in line with 2 recently published hyposerotonergic animal models, TPH2 R439H knockin mice [17], and Pet-1 deficient animals [89, 177].
It was recently reported that the absence of brain serotonin leads to increased male-male mounting behavior in a 30 minutes social interactions task [125]. This phenotype was not prominent during 10 minutes of the resident-intruder test performed in this study. Moreover, in several cases I had to interrupt the test due to the extreme aggressiveness of *Tph2*-deficient animals. It can not be excluded that defensive behavior of serotonin-deficient animals was misinterpreted in the study of Liu et al. [125].

DEPRESSION-LIKE PHENOTYPE IN *Tph2*-DEFICIENT MICE A role of serotonin in the etiology of depressive disorders was suggested more than 50 years ago [28]. Later on formulation of the monoami-

nergic theory of depression led to the development of antidepressive drugs which increase the monoaminergic activity [128]. Moreover, severely depressed patients treated with Trp or 5-HTP show symptomatic improvement [206, 165], whereas, giving tryptophan-free diet to depressed individuals elicits a relapse in patients getting treatment with antidepressants [139, 179]. Mice depleted in brain serotonin exhibited a lack of motivation to struggle in the FST that can be interpreted as a depression-like phenotype, supporting the monoaminergic theory of depression Figure 20.

There is some discrepancy regarding this phenotype between these results and recent data showing a slight anti-depressive effect in *Tph2*-deficient mice on the second day of FST [176]. These conflicting findings could be due to several reasons, such as different analysis methods - automated [176] versus manual (this study) [14] - or the two day FST protocol [176], which is commonly used for identifying a depressive state in rats versus the one day protocol usually performed for mice [46, 56]. Moreover, the study of depression-like behavior by Savelieva et al. [176] was performed on a mixed genetic background (C57Bl/6Jx129S5/S), that may have masked the behavioral effect of *Tph2* gene ablation. Interestingly, there are reports that show an increased immobility time in the TST in another genetic model of central serotonin depletion — heterozygous *VMAT2*-knockout mice [69]. However, in these mice levels of other neurotransmitters are also changed and, therefore, the altered behavior in TST could not be interpreted as only due to the depletion of central serotonin. Surprisingly, when *VMAT2* was ablated only in SERT-positive neurons, the behavior in TST was reversed: $Vmat2^{Sert-cre}$ mice showed a clear anti-depressive phenotype in the TST [146]. However, these animals were also on a mixed genetic background that may have veiled the effect of central serotonin ablation. In the first description of *Tph2*-deficient mice [176] no differences in the TST was observed at the first day of experiment. Although both tests, TST and FST, are widely used for the screening of antidepressants, the validity of these tasks to evaluate symptoms of intrinsic depressive behavior is not so clear [45]. Moreover, the sensitivity of these two tests to pharmacological drugs is not identical, indicating that different neurochemical pathways may mediate the performance in these tasks [13]. Additionally, mice being in two different inescapable situations (wet conditions in FST and dry in TST) could use different strategies to struggle.

Several previous studies failed to detect any drastic alteration in depression-like behavior in models of serotonin depletion after PCPA treatment [72, 149, 166]. The clear depression-like phenotype observed in the FST in *Tph2*-deficient mice can be a consequence of a life long depletion in serotonergic transduction versus short term effects of PCPA.

DECREASED ANXIETY-LIKE BEHAVIOR IN *Tph2*-DEFICIENT MICE

The behavioral evaluation performed in this study showed that *Tph2*-deficient mice have decreased level of aversive behavior in approach-avoidance-conflict tests Figure 19. It correlates with the hypothesis that enhanced serotonergic transmission in the brain facilitates anxiety, whereas a decrease in extracellular 5-HT leads to reduced anxious behavior. This hypothesis, formulated in early the 1970-ies [217] was further refined employing animal models with 5-HT depletion by serotonin synthesis inhibition or lesions of serotonergic neurons [8, 27, 44, 187]. Furthermore, studies in *Sert* overexpressing and *Sert*-deficient mice [9, 94, 100], 5-HT_{1A}-deficient animals [76], as well as in very recently published hyposerotonergic mouse models including *Lmx1b*-, *Pet1*-, or *Vmat2*-deficient animals [48, 107, 146] correlate with this hypothesis. Despite being in line with the low-anxiety phenotype, observed in the EPM and NSF tests, the results of the MBT poorly correlate with literature data from other genetic models affecting the serotonergic system [176, 177, 226]. The opposite effects observed in this study may originate from the differences in the genetic background (pure C57Bl/6 used in this study vs. mixed in other studies) — a factor which may strongly affect serotonin-related behavior, as already shown in *Sert*-knockout mice [94]. On the other hand, the experimental setup used in this study was not identical to the one of other studies: the protocols differ in several aspects, such as amount of marbles, cage parameters, and test conditions. Moreover, one can not exclude, that increased locomotion, unexpectedly observed during MBT and not reported in other studies, had an impact on results of this test in our experiments.

UNAFFECTED MEMORY IN *Tph2*-DEFICIENT MICE

Serotonin fibers form a dense network in the hippocampus. Most of the serotonergic fibers originate from median raphe, although both median and dorsal raphe innervate the ventral part of the hippocampus [68, 77]. Furthermore, serotonin fibers form dense projections to the hippocampus during gestation. There is as well a notable expression of 5-HT_{1A}, 5-HT_{2A}, and 5-HT_{2C} receptors in hippocampus [49, 108, 156]. Serotonin is considered to play an important role in memory formation [31, 174, 218]. However, evaluation of hippocampal function in serotonergic lesion models revealed controversial results [1, 97, 163]. In order to clarify the role of central serotonin in memory formation spatial memory in the water maze task and the Y-maze test was assessed in $Tph2^{-/-}$ mice. Central serotonin ablation did not lead to dramatic changes in spatial navigation in these paradigms, suggesting that 5-HT has no direct effect on memory formation, or its effects are masked by the compensatory mechanisms under the condition of life-long central serotonin depletion.

Unperturbed precursor cell proliferation and survival at baseline and after fluoxetine treatment in *Tph2*-deficient mice Neurogenesis, formation of new granule cells, and memory formation are closely connected [104]. Newly developed cells are formed from the precursor cells in the subgranular zone (SGZ) [5, 32, 103]. Numerous works explored the connection between hippocampal function and 5-HT_{1A} receptor deficiency or overexpression. Acute deletion of 5-HT through pharmacological treatment [15, 108] or 5-HT synthesis inhibition or lesion approaches by injecting the neurotoxin 5,7-DHT [26] revealed controversial results which may arise due to further severe impairments caused by the drug treatment. However, until now there was no report elucidating how specific ablation of central serotonin affects hippocampal neurogenesis. It is suggested that the antidepressant effect of some antipsychotics is accomplished through hippocampal neurogenesis [175]. Accordingly, a positive effect of FLX treatment on newly generated cells was reported [133, 132, 175]. FLX is one of the mostly used antidepressant, and it inhibits primarily the serotonin transporter thus increasing the amount of 5-HT in the synaptic cleft. Surprisingly, at baseline neither proliferation nor survival of precursor cells were affected by life long absence of serotonin Figure 24. Furthermore, no effect of FLX treatment on neuronal cell proliferation or on survival was observed neither in control nor in *Tph2*$^{-/-}$ mice. It is known that neurogenesis is decreased with age [18, 112, 180]. Taking into the account the age of the animals used in the study, I can not exclude that the effect of FLX or central serotonin ablation was very subtle and undetectable in the small numbers of proliferating/surviving cells seen at this age. Further experiments with younger mice need to be performed in order to clarify the consequence of central serotonin loss at baseline and after FLX treatment.

Normalised anxiety- and depression-like behavior in *Tph2*-deficient mice after fluoxetine treatment Antidepressive drugs were discovered before their mechanism of action was identified. However, even today we cannot explain why treatment with SSRI takes 2-3 weeks before one can see any behavior changes in patients. Moreover, there are some patients who do not react or show the opposite to expected behavioral responses to these drug. *Tph2*$^{-/-}$ mice represent an exclusive model to identify probable targets of SSRIs which are independent of serotonin reuptake. In this study I showed that 2 weeks of oral FLX treatment reduces immobility time in the FST in *Tph2*-deficient mice indicating normalization of depression-like behavior Figure 22. Furthermore, SSRI treatment in *Tph2*$^{-/-}$ mice led to the stabilization of anxiety-like behavior in EPM Figure 22. These data strongly argue for existance of FLX targets be-

yond the serotonin system, and further experiments need to be done in order to unravel these pathways. One of the possible targets could be GSK3β. In R439H TPH2 knockin mice GSK3β was shown to be responsible for the observed behavior phenotype and antidepressant actions [17]. In other studies it was shown that FLX can bind directly to various serotonergic receptors (5-HT_{1A} or 5-HT_{2C}) with higher or lower affinity. Furthermore, at high concentrations it can block the noradrenaline transporter. In case of complete central serotonin deficiency these secondary targets could become more important for FLX action.

ADEQUATE BEHAVIORAL RESPONSES IN MICE WITH GRADUALLY DECREASED TPH2 ACTIVITY IS ASSOCIATED WITH REDUCED SEROTONIN TURNOVER AND 5-HT1A RECEPTOR SENSITIZATION Next I aimed to clarify the consequences of gradual reduction of TPH2 activity in mice. For this purpose, a new mouse model carrying one hypomorphic *Tph2* allele and one *Tph2* null allele ($Tph2^{G/-}$ mice) was generated. For comparison wild type C57Bl/6 mice ($Tph2^{C/C}$), and two previously characterized strains with around 50% reduction in TPH2 activity (congenic C57Bl/6 mice homozygous for the *Tph2* 1473G allele ($Tph2^{G/G}$) [194] and $Tph2^{C/-}$ mice, carrying one 1473C allele and one null allele [4, 140] were used.

First I addressed the question whether a gradual decrease in TPH2 activity leads to alterations in manifestation of anxiety- and depression-like behavior similiar to $Tph2^{-/-}$ mice.

Evaluation of mouse behavior in three tests for anxiety (EPM, MBT, and NSF) and FST for depression-like behavior revealed no difference in $Tph2^{C/-}$ and $Tph2^{G/G}$ mice in comparison to $Tph2^{C/C}$ animals Figure 25. Surprisingly, a further decrease in TPH2 activity (in $Tph2^{G/-}$ mice) did not have any impact on mouse behavior in these paradigms, either.

Interestingly, the measurement of brain serotonin levels revealed only a subtle reduction by around 20% in $Tph2^{G/-}$ mice and almost unaffected levels of central serotonin in $Tph2^{C/-}$ and $Tph2^{G/G}$ mice Table 11. These findings suggest the existence of compensatory mechanisms involved in the normalisation of brain serotonin levels and the maintenance of intact behavior in conditions of life-long reduced TPH2 activity. In the search for such mechanisms serotonin metabolism in models with partially reduced TPH2 activity was assessed. Evaluation of *in vivo* serotonin production clearly indicated that in all studied models the serotonin synthesis rate, despite being reduced, did not correspond to the expected values predicted from the *in vitro* measurements of TPH2 activity ($Tph2^{G/G}$ mice) [194, 224] or *Tph2* mRNA expression levels ($Tph2^{C/-}$ mice) Figure 15.

Tryptophan (Trp) availability is a critical factor affecting serotonin

synthesis rate, and under normal physiological conditions TPH2 is only 50% saturated with its substrate [63]. Therefore, brain serotonin levels are dependent on the plasma concentration of free Trp and on the plasma levels of the other large neutral amino acids which compete with Trp for the large amino acid transporter at the blood brain barrier. Indeed, administration of Trp in mice with partial TPH2 inactivation rendered the 5-HT synthesis rate strictly correlated to the predicted TPH2 activity in these animals Figure 27.
Nevertheless, the serotonin levels in the brain did not reflect its *in vivo* synthesis rate in the studied mouse models neither at baseline nor after Trp administration Figure 27. The evaluation of serotonin turnover revealed that 5-HT content is mostly regulated at its degradation level, which was stepwise decreased in mice with a gradual reduction in 5-HT synthesis rate. Recently, a similar observation was made in *Tph2*$^{G/G}$ congenic mice by another group [183]. However, analysis of mRNA expression and in vitro activity of the serotonin degrading enzymes MAO-A and MAO-B failed to identify any substantial differences between the strains that could be responsible for the lower 5-HT degradation rate [183]. My data support these findings since treatment with 5-HTP or Trp drastically increased not only serotonin levels in the brain, but also lead to a higher accumulation of its degradation product, 5-HIAA, arguing that *in vivo* MAO processivity mostly depends on substrate availability. The amount of 5-HT readily available for MAO-mediated degradation is, however, dependent not only on the serotonin synthesis rate, but also on the activity of the vesicular monoamine transporter, VMAT2. The balance between packaging of 5-HT into the synaptic vesicles, where it is protected from MAO-mediated oxygenation, and degradation of the monoamine, which remains in the cytosol, provides a fine tuning of the serotonin concentration in the cell. The monoamine storage in vesicles is regulated by a G-protein dependent feedback loop that allows modulating VMAT-mediated uptake based on the degree of vesicular filling [95]. This regulation of VMAT activity was shown to be dependent on the G-protein alpha-subunits of Go2 and Gq in neurons and platelets, respectively [96]. In this respect, it is conceivable that the Go2 dependent regulation of VMAT2 activity plays a role in the process of elimination of excessive serotonin, and, thus, in the stabilization of intraneuronal serotonin concentration.
Another potential mechanism facilitating an adaptation of the serotonergic system to reduced TPH2 activity in mice is the modulation of the 5-HT$_{1A}$ autoreceptor function on serotonergic neurons. 5-HT$_{1A}$ is a key component of a negative feedback loop implicated in the inhibition of 5-HT release upon activation of these cells [54]. The desensitization of 5-HT$_{1A}$ receptors is a well-known phenomenon in models with permanently increased 5-HT transmission triggered by genetic ablation of SERT [60, 120] or its pharmacological inhibition by SSRIs,

such as FLX [115, 119]. Surprisingly, attempts to identify an increase in 5-HT_{1A} expression or receptor density in different hyposerotonergic models revealed either little or no difference [78, 99, 110]. To study autoreceptor function in mice with reduced TPH2 activity, I evaluated the hypothermic response to the 5-HT_{1A} receptor agonist 8-OH DPAT, which is known to be mediated by 5-HT_{1A} auto- but not heteroreceptors [168]. Analysis of 8-OH-DPAT induced hypothermia clearly indicated a functional sensitization of 5-HT1A autoreceptor in mice with life-long reduced TPH2 activity Figure 28. Whether this sensitisation occurs at the level of receptor expression, trafficking, or its downstream signalling needs to be clarified [54].
Several studies revealed a link between reduced 5-HT_{1A} function and increased anxiety-like behavior in mice [76, 87, 154], whereas activation of 5-HT_{1A} autoreceptors via agonist infusion decreases anxiety-like behavior as measured in different behavior paradigms [50, 170]. Whether autoreceptor hypersensitivity observed in this study plays a role in the maintenance of normal behavior response in anxiety-related tests per se, or is just a marker of a renewed steady-state of the serotonergic system established in conditions of life-long reduced serotonin synthesis has to be further elucidated.
Since 2004, when a functional C1473G polymorphism was discovered in the mouse *Tph2* gene, several studies aimed to elucidate its implication in behavior. Congenic mice carrying this SNP on a C57Bl/6 genetic background were independently generated by several groups [20, 151, 194]. Evaluation of serotonin content and metabolism revealed a similar profile in all these mice, showing around 30% reduction in serotonin *in vivo* synthesis rate in this and other [20, 183] studies. However, this decrease resulted in little or no (ranging from 0 to 10 %) decrease in serotonin levels in different brain areas and in the whole brain [20, 183, 194], whereby the small difference between the studies in the serotonin content may arise from the time of the day, and the food consumption and composition, which may differ between the labs. However, extensive evaluation of behavioral traits in these mouse strains revealed controversial results, such as a lack of difference in anxiety- (EPM, MBT and NSF) and depression-like behavior (TST [183], FST performed in this study and by others [194]); lowered aggression, no differences in anxiety in the open field test, and decreased immobility time in the FST [151]; elevated anxiety (EPM, elevated zero maze, light-dark exploration test, and novelty induced hypophagia), but no difference in the depression-like behavior at baseline and after chronic mild stress in TST, FST, sucrose preference test, and learned helplessness [20]. This inconsistency in behavior data obtained from congenic mice carrying the Tph21473G allele on a pure genetic background could be partially due to different number of generations (3 to 8) the animals were backcrossed [20, 151, 183]. It can also not be excluded that additional genetic traits

may have been inherited with the *Tph2* polymorphic allele during the backcrossing by the different groups resulting in elevated anxiety [20] or more pronounced depression-like behavior [62]. Moreover, the observed discrepancy in the outcome of behavior testing could lie in some small but essential methodological differences such as the illumination used during anxiety tests, the housing conditions (group and cage sizes, enrichment, lightning) and the testing conditions (for example diameter and height of the beaker used in FST) [160].
This work has implications for human antidepressant therapy. SSRIs and other measures which increase brain serotonin levels, such as food supplementation with Trp, are widely used in humans for the treatment of anxiety and depression-like disorders. Here I evaluated the effect of such treatments on serotonin levels in brains of mice with reduced TPH2 activity. Trp administration led to a substantial elevation of brain serotonin levels in $Tph2^{C/C}$, $Tph2^{G/G}$, and $Tph2^{C/-}$ Figure 27. However, in $Tph2^{G/-}$ mice it resulted only in a mild increase in serotonin content, which remained clearly below the level of untreated WT mice. Even more drastic differences between the mouse strains were observed after the chronic treatment with the SSRI FLX Table 11. While control animals showed a slight decrease in brain serotonin levels in line with previous findings [7], this drop was much more pronounced in mice with reduced TPH2 activity reaching a 60% reduction in overall serotonin levels in $Tph2^{G/-}$ mice compared to untreated controls. Thus, the outcome of antidepressant therapy may be dramatically affected in conditions of reduced TPH2 activity, leading to inefficiency or possibly even to adverse effects of SSRIs.

6

CONCLUSIONS

In the first part of this study I showed that serotonin is not required for embryonic growth, but is crucial for postnatal development. Postnatal growth retardation in central serotonin deficient mice was not due to dysfunction of the hypothalamo-pituitary-adrenal axis, impaired thermoregulation, or improper food digestion but could originate from impaired pups' ultrasonic vocalisation.
In the second part of this study I showed that a partial decrease in brain serotonin has no influence on behavior while complete brain serotonin ablation leads to strong behavioral consequences determined by exaggerated aggression, increased depression-like beha- vior, and decreased anxiety. In conditions of reduced serotonin production a lowered serotonin degradation rate and 5-HT_{1A} receptor sensitization contribute to the maintenance of brain serotonin at le- vels which are sufficient for adequate behavior responses.
Moreover, my data point out that the antidepressant fluoxetine may implement at least part of its actions through serotonin-independent mechanisms. Furthermore, fluoxetine treatment leads to a drastic reduction in serotonin levels in the brain in conditions of reduced TPH2 activity, which may negatively affect the outcome of antidepressant therapy.

7

ABBREVIATIONS

5-HIAA — 5-hydroxyinole acetic acid
5-HT — serotonin
5-HTP — 5-hydroxytryptophan
A — amygdala
AADC — aromatic aminoacid decarboxylase
ADH — alcohol dehydrogenase
BDNF — brain-derived neurotrophic factor
BH_4 — tetrahydrobiopterin
BrdU — bromodeoxyuridine
BS — brain stem
C — cortex
CNS — central nervous system
DA — dopamine
DOPAC — 3,4-dihydroxyphenylacetic acid
EPM — elevated plus maze
FC — frontal cortex
FLX — fluoxetine
FST — forced swim test
GABA — gamma-aminobutyric acid
GH — growth hormone
HFD — high fat diet
HIOMT — hydroxy-indole-O-methyl transferase
Hip — hippocampus
HPA — hypothalamo-pituitary-adrenal axis
HPLC — high-performance liquid chromatography
HVA — homovanillic acid
IGF — insulin-like growth factor
LSD — lysergic acid diethylamine
MAO — monoamine oxidase
MBT — marble burying test
MWM — Morris water maze
NA — noradrenaline
NE — norepinephrine
ND — normal diet
NM — normetanephrine
NSD — 3-hydroxybenzylhydrazine
NSF — novelty suppressed feeding
Ob — olfactory balb
OF — open field
PAH — phenylalanine hydroxylase

PCPA — parachlorphenylalanine
PFA — paraformaldehyde
PVN — paraventricular nuclei
RI — resident intruder
RR — rostral raphe
SD — standard diet
SERT — selective serotonin transporter
SGZ — subgranular zone
SN — substancia nigra
SNAT — serotonin N-acetyltransferase
SNP — single nucleotide polymorphism
SSRI — selective serotonin transporter inhibitor
SST — somatostatin
T — thalamus
TBP — TATA box binding protein
TH — tyrosine hydroxylase
TPH — tryptophan hydroxylase
Trp — tryptophan
TST — tail suspension test
USV — ultrasonic vocalisation
VMAT — vesicular monoamine transporter

BIBLIOGRAPHY

[1] W. Adams, S. Kusljic, and M. van den Buuse. Serotonin depletion in the dorsal and ventral hippocampus: effects on locomotor hyperactivity, prepulse inhibition and learning and memory. *Neuropharmacology*, 55(6):1048–55, 2008.

[2] Y. Ago, Y. Koyama, A. Baba, and T. Matsuda. Regulation by 5-ht1a receptors of the in vivo release of 5-ht and da in mouse frontal cortex. *Neuropharmacology*, 45(8):1050–6, 2003.

[3] P. R. Albert, Q. Y. Zhou, H. H. Van Tol, J. R. Bunzow, and O. Civelli. Cloning, functional expression, and mrna tissue distribution of the rat 5-hydroxytryptamine1a receptor gene. *J Biol Chem*, 265(10):5825–32, 1990.

[4] N. Alenina, D. Kikic, M. Todiras, V. Mosienko, F. Qadri, R. Plehm, P. Boye, L. Vilianovitch, R. Sohr, K. Tenner, H. Hortnagl, and M. Bader. Growth retardation and altered autonomic control in mice lacking brain serotonin. *Proc Natl Acad Sci U S A*, 106(25):10332–7, 2009.

[5] J. Altman and G. D. Das. Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *J Comp Neurol*, 124(3):319–35, 1965.

[6] C. Alvarez, T. Vitalis, E. A. Fon, N. Hanoun, M. Hamon, I. Seif, R. Edwards, P. Gaspar, and O. Cases. Effects of genetic depletion of monoamines on somatosensory cortical development. *Neuroscience*, 115(3):753–64, 2002.

[7] J. C. Alvarez, M. Sanceaume, C. Advenier, and O. Spreux-Varoquaux. Differential changes in brain and platelet 5-ht concentrations after steady-state achievement and repeated administration of antidepressant drugs in mice. *Eur Neuropsychopharmacol*, 10(1):31–6, 1999.

[8] T. G. Andrade, C. E. Macedo, Jr. Zangrossi, H., and F. G. Graeff. Anxiolytic-like effects of median raphe nucleus lesion in the elevated t-maze. *Behav Brain Res*, 153(1):55–60, 2004.

[9] M. S. Ansorge, M. Zhou, A. Lira, R. Hen, and J. A. Gingrich. Early-life blockade of the 5-ht transporter alters emotional behavior in adult mice. *Science*, 306(5697):879–81, 2004.

[10] C. H. Asbreuk, C. F. Vogelaar, A. Hellemons, M. P. Smidt, and J. P. Burbach. Cns expression pattern of lmx1b and coexpression

with ptx genes suggest functional cooperativity in the development of forebrain motor control systems. *Mol Cell Neurosci*, 21(3):410–20, 2002.

[11] E. Audero, E. Coppi, B. Mlinar, T. Rossetti, A. Caprioli, M. A. Banchaabouchi, R. Corradetti, and C. Gross. Sporadic autonomic dysregulation and death associated with excessive serotonin autoinhibition. *Science*, 321(5885):130–3, 2008.

[12] G. Baccini, B. Mlinar, E. Audero, C. T. Gross, and R. Corradetti. Impaired chemosensitivity of mouse dorsal raphe serotonergic neurons overexpressing serotonin 1a (htr1a) receptors. *PLoS One*, 7(9):e45072, 2012.

[13] F. Bai, X. Li, M. Clay, T. Lindstrom, and P. Skolnick. Intra- and interstrain differences in models of "behavioral despair". *Pharmacol Biochem Behav*, 70(2-3):187–92, 2001.

[14] M. Baker. Animal models: inside the minds of mice and men. *Nature*, 475(7354):123–8, 2011.

[15] M. Banasr, M. Hery, R. Printemps, and A. Daszuta. Serotonin-induced increases in adult cell proliferation and neurogenesis are mediated through different and common 5-ht receptor subtypes in the dentate gyrus and the subventricular zone. *Neuropsychopharmacology*, 29(3):450–60, 2004.

[16] N. M. Barnes and T. Sharp. A review of central 5-ht receptors and their function. *Neuropharmacology*, 38(8):1083–152, 1999.

[17] J. M. Beaulieu, X. Zhang, R. M. Rodriguiz, T. D. Sotnikova, M. J. Cools, W. C. Wetsel, R. R. Gainetdinov, and M. G. Caron. Role of gsk3 beta in behavioral abnormalities induced by serotonin deficiency. *Proc Natl Acad Sci U S A*, 105(4):1333–8, 2008.

[18] N. M. Ben Abdallah, L. Slomianka, A. L. Vyssotski, and H. P. Lipp. Early age-related changes in adult hippocampal neurogenesis in c57 mice. *Neurobiol Aging*, 31(1):151–61, 2010.

[19] D. Bengel, O. Johren, A. M. Andrews, A. Heils, R. Mossner, G. L. Sanvitto, J. M. Saavedra, K. P. Lesch, and D. L. Murphy. Cellular localization and expression of the serotonin transporter in mouse brain. *Brain Res*, 778(2):338–45, 1997.

[20] S. M. Berger, T. Weber, S. Perreau-Lenz, M. A. Vogt, S. E. Gartside, C. Maser-Gluth, L. Lanfumey, P. Gass, R. Spanagel, and D. Bartsch. A functional tph2 c1473g polymorphism causes an anxiety phenotype via compensatory changes in the serotonergic system. *Neuropsychopharmacology*, 37(9):1986–98, 2012.

[21] J. M. Bessa, A. R. Mesquita, M. Oliveira, J. M. Pego, J. J. Cerqueira, J. A. Palha, O. F. Almeida, and N. Sousa. A trans-dimensional approach to the behavioral aspects of depression. *Front Behav Neurosci*, 3:1, 2009.

[22] M. A. Bicalho, G. J. Pimenta, F. S. Neves, H. Correa, E. N. de Moraes, L. De Marco, and M. A. Romano-Silva. Genotyping of the g1463a (arg441his) tph2 polymorphism in a geriatric population of patients with major depression. *Mol Psychiatry*, 11(9 number):799–800, 2006.

[23] R. D. Blakely. Overview: a rare opportunity or just one less reason to be depressed. *Neuron*, 48(5 number):701–2; author reply 705–6, 2005.

[24] P. Blier and C. de Montigny. Electrophysiological investigation of the adaptive response of the 5-ht system to the administration of 5-ht1a receptor agonists. *J Cardiovasc Pharmacol*, 15 Suppl 7:S42–8, 1990.

[25] S. E. Breidenthal, D. J. White, and C. E. Glatt. Identification of genetic variants in the neuronal form of tryptophan hydroxylase (tph2). *Psychiatr Genet*, 14(2):69–72, 2004.

[26] J. M. Brezun and A. Daszuta. Depletion in serotonin decreases neurogenesis in the dentate gyrus and the subventricular zone of adult rats. *Neuroscience*, 89(4):999–1002, 1999.

[27] M. Briley, P. Chopin, and C. Moret. Effect of serotonergic lesion on "anxious" behaviour measured in the elevated plus-maze test in the rat. *Psychopharmacology (Berl)*, 101(2):187–9, 1990.

[28] B. B. Brodie, A. Pletscher, and P. A. Shore. Evidence that serotonin has a role in brain function. *Science*, 122(3177):968, 1955.

[29] S. M. Brown, E. Peet, S. B. Manuck, D. E. Williamson, R. E. Dahl, R. E. Ferrell, and A. R. Hariri. A regulatory variant of the human tryptophan hydroxylase-2 gene biases amygdala reactivity. *Mol Psychiatry*, 10(9):884–8, 805, 2005.

[30] D. Brunner, M. C. Buhot, R. Hen, and M. Hofer. Anxiety, motor activation, and maternal-infant interactions in 5ht1b knockout mice. *Behav Neurosci*, 113(3):587–601, 1999.

[31] M. C. Buhot, S. Martin, and L. Segu. Role of serotonin in memory impairment. *Ann Med*, 32(3):210–21, 2000.

[32] H. A. Cameron, C. S. Woolley, B. S. McEwen, and E. Gould. Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat. *Neuroscience*, 56(2):337–44, 1993.

[33] T. Canli, E. Congdon, L. Gutknecht, R. T. Constable, and K. P. Lesch. Amygdala responsiveness is modulated by tryptophan hydroxylase-2 gene variation. *J Neural Transm*, 112(11):1479–85, 2005.

[34] L. Carnevali, F. Mastorci, E. Audero, G. Graiani, S. Rossi, E. Macchi, S. Callegari, A. Bartolomucci, E. Nalivaiko, F. Quaini, C. Gross, and A. Sgoifo. Stress-induced susceptibility to sudden cardiac death in mice with altered serotonin homeostasis. *PLoS One*, 7(7):e41184, 2012.

[35] G. L. Chen, E. J. Vallender, and G. M. Miller. Functional characterization of the human tph2 5′ regulatory region: untranslated region and polymorphisms modulate gene expression in vitro. *Hum Genet*, 122(6):645–57, 2008.

[36] S. Cichon, I. Winge, M. Mattheisen, A. Georgi, A. Karpushova, J. Freudenberg, Y. Freudenberg-Hua, G. Babadjanova, A. Van Den Bogaert, L. I. Abramova, S. Kapiletti, P. M. Knappskog, J. McKinney, W. Maier, R. A. Jamra, T. G. Schulze, J. Schumacher, P. Propping, M. Rietschel, J. Haavik, and M. M. Nothen. Brain-specific tryptophan hydroxylase 2 (tph2): a functional pro206ser substitution and variation in the 5′-region are associated with bipolar affective disorder. *Hum Mol Genet*, 17(1):87–97, 2008.

[37] E. F. Coccaro. Impulsive aggression and central serotonergic system function in humans: an example of a dimensional brain-behavior relationship. *Int Clin Psychopharmacol*, 7(1):3–12, 1992.

[38] E. F. Coccaro, R. J. Kavoussi, and R. L. Hauger. Serotonin function and antiaggressive response to fluoxetine: a pilot study. *Biol Psychiatry*, 42(7):546–52, 1997.

[39] G. G. Collins and M. B. Youdim. Multiple forms of human brain monoamine oxidase: substrate specificities. *Biochem J*, 117(2):43P, 1970.

[40] H. Coon, D. Dunn, J. Lainhart, J. Miller, C. Hamil, A. Battaglia, R. Tancredi, M. F. Leppert, R. Weiss, and W. McMahon. Possible association between autism and variants in the brain-expressed tryptophan hydroxylase gene (tph2). *Am J Med Genet B Neuropsychiatr Genet*, 135B(1):42–6, 2005.

[41] S. L. Coon, K. Mazuruk, M. Bernard, P. H. Roseboom, D. C. Klein, and I. R. Rodriguez. The human serotonin n-acetyltransferase (ec 2.3.1.87) gene (aanat): structure, chromosomal localization, and tissue expression. *Genomics*, 34(1):76–84, 1996.

[42] F. Cote, C. Fligny, E. Bayard, J. M. Launay, M. D. Gershon, J. Mallet, and G. Vodjdani. Maternal serotonin is crucial for murine embryonic development. *Proc Natl Acad Sci U S A*, 104(1):329–34, 2007.

[43] F. Cote, E. Thevenot, C. Fligny, Y. Fromes, M. Darmon, M. A. Ripoche, E. Bayard, N. Hanoun, F. Saurini, P. Lechat, L. Dandolo, M. Hamon, J. Mallet, and G. Vodjdani. Disruption of the nonneuronal tph1 gene demonstrates the importance of peripheral serotonin in cardiac function. *Proc Natl Acad Sci U S A*, 100(23):13525–30, 2003.

[44] M. A. Critchley, K. Njung'e, and S. L. Handley. Actions and some interactions of 5-ht1a ligands in the elevated x-maze and effects of dorsal raphe lesions. *Psychopharmacology (Berl)*, 106(4):484–90, 1992.

[45] J. F. Cryan and A. Holmes. The ascent of mouse: advances in modelling human depression and anxiety. *Nat Rev Drug Discov*, 4(9):775–90, 2005.

[46] J. F. Cryan and C. Mombereau. In search of a depressed mouse: utility of models for studying depression-related behavior in genetically modified mice. *Mol Psychiatry*, 9(4):326–57, 2004.

[47] A. Dahlstrom and K. Fuxe. Localization of monoamines in the lower brain stem. *Experientia*, 20(7):398–9, 1964.

[48] J. X. Dai, H. L. Han, M. Tian, J. Cao, J. B. Xiu, N. N. Song, Y. Huang, T. L. Xu, Y. Q. Ding, and L. Xu. Enhanced contextual fear memory in central serotonin-deficient mice. *Proc Natl Acad Sci U S A*, 105(33):11981–6, 2008.

[49] T. Dang, D. H. Avery, and J. Russo. Within-session mood changes from tms in depressed patients. *J Neuropsychiatry Clin Neurosci*, 19(4):458–63, 2007.

[50] R. M. De Almeida, M. Giovenardi, H. Charchat, and A. B. Lucion. 8-oh-dpat in the median raphe nucleus decreases while in the medial septal area it may increase anxiety in female rats. *Neurosci Biobehav Rev*, 23(2):259–64, 1998.

[51] V. De Luca, D. Hlousek, O. Likhodi, H. H. Van Tol, J. L. Kennedy, and A. H. Wong. The interaction between tph2 promoter haplotypes and clinical-demographic risk factors in suicide victims with major psychoses. *Genes Brain Behav*, 5(1):107–10, 2006.

[52] V. De Luca, D. J. Mueller, S. Tharmalingam, N. King, and J. L. Kennedy. Analysis of the novel tph2 gene in bipolar disorder and suicidality. *Mol Psychiatry*, 9(10):896–7, 2004.

[53] R. Delorme, C. M. Durand, C. Betancur, M. Wagner, S. Ruhrmann, H. J. Grabe, G. Nygren, C. Gillberg, M. Leboyer, T. Bourgeron, P. Courtet, F. Jollant, C. Buresi, J. M. Aubry, P. Baud, G. Bondolfi, G. Bertschy, N. Perroud, and A. Malafosse. No human tryptophan hydroxylase-2 gene r441h mutation in a large cohort of psychiatric patients and control subjects. *Biol Psychiatry*, 60(2):202–3, 2006.

[54] L. Descarries and M. Riad. Effects of the antidepressant fluoxetine on the subcellular localization of 5-ht1a receptors and sert. *Philos Trans R Soc Lond B Biol Sci*, 367(1601):2416–25, 2012.

[55] Y. Q. Ding, U. Marklund, W. Yuan, J. Yin, L. Wegman, J. Ericson, E. Deneris, R. L. Johnson, and Z. F. Chen. Lmx1b is essential for the development of serotonergic neurons. *Nat Neurosci*, 6(9):933–8, 2003.

[56] R. C. Drugan, A. L. Morrow, R. Weizman, A. Weizman, S. I. Deutsch, J. N. Crawley, and S. M. Paul. Stress-induced behavioral depression in the rat is associated with a decrease in gaba receptor-mediated chloride ion flux and brain benzodiazepine receptor occupancy. *Brain Res*, 487(1):45–51, 1989.

[57] L. E. Dyck. Release of monoamines from striatal slices by phenelzine and beta-phenylethylamine. *Prog Neuropsychopharmacol Biol Psychiatry*, 7(4-6):797–800, 1983.

[58] J. D. Erickson and L. E. Eiden. Functional identification and molecular cloning of a human brain vesicle monoamine transporter. *J Neurochem*, 61(6):2314–7, 1993.

[59] J. D. Erickson, M. K. Schafer, T. I. Bonner, L. E. Eiden, and E. Weihe. Distinct pharmacological properties and distribution in neurons and endocrine cells of two isoforms of the human vesicular monoamine transporter. *Proc Natl Acad Sci U S A*, 93(10):5166–71, 1996.

[60] V. Fabre, C. Beaufour, A. Evrard, A. Rioux, N. Hanoun, K. P. Lesch, D. L. Murphy, L. Lanfumey, M. Hamon, and M. P. Martres. Altered expression and functions of serotonin 5-ht1a and 5-ht1b receptors in knock-out mice lacking the 5-ht transporter. *Eur J Neurosci*, 12(7):2299–310, 2000.

[61] S. J. Farley, B. M. McKay, J. F. Disterhoft, and C. Weiss. Reevaluating hippocampus-dependent learning in fvb/n mice. *Behav Neurosci*, 125(6):871–8, 2011.

[62] S. P. Fernandez and P. Gaspar. Investigating anxiety and depressive-like phenotypes in genetic mouse models of serotonin depletion. *Neuropharmacology*, 62(1):144–54, 2012.

[63] J. D. Fernstrom and R. J. Wurtman. Brain serotonin content: physiological dependence on plasma tryptophan levels. *Science*, 173(992):149–52, 1971.

[64] P. F. Ferrari, A. M. van Erp, W. Tornatzky, and K. A. Miczek. Accumbal dopamine and serotonin in anticipation of the next aggressive episode in rats. *Eur J Neurosci*, 17(2):371–8, 2003.

[65] P. F. Fitzpatrick. Tetrahydropterin-dependent amino acid hydroxylases. *Annu Rev Biochem*, 68:355–81, 1999.

[66] E. A. Fon, E. N. Pothos, B. C. Sun, N. Killeen, D. Sulzer, and R. H. Edwards. Vesicular transport regulates monoamine storage and release but is not essential for amphetamine action. *Neuron*, 19(6):1271–83, 1997.

[67] L. Franchini, A. Serretti, M. Gasperini, and E. Smeraldi. Familial concordance of fluvoxamine response as a tool for differentiating mood disorder pedigrees. *J Psychiatr Res*, 32(5):255–9, 1998.

[68] T. F. Freund, A. I. Gulyas, L. Acsady, T. Gorcs, and K. Toth. Serotonergic control of the hippocampus via local inhibitory interneurons. *Proc Natl Acad Sci U S A*, 87(21):8501–5, 1990.

[69] M. Fukui, R. M. Rodriguiz, J. Zhou, S. X. Jiang, L. E. Phillips, M. G. Caron, and W. C. Wetsel. Vmat2 heterozygous mutant mice display a depressive-like phenotype. *J Neurosci*, 27(39):10520–9, 2007.

[70] D. Fyodorov, T. Nelson, and E. Deneris. Pet-1, a novel ets domain factor that can activate neuronal nachr gene transcription. *J Neurobiol*, 34(2):151–63, 1998.

[71] H. A. Garriock, J. J. Allen, P. Delgado, Z. Nahaz, M. A. Kling, L. Carpenter, M. Burke, W. Burke, T. Schwartz, L. B. Marangell, M. Husain, R. P. Erickson, and F. A. Moreno. Lack of association of tph2 exon xi polymorphisms with major depression and treatment resistance. *Mol Psychiatry*, 10(11):976–7, 2005.

[72] E. C. Gavioli, C. W. Vaughan, G. Marzola, R. Guerrini, V. A. Mitchell, S. Zucchini, T. C. De Lima, G. A. Rae, S. Salvadori, D. Regoli, and G. Calo. Antidepressant-like effects of the nociceptin/orphanin fq receptor antagonist ufp-101: new evidence from rats and mice. *Naunyn Schmiedebergs Arch Pharmacol*, 369(6):547–53, 2004.

[73] R. Gizatullin, G. Zaboli, E. G. Jonsson, M. Asberg, and R. Leopardi. The tryptophan hydroxylase (tph) 2 gene unlike tph-1 exhibits no association with stress-induced depression. *J Affect Disord*, 107(1-3):175–9, 2008.

[74] C. E. Glatt, E. Carlson, T. R. Taylor, N. Risch, V. I. Reus, and C. A. Schaefer. Response to zhang et al. (2005): loss-of-function mutation in tryptophan hydroxylase-2 identified in unipolar major depression. neuron 45, 11-16. *Neuron*, 48(5):704–5; author reply 705–6, 2005.

[75] M. Grohmann, P. Hammer, M. Walther, N. Paulmann, A. Buttner, W. Eisenmenger, T. C. Baghai, C. Schule, R. Rupprecht, M. Bader, B. Bondy, P. Zill, J. Priller, and D. J. Walther. Alternative splicing and extensive rna editing of human tph2 transcripts. *PLoS One*, 5(1):e8956, 2010.

[76] C. Gross, L. Santarelli, D. Brunner, X. Zhuang, and R. Hen. Altered fear circuits in 5-ht(1a) receptor ko mice. *Biol Psychiatry*, 48(12):1157–63, 2000.

[77] A. I. Gulyas, L. Acsady, and T. F. Freund. Structural basis of the cholinergic and serotonergic modulation of gabaergic neurons in the hippocampus. *Neurochem Int*, 34(5):359–72, 1999.

[78] L. Gutknecht, N. Araragi, S. Merker, J. Waider, F. M. Sommerlandt, B. Mlinar, G. Baccini, U. Mayer, F. Proft, M. Hamon, A. G. Schmitt, R. Corradetti, L. Lanfumey, and K. P. Lesch. Impacts of brain serotonin deficiency following tph2 inactivation on development and raphe neuron serotonergic specification. *PLoS One*, 7(8):e43157, 2012.

[79] L. Gutknecht, C. Jacob, A. Strobel, C. Kriegebaum, J. Muller, Y. Zeng, C. Markert, A. Escher, J. Wendland, A. Reif, R. Mossner, C. Gross, B. Brocke, and K. P. Lesch. Tryptophan hydroxylase-2 gene variation influences personality traits and disorders related to emotional dysregulation. *Int J Neuropsychopharmacol*, 10(3):309–20, 2007.

[80] L. Gutknecht, J. Waider, S. Kraft, C. Kriegebaum, B. Holtmann, A. Reif, A. Schmitt, and K. P. Lesch. Deficiency of brain 5-ht synthesis but serotonergic neuron formation in tph2 knockout mice. *J Neural Transm*, 115(8):1127–32, 2008.

[81] J. Haavik, N. Blau, and B. Thony. Mutations in human monoamine-related neurotransmitter pathway genes. *Hum Mutat*, 29(7):891–902, 2008.

[82] F. Haghighi, H. Bach-Mizrachi, Y. Y. Huang, V. Arango, S. Shi, A. J. Dwork, G. Rosoklija, H. T. Sheng, I. Morozova, J. Ju, J. J. Russo, and J. J. Mann. Genetic architecture of the human tryptophan hydroxylase 2 gene: existence of neural isoforms and relevance for major depression. *Mol Psychiatry*, 13(8):813–20, 2008.

[83] H. P. Harding, H. Zeng, Y. Zhang, R. Jungries, P. Chung, H. Plesken, D. D. Sabatini, and D. Ron. Diabetes mellitus and exocrine pancreatic dysfunction in perk-/- mice reveals a role for translational control in secretory cell survival. *Mol Cell*, 7(6):1153–63, 2001.

[84] M. Harvey, B. Gagne, M. Labbe, and N. Barden. Polymorphisms in the neuronal isoform of tryptophan hydroxylase 2 are associated with bipolar disorder in french canadian pedigrees. *Psychiatr Genet*, 17(1):17–22, 2007.

[85] M. Harvey, E. Shink, M. Tremblay, B. Gagne, C. Raymond, M. Labbe, D. J. Walther, M. Bader, and N. Barden. Support for the involvement of tph2 gene in affective disorders. *Mol Psychiatry*, 9(11):980–1, 2004.

[86] A. J. Heinz, A. Beck, A. Meyer-Lindenberg, P. Sterzer, and A. Heinz. Cognitive and neurobiological mechanisms of alcohol-related aggression. *Nat Rev Neurosci*, 12(7):400–13, 2011.

[87] L. K. Heisler, H. M. Chu, T. J. Brennan, J. A. Danao, P. Bajwa, L. H. Parsons, and L. H. Tecott. Elevated anxiety and antidepressant-like responses in serotonin 5-ht1a receptor mutant mice. *Proc Natl Acad Sci U S A*, 95(25):15049–54, 1998.

[88] T. Hendricks, N. Francis, D. Fyodorov, and E. S. Deneris. The ets domain factor pet-1 is an early and precise marker of central serotonin neurons and interacts with a conserved element in serotonergic genes. *J Neurosci*, 19(23):10348–56, 1999.

[89] T. J. Hendricks, D. V. Fyodorov, L. J. Wegman, N. B. Lelutiu, E. A. Pehek, B. Yamamoto, J. Silver, E. J. Weeber, J. D. Sweatt, and E. S. Deneris. Pet-1 ets gene plays a critical role in 5-ht neuron development and is required for normal anxiety-like and aggressive behavior. *Neuron*, 37(2):233–47, 2003.

[90] S. Henningsson, K. Annerbrink, M. Olsson, C. Allgulander, S. Andersch, I. Sjodin, D. Gustafson, I. Skoog, E. Eriksson, and L. Westberg. Absence of the arg441his polymorphism in the tryptophan hydroxylase 2 gene in adults with anxiety disorders and depression. *Am J Med Genet B Neuropsychiatr Genet*, 144B(6):816–7, 2007.

[91] D. P. Henry and R. R. Bowsher. An improved radioenzymatic assay for plasma norepinephrine using purified phenylethanolamine n-methyltransferase. *Life Sci*, 38(16):1473–83, 1986.

[92] M. R. Hodges, M. Wehner, J. Aungst, J. C. Smith, and G. B. Richerson. Transgenic mice lacking serotonin neurons have se-

vere apnea and high mortality during development. *J Neurosci*, 29(33):10341–9, 2009.

[93] M. A. Hofer. Toward a developmental basis for disease predisposition: the effects of early maternal separation on brain, behavior, and cardiovascular system. *Res Publ Assoc Res Nerv Ment Dis*, 59:209–28, 1981.

[94] A. Holmes, Q. Lit, D. L. Murphy, E. Gold, and J. N. Crawley. Abnormal anxiety-related behavior in serotonin transporter null mutant mice: the influence of genetic background. *Genes Brain Behav*, 2(6):365–80, 2003.

[95] M. Holtje, B. von Jagow, I. Pahner, M. Lautenschlager, H. Hortnagl, B. Nurnberg, R. Jahn, and G. Ahnert-Hilger. The neuronal monoamine transporter vmat2 is regulated by the trimeric gtpase go(2). *J Neurosci*, 20(6):2131–41, 2000.

[96] M. Holtje, S. Winter, D. Walther, I. Pahner, H. Hortnagl, O. P. Ottersen, M. Bader, and G. Ahnert-Hilger. The vesicular monoamine content regulates vmat2 activity through galphaq in mouse platelets. evidence for autoregulation of vesicular transmitter uptake. *J Biol Chem*, 278(18):15850–8, 2003.

[97] L. Hritcu, M. Clicinschi, and T. Nabeshima. Brain serotonin depletion impairs short-term memory, but not long-term memory in rats. *Physiol Behav*, 91(5):652–7, 2007.

[98] A. Illi, E. Setala-Soikkeli, M. Viikki, O. Poutanen, H. Huhtala, N. Mononen, T. Lehtimaki, E. Leinonen, and O. Kampman. 5-htr1a, 5-htr2a, 5-htr6, tph1 and tph2 polymorphisms and major depression. *Neuroreport*, 20(12):1125–8, 2009.

[99] J. P. Jacobsen, W. B. Siesser, B. D. Sachs, S. Peterson, M. J. Cools, V. Setola, J. H. Folgering, G. Flik, and M. G. Caron. Deficient serotonin neurotransmission and depression-like serotonin biomarker alterations in tryptophan hydroxylase 2 (tph2) loss-of-function mice. *Mol Psychiatry*, 2011.

[100] K. A. Jennings, M. K. Loder, W. J. Sheward, Q. Pei, R. M. Deacon, M. A. Benson, H. J. Olverman, N. D. Hastie, A. J. Harmar, S. Shen, and T. Sharp. Increased expression of the 5-ht transporter confers a low-anxiety phenotype linked to decreased 5-ht transmission. *J Neurosci*, 26(35):8955–64, 2006.

[101] J. P. Johnston. Some observations upon a new inhibitor of monoamine oxidase in brain tissue. *Biochem Pharmacol*, 17(7):1285–97, 1968.

[102] A. V. Juorio and A. A. Boulton. The effect of some precursor amino acids and enzyme inhibitors on the mouse striatal concentration of tyramines and homovanillic acid. *J Neurochem*, 39(3):859–63, 1982.

[103] M. S. Kaplan and J. W. Hinds. Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs. *Science*, 197(4308):1092–4, 1977.

[104] G. Kempermann. Why new neurons? possible functions for adult hippocampal neurogenesis. *J Neurosci*, 22(3):635–8, 2002.

[105] K. S. Kim, T. C. Wessel, D. M. Stone, C. H. Carver, T. H. Joh, and D. H. Park. Molecular cloning and characterization of cdna encoding tryptophan hydroxylase from rat central serotonergic neurons. *Brain Res Mol Brain Res*, 9(4):277–83, 1991.

[106] Y. K. Kim, H. J. Lee, J. C. Yang, J. A. Hwang, and H. K. Yoon. A tryptophan hydroxylase 2 gene polymorphism is associated with panic disorder. *Behav Genet*, 39(2):170–5, 2009.

[107] V. Kiyasova, S. P. Fernandez, J. Laine, L. Stankovski, A. Muzerelle, S. Doly, and P. Gaspar. A genetically defined morphologically and functionally unique subset of 5-ht neurons in the mouse raphe nuclei. *J Neurosci*, 31(8):2756–68, 2011.

[108] F. Klempin, H. Babu, D. De Pietri Tonelli, E. Alarcon, K. Fabel, and G. Kempermann. Oppositional effects of serotonin receptors 5-ht1a, 2, and 2c in the regulation of adult hippocampal neurogenesis. *Front Mol Neurosci*, 3, 2010.

[109] R. Koopman, G. Schaart, and M. K. Hesselink. Optimisation of oil red o staining permits combination with immunofluorescence and automated quantification of lipids. *Histochem Cell Biol*, 116(1):63–8, 2001.

[110] C. Kriegebaum, N. N. Song, L. Gutknecht, Y. Huang, A. Schmitt, A. Reif, Y. Q. Ding, and K. P. Lesch. Brain-specific conditional and time-specific inducible tph2 knockout mice possess normal serotonergic gene expression in the absence of serotonin during adult life. *Neurochem Int*, 57(5):512–7, 2010.

[111] C. M. Kuhn and S. M. Schanberg. Responses to maternal separation: mechanisms and mediators. *Int J Dev Neurosci*, 16(3-4):261–70, 1998.

[112] H. G. Kuhn, H. Dickinson-Anson, and F. H. Gage. Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. *J Neurosci*, 16(6):2027–33, 1996.

[113] A. V. Kulikov, D. V. Osipova, V. S. Naumenko, and N. K. Popova. Association between tph2 gene polymorphism, brain tryptophan hydroxylase activity and aggressiveness in mouse strains. *Genes Brain Behav*, 4(8):482–5, 2005.

[114] A. M. Laporte, L. Lima, H. Gozlan, and M. Hamon. Selective in vivo labelling of brain 5-ht1a receptors by [3h]way 100635 in the mouse. *Eur J Pharmacol*, 271(2-3):505–14, 1994.

[115] E. Le Poul, N. Laaris, E. Doucet, A. M. Laporte, M. Hamon, and L. Lanfumey. Early desensitization of somato-dendritic 5-ht1a autoreceptors in rats treated with fluoxetine or paroxetine. *Naunyn Schmiedebergs Arch Pharmacol*, 352(2):141–8, 1995.

[116] C. Lebrand, O. Cases, R. Wehrle, R. D. Blakely, R. H. Edwards, and P. Gaspar. Transient developmental expression of monoamine transporters in the rodent forebrain. *J Comp Neurol*, 401(4):506–24, 1998.

[117] J. W. Lenders, G. Eisenhofer, N. G. Abeling, W. Berger, D. L. Murphy, C. H. Konings, L. M. Wagemakers, I. J. Kopin, F. Karoum, A. H. van Gennip, and H. G. Brunner. Specific genetic deficiencies of the a and b isoenzymes of monoamine oxidase are characterized by distinct neurochemical and clinical phenotypes. *J Clin Invest*, 97(4):1010–9, 1996.

[118] P. Levitt and P. Rakic. The time of genesis, embryonic origin and differentiation of the brain stem monoamine neurons in the rhesus monkey. *Brain Res*, 256(1):35–57, 1982.

[119] Q. Li, N. A. Muma, G. Battaglia, and L. D. Van de Kar. A desensitization of hypothalamic 5-ht1a receptors by repeated injections of paroxetine: reduction in the levels of g(i) and g(o) proteins and neuroendocrine responses, but not in the density of 5-ht1a receptors. *J Pharmacol Exp Ther*, 282(3):1581–90, 1997.

[120] Q. Li, C. Wichems, A. Heils, L. D. Van De Kar, K. P. Lesch, and D. L. Murphy. Reduction of 5-hydroxytryptamine (5-ht)(1a)-mediated temperature and neuroendocrine responses and 5-ht(1a) binding sites in 5-ht transporter knockout mice. *J Pharmacol Exp Ther*, 291(3):999–1007, 1999.

[121] H. G. Lidov and M. E. Molliver. An immunohistochemical study of serotonin neuron development in the rat: ascending pathways and terminal fields. *Brain Res Bull*, 8(4):389–430, 1982.

[122] Y. M. Lin, S. C. Chao, T. M. Chen, T. J. Lai, J. S. Chen, and H. S. Sun. Association of functional polymorphisms of the human tryptophan hydroxylase 2 gene with risk for bipolar disorder in han chinese. *Arch Gen Psychiatry*, 64(9):1015–24, 2007.

[123] A. Lira, M. Zhou, N. Castanon, M. S. Ansorge, J. A. Gordon, J. H. Francis, M. Bradley-Moore, J. Lira, M. D. Underwood, V. Arango, H. F. Kung, M. A. Hofer, R. Hen, and J. A. Gingrich. Altered depression-related behaviors and functional changes in the dorsal raphe nucleus of serotonin transporter-deficient mice. *Biol Psychiatry*, 54(10):960–71, 2003.

[124] C. Liu, T. Maejima, S. C. Wyler, G. Casadesus, S. Herlitze, and E. S. Deneris. Pet-1 is required across different stages of life to regulate serotonergic function. *Nat Neurosci*, 13(10):1190–8, 2010.

[125] Y. Liu, Y. Jiang, Y. Si, J. Y. Kim, Z. F. Chen, and Y. Rao. Molecular regulation of sexual preference revealed by genetic studies of 5-ht in the brains of male mice. *Nature*, 472(7341):95–9, 2011.

[126] K. J. Livak and T. D. Schmittgen. Analysis of relative gene expression data using real-time quantitative pcr and the 2(-delta delta c(t)) method. *Methods*, 25(4):402–8, 2001.

[127] V. A. Lopez, S. Detera-Wadleigh, I. Cardona, L. Kassem, and F. J. McMahon. Nested association between genetic variation in tryptophan hydroxylase ii, bipolar affective disorder, and suicide attempts. *Biol Psychiatry*, 61(2):181–6, 2007.

[128] F. Lopez-Munoz and C. Alamo. Monoaminergic neurotransmission: the history of the discovery of antidepressants from 1950s until today. *Curr Pharm Des*, 15(14):1563–86, 2009.

[129] J. J. Lucas and R. Hen. New players in the 5-ht receptor field: genes and knockouts. *Trends Pharmacol Sci*, 16(7):246–52, 1995.

[130] I. Lucki. The spectrum of behaviors influenced by serotonin. *Biol Psychiatry*, 44(3):151–62, 1998.

[131] J. W. Maas. Neurochemical differences between two strains of mice. *Science*, 137:621–2, 1962.

[132] J. E. Malberg and R. S. Duman. Cell proliferation in adult hippocampus is decreased by inescapable stress: reversal by fluoxetine treatment. *Neuropsychopharmacology*, 28(9):1562–71, 2003.

[133] J. E. Malberg, A. J. Eisch, E. J. Nestler, and R. S. Duman. Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. *J Neurosci*, 20(24):9104–10, 2000.

[134] J. J. Mann, D. Currier, L. Murphy, Y. Y. Huang, H. Galfalvy, D. Brent, L. Greenhill, and M. Oquendo. No association between a tph2 promoter polymorphism and mood disorders or monoamine turnover. *J Affect Disord*, 106(1-2):117–21, 2008.

[135] T. A. Mathews, D. E. Fedele, F. M. Coppelli, A. M. Avila, D. L. Murphy, and A. M. Andrews. Gene dose-dependent alterations in extraneuronal serotonin but not dopamine in mice with reduced serotonin transporter expression. *J Neurosci Methods*, 140(1-2):169–81, 2004.

[136] S. Matthes, V. Mosienko, S. Bashammakh, N. Alenina, and M. Bader. Tryptophan hydroxylase as novel target for the treatment of depressive disorders. *Pharmacology*, 85(2):95–109, 2010.

[137] J. A. McKinney, B. Turel, I. Winge, P. M. Knappskog, and J. Haavik. Functional properties of missense variants of human tryptophan hydroxylase 2. *Hum Mutat*, 30(5):787–94, 2009.

[138] S. Migliarini, G. Pacini, B. Pelosi, G. Lunardi, and M. Pasqualetti. Lack of brain serotonin affects postnatal development and serotonergic neuronal circuitry formation. *Mol Psychiatry*, 2012.

[139] H. L. Miller, P. L. Delgado, R. M. Salomon, J. Licinio, L. C. Barr, and D. S. Charney. Acute tryptophan depletion: a method of studying antidepressant action. *J Clin Psychiatry*, 53 Suppl:28–35, 1992.

[140] V. Mosienko, B. Bert, D. Beis, S. Matthes, H. Fink, M. Bader, and N. Alenina. Exaggerated aggression and decreased anxiety in mice deficient in brain serotonin. *Transl Psychiatry*, 2:e122, 2012.

[141] R. Mossner, S. Walitza, F. Geller, A. Scherag, L. Gutknecht, C. Jacob, L. Bogusch, H. Remschmidt, M. Simons, B. Herpertz-Dahlmann, C. Fleischhaker, E. Schulz, A. Warnke, A. Hinney, C. Wewetzer, and K. P. Lesch. Transmission disequilibrium of polymorphic variants in the tryptophan hydroxylase-2 gene in children and adolescents with obsessive-compulsive disorder. *Int J Neuropsychopharmacol*, 9(4):437–42, 2006.

[142] K. Mouri, A. Hishimoto, M. Fukutake, K. Shiroiwa, M. Asano, Y. Nagasaki, Y. Ueno, O. Shirakawa, N. Nishiguchi, and K. Maeda. Tph2 is not a susceptibility gene for suicide in japanese population. *Prog Neuropsychopharmacol Biol Psychiatry*, 2009.

[143] D. V. Muralidhara and P. S. Shetty. Effects of preweaning nutritional deprivation on basal metabolism and thermoregulatory thermogenesis in the rat. *Br J Nutr*, 56(3):615–23, 1986.

[144] A. Nantel-Vivier, R. O. Pihl, S. N. Young, S. Parent, S. A. Belanger, R. Sutton, M. E. Dubois, R. E. Tremblay, and J. R. Seguin. Serotonergic contribution to boys' behavioral regulation. *PLoS One*, 6(6):e20304, 2011.

[145] N. Narboux-Neme, G. Angenard, V. Mosienko, F. Klempin, P. M. Pitychoutis, E. Deneris, M. Bader, B. Giros, N. Alenina, and P. Gaspar. Postnatal growth defects in mice with constitutive depletion of central serotonin. *ACS Chem Neurosci*, 4(1):171–81, 2013.

[146] N. Narboux-Neme, C. Sagne, S. Doly, S. L. Diaz, C. B. Martin, G. Angenard, M. P. Martres, B. Giros, M. Hamon, L. Lanfumey, P. Gaspar, and R. Mongeau. Severe serotonin depletion after conditional deletion of the vesicular monoamine transporter 2 gene in serotonin neurons: Neural and behavioral consequences. *Neuropsychopharmacology*, 2011.

[147] K. B. Neal, L. J. Parry, and J. C. Bornstein. Strain-specific genetics, anatomy and function of enteric neural serotonergic pathways in inbred mice. *J Physiol*, 587(Pt 3):567–86, 2009.

[148] K. Njung'e and S. L. Handley. Evaluation of marble-burying behavior as a model of anxiety. *Pharmacol Biochem Behav*, 38(1):63–7, 1991.

[149] O. F. O'Leary, A. J. Bechtholt, J. J. Crowley, T. E. Hill, M. E. Page, and I. Lucki. Depletion of serotonin and catecholamines block the acute behavioral response to different classes of antidepressant drugs in the mouse tail suspension test. *Psychopharmacology (Berl)*, 192(3):357–71, 2007.

[150] R. L. O'Reilly, L. Bogue, and S. M. Singh. Pharmacogenetic response to antidepressants in a multicase family with affective disorder. *Biol Psychiatry*, 36(7):467–71, 1994.

[151] D. V. Osipova, A. V. Kulikov, and N. K. Popova. C1473g polymorphism in mouse tph2 gene is linked to tryptophan hydroxylase-2 activity in the brain, intermale aggression, and depressive-like behavior in the forced swim test. *J Neurosci Res*, 87(5):1168–74, 2009.

[152] I. H. Page. The discovery of serotonin. *Perspect Biol Med*, 20(1):1–8, 1976.

[153] A. Papageorgiou and C. Denef. Stimulation of growth hormone release by 5-hydroxytryptamine (5-ht) in cultured rat anterior pituitary cell aggregates: evidence for mediation by 5-ht2b, 5-ht7, 5-ht1b, and ketanserin-sensitive receptors. *Endocrinology*, 148(9):4509–22, 2007.

[154] C. L. Parks, P. S. Robinson, E. Sibille, T. Shenk, and M. Toth. Increased anxiety of mice lacking the serotonin1a receptor. *Proc Natl Acad Sci U S A*, 95(18):10734–9, 1998.

[155] P. D. Patel, C. Pontrello, and S. Burke. Robust and tissue-specific expression of tph2 versus tph1 in rat raphe and pineal gland. *Biol Psychiatry*, 55(4):428–33, 2004.

[156] T. D. Patel and F. C. Zhou. Ontogeny of 5-ht1a receptor expression in the developing hippocampus. *Brain Res Dev Brain Res*, 157(1):42–57, 2005.

[157] S. Pellow, P. Chopin, S. E. File, and M. Briley. Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J Neurosci Methods*, 14(3):149–67, 1985.

[158] E. J. Peters, S. L. Slager, G. D. Jenkins, M. S. Reinalda, H. A. Garriock, S. I. Shyn, J. B. Kraft, P. J. McGrath, and S. P. Hamilton. Resequencing of serotonin-related genes and association of tagging snps to citalopram response. *Pharmacogenet Genomics*, 19(1):1–10, 2009.

[159] E. J. Peters, S. L. Slager, P. J. McGrath, J. A. Knowles, and S. P. Hamilton. Investigation of serotonin-related genes in antidepressant response. *Mol Psychiatry*, 9(9):879–89, 2004.

[160] B. Petit-Demouliere, F. Chenu, and M. Bourin. Forced swimming test in mice: a review of antidepressant activity. *Psychopharmacology (Berl)*, 177(3):245–55, 2005.

[161] G. C. Petzold, A. Hagiwara, and V. N. Murthy. Serotonergic modulation of odor input to the mammalian olfactory bulb. *Nat Neurosci*, 12(6):784–91, 2009.

[162] H. Pfaar, A. von Holst, D. M. Vogt Weisenhorn, C. Brodski, J. Guimera, and W. Wurst. mpet-1, a mouse ets-domain transcription factor, is expressed in central serotonergic neurons. *Dev Genes Evol*, 212(1):43–6, 2002.

[163] A. Piechal, K. Blecharz-Klin, E. Wyszogrodzka, P. Kolomanska, P. Rok-Bujko, P. Krzascik, W. Kostowski, E. Widy-Tyszkiewicz, M. Filip, and R. Stefanski. Neonatal serotonin (5-ht) depletion does not affect spatial learning and memory in rats. *Pharmacol Rep*, 64(2):266–74, 2012.

[164] R. D. Porsolt, A. Bertin, and M. Jalfre. Behavioral despair in mice: a primary screening test for antidepressants. *Arch Int Pharmacodyn Ther*, 229(2):327–36, 1977.

[165] L. H. Price, R. T. Malison, C. J. McDougle, G. H. Pelton, and G. R. Heninger. The neurobiology of tryptophan depletion in depression: effects of intravenous tryptophan infusion. *Biol Psychiatry*, 43(5):339–47, 1998.

[166] J. P. Redrobe and M. Bourin. Dose-dependent influence of buspirone on the activities of selective serotonin reuptake inhibitors in the mouse forced swimming test. *Psychopharmacology (Berl)*, 138(2):198–206, 1998.

[167] M. Reuter, C. Esslinger, C. Montag, S. Lis, B. Gallhofer, and P. Kirsch. A functional variant of the tryptophan hydroxylase 2 gene impacts working memory: a genetic imaging study. *Biol Psychol*, 79(1):111–7, 2008.

[168] J. W. Richardson-Jones, C. P. Craige, T. H. Nguyen, H. F. Kung, A. M. Gardier, A. Dranovsky, D. J. David, B. P. Guiard, S. G. Beck, R. Hen, and E. D. Leonardo. Serotonin-1a autoreceptors are necessary and sufficient for the normal formation of circuits underlying innate anxiety. *J Neurosci*, 31(16):6008–18, 2011.

[169] S. Roche and P. McKeon. Support for tryptophan hydroxylase-2 as a susceptibility gene for bipolar affective disorder. *Psychiatr Genet*, 19(3):142–6, 2009.

[170] A. Romaniuk, M. Koprowska, M. Krotewicz, M. Strzelczuk, and M. Wieczorek. Effects of 8-ohdpat administration into the dorsal raphe nucleus and dorsal hippocampus on fear behavior and regional brain monoamines distribution in rats. *Behav Brain Res*, 120(1):47–57, 2001.

[171] R. Sachidanandam, D. Weissman, S. C. Schmidt, J. M. Kakol, L. D. Stein, G. Marth, S. Sherry, J. C. Mullikin, B. J. Mortimore, D. L. Willey, S. E. Hunt, C. G. Cole, P. C. Coggill, C. M. Rice, Z. Ning, J. Rogers, D. R. Bentley, P. Y. Kwok, E. R. Mardis, R. T. Yeh, B. Schultz, L. Cook, R. Davenport, M. Dante, L. Fulton, L. Hillier, R. H. Waterston, J. D. McPherson, B. Gilman, S. Schaffner, W. J. Van Etten, D. Reich, J. Higgins, M. J. Daly, B. Blumenstiel, J. Baldwin, N. Stange-Thomann, M. C. Zody, L. Linton, E. S. Lander, and D. Altshuler. A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms. *Nature*, 409(6822):928–33, 2001.

[172] S. A. Sakowski, T. J. Geddes, and D. M. Kuhn. Mouse tryptophan hydroxylase isoform 2 and the role of proline 447 in enzyme function. *J Neurochem*, 96(3):758–65, 2006.

[173] S. A. Sakowski, T. J. Geddes, D. M. Thomas, E. Levi, J. S. Hatfield, and D. M. Kuhn. Differential tissue distribution of tryptophan hydroxylase isoforms 1 and 2 as revealed with monospecific antibodies. *Brain Res*, 1085(1):11–8, 2006.

[174] C. D. Sanberg, F. L. Jones, V. H. Do, Jr. Dieguez, D., and B. E. Derrick. 5-ht1a receptor antagonists block perforant path-

dentate ltp induced in novel, but not familiar, environments. *Learn Mem*, 13(1):52–62, 2006.

[175] L. Santarelli, M. Saxe, C. Gross, A. Surget, F. Battaglia, S. Dulawa, N. Weisstaub, J. Lee, R. Duman, O. Arancio, C. Belzung, and R. Hen. Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. *Science*, 301(5634):805–9, 2003.

[176] K. V. Savelieva, S. Zhao, V. M. Pogorelov, I. Rajan, Q. Yang, E. Cullinan, and T. H. Lanthorn. Genetic disruption of both tryptophan hydroxylase genes dramatically reduces serotonin and affects behavior in models sensitive to antidepressants. *PLoS One*, 3(10):e3301, 2008.

[177] T. L. Schaefer, C. V. Vorhees, and M. T. Williams. Mouse plasmacytoma-expressed transcript 1 knock out induced 5-ht disruption results in a lack of cognitive deficits and an anxiety phenotype complicated by hypoactivity and defensiveness. *Neuroscience*, 164(4):1431–43, 2009.

[178] K. Scheuch, M. Lautenschlager, M. Grohmann, S. Stahlberg, J. Kirchheiner, P. Zill, A. Heinz, D. J. Walther, and J. Priller. Characterization of a functional promoter polymorphism of the human tryptophan hydroxylase 2 gene in serotonergic raphe neurons. *Biol Psychiatry*, 62(11):1288–94, 2007.

[179] K. Schruers and E. Griez. The effects of tryptophan depletion on mood and psychiatric symptoms. *J Affect Disord*, 74(3):305, 2003.

[180] T. Seki and Y. Arai. Age-related production of new granule cells in the adult dentate gyrus. *Neuroreport*, 6(18):2479–82, 1995.

[181] A. N. Sferruzzi-Perri, J. A. Owens, K. G. Pringle, and C. T. Roberts. The neglected role of insulin-like growth factors in the maternal circulation regulating fetal growth. *J Physiol*, 589(Pt 1):7–20, 2011.

[182] K. Sheehan, N. Lowe, A. Kirley, C. Mullins, M. Fitzgerald, M. Gill, and Z. Hawi. Tryptophan hydroxylase 2 (tph2) gene variants associated with adhd. *Mol Psychiatry*, 10(10):944–9, 2005.

[183] W. B. Siesser, X. Zhang, J. P. Jacobsen, T. D. Sotnikova, R. R. Gainetdinov, and M. G. Caron. Tryptophan hydroxylase 2 genotype determines brain serotonin synthesis but not tissue content in c57bl/6 and balb/c congenic mice. *Neurosci Lett*, 481(1):6–11, 2010.

[184] A. Sjoerdsma and M. G. Palfreyman. History of serotonin and serotinin disorders. *Ann N Y Acad Sci*, 600:1–7; discussion 7–8, 1990.

[185] A. Slominski, T. W. Fischer, M. A. Zmijewski, J. Wortsman, I. Semak, B. Zbytek, R. M. Slominski, and D. J. Tobin. On the role of melatonin in skin physiology and pathology. *Endocrine*, 27(2):137–48, 2005.

[186] G. A. Smythe, J. F. Brandstater, and L. Lazarus. Serotoninergic control of rat growth hormone secretion. *Neuroendocrinology*, 17(3):245–57, 1975.

[187] B. Soderpalm and J. A. Engel. Serotonergic involvement in conflict behaviour. *Eur Neuropsychopharmacol*, 1(1):7–13, 1990.

[188] N. N. Song, J. B. Xiu, Y. Huang, J. Y. Chen, L. Zhang, L. Gutknecht, K. P. Lesch, H. Li, and Y. Q. Ding. Adult raphe-specific deletion of lmx1b leads to central serotonin deficiency. *PLoS One*, 6(1):e15998, 2011.

[189] J. S. Sprouse and G. K. Aghajanian. Responses of hippocampal pyramidal cells to putative serotonin 5-ht1a and 5-ht1b agonists: a comparative study with dorsal raphe neurons. *Neuropharmacology*, 27(7):707–15, 1988.

[190] B. Stanley, A. Molcho, M. Stanley, R. Winchel, M. J. Gameroff, B. Parsons, and J. J. Mann. Association of aggressive behavior with altered serotonergic function in patients who are not suicidal. *Am J Psychiatry*, 157(4):609–14, 2000.

[191] D. J. Stein, H. G. Westenberg, and M. R. Liebowitz. Social anxiety disorder and generalized anxiety disorder: serotonergic and dopaminergic neurocircuitry. *J Clin Psychiatry*, 63 Suppl 6:12–9, 2002.

[192] M. Stuart, L. Lazarus, G. A. Smythe, S. Moore, and V. Sara. Biogenic amine control of growth hormone secretion in the fetal and neonatal rat. *Neuroendocrinology*, 22(4):337–42, 1976.

[193] E. Tanaka and R. A. North. Actions of 5-hydroxytryptamine on neurons of the rat cingulate cortex. *J Neurophysiol*, 69(5):1749–57, 1993.

[194] K. Tenner, F. Qadri, B. Bert, J. P. Voigt, and M. Bader. The mtph2 c1473g single nucleotide polymorphism is not responsible for behavioural differences between mouse strains. *Neurosci Lett*, 431(1):21–5, 2008.

[195] K. Tenner, D. Walther, and M. Bader. Influence of human tryptophan hydroxylase 2 n- and c-terminus on enzymatic activity and oligomerization. *J Neurochem*, 102(6):1887–94, 2007.

[196] D. M. Thomas, M. Angoa Perez, D. M. Francescutti-Verbeem, M. M. Shah, and D. M. Kuhn. The role of endogenous serotonin in methamphetamine-induced neurotoxicity to dopamine nerve endings of the striatum. *J Neurochem*, 115(3):595–605, 2010.

[197] S. Trowbridge, N. Narboux-Neme, and P. Gaspar. Genetic models of serotonin (5-ht) depletion: what do they tell us about the developmental role of 5-ht? *Anat Rec (Hoboken)*, 294(10):1615–23, 2011.

[198] S. J. Tsai, C. J. Hong, Y. J. Liou, Y. W. Yu, T. J. Chen, S. J. Hou, and F. C. Yen. Tryptophan hydroxylase 2 gene is associated with major depression and antidepressant treatment response. *Prog Neuropsychopharmacol Biol Psychiatry*, 33(4):637–41, 2009.

[199] B. M. Twarog and I. H. Page. Serotonin content of some mammalian tissues and urine and a method for its determination. *Am J Physiol*, 175(1):157–61, 1953.

[200] M. V. Tzvetkov, J. Brockmoller, I. Roots, and J. Kirchheiner. Common genetic variations in human brain-specific tryptophan hydroxylase-2 and response to antidepressant treatment. *Pharmacogenet Genomics*, 18(6):495–506, 2008.

[201] L. Valzelli, E. Giacalone, and S. Garattini. Pharmacological control of aggressive behavior in mice. *Eur J Pharmacol*, 2(2):144–6, 1967.

[202] A. Van Den Bogaert, S. De Zutter, L. Heyrman, J. Mendlewicz, R. Adolfsson, C. Van Broeckhoven, and J. Del-Favero. Response to zhang et al (2005): loss-of-function mutation in tryptophan hydroxylase-2 identified in unipolar major depression. neuron 45, 11-16. *Neuron*, 48(5):704; author reply 705–6, 2005.

[203] A. Van Den Bogaert, K. Sleegers, S. De Zutter, L. Heyrman, K. F. Norrback, R. Adolfsson, C. Van Broeckhoven, and J. Del-Favero. Association of brain-specific tryptophan hydroxylase, tph2, with unipolar and bipolar disorder in a northern swedish, isolated population. *Arch Gen Psychiatry*, 63(10):1103–10, 2006.

[204] A. M. van Erp and K. A. Miczek. Aggressive behavior, increased accumbal dopamine, and decreased cortical serotonin in rats. *J Neurosci*, 20(24):9320–5, 2000.

[205] E. S. van Kleef, P. Gaspar, and A. Bonnin. Insights into the complex influence of 5-ht signaling on thalamocortical axonal system development. *Eur J Neurosci*, 35(10):1563–72, 2012.

[206] H. M. van Praag. In search of the mode of action of antidepressants. 5-htp/tyrosine mixtures in depressions. *Neuropharmacology*, 22(3 Spec No):433–40, 1983.

[207] M. Vaswani, F. K. Linda, and S. Ramesh. Role of selective serotonin reuptake inhibitors in psychiatric disorders: a comprehensive review. *Prog Neuropsychopharmacol Biol Psychiatry*, 27(1):85–102, 2003.

[208] J. Veenstra-VanderWeele, G. M. Anderson, and Jr. Cook, E. H. Pharmacogenetics and the serotonin system: initial studies and future directions. *Eur J Pharmacol*, 410(2-3):165–181, 2000.

[209] C. V. Vorhees and M. T. Williams. Morris water maze: procedures for assessing spatial and related forms of learning and memory. *Nat Protoc*, 1(2):848–58, 2006.

[210] J. Waider, N. Araragi, L. Gutknecht, and K. P. Lesch. Tryptophan hydroxylase-2 (tph2) in disorders of cognitive control and emotion regulation: a perspective. *Psychoneuroendocrinology*, 36(3):393–405, 2011.

[211] S. Walitza, T. J. Renner, A. Dempfle, K. Konrad, Ch Wewetzer, A. Halbach, B. Herpertz-Dahlmann, H. Remschmidt, J. Smidt, M. Linder, L. Flierl, U. Knolker, S. Friedel, H. Schafer, C. Gross, J. Hebebrand, A. Warnke, and K. P. Lesch. Transmission disequilibrium of polymorphic variants in the tryptophan hydroxylase-2 gene in attention-deficit/hyperactivity disorder. *Mol Psychiatry*, 10(12):1126–32, 2005.

[212] J. A. Wallace and J. M. Lauder. Development of the serotonergic system in the rat embryo: an immunocytochemical study. *Brain Res Bull*, 10(4):459–79, 1983.

[213] D. J. Walther and M. Bader. A unique central tryptophan hydroxylase isoform. *Biochem Pharmacol*, 66(9):1673–80, 2003.

[214] D. J. Walther, J. U. Peter, S. Bashammakh, H. Hortnagl, M. Voits, H. Fink, and M. Bader. Synthesis of serotonin by a second tryptophan hydroxylase isoform. *Science*, 299(5603):76, 2003.

[215] Y. M. Wang, R. R. Gainetdinov, F. Fumagalli, F. Xu, S. R. Jones, C. B. Bock, G. W. Miller, R. M. Wightman, and M. G. Caron. Knockout of the vesicular monoamine transporter 2 gene results in neonatal death and supersensitivity to cocaine and amphetamine. *Neuron*, 19(6):1285–96, 1997.

[216] A. Weller, A. C. Leguisamo, L. Towns, S. Ramboz, E. Bagiella, M. Hofer, R. Hen, and D. Brunner. Maternal effects in infant and adult phenotypes of 5ht1a and 5ht1b receptor knockout mice. *Dev Psychobiol*, 42(2):194–205, 2003.

[217] C. D. Wise, B. D. Berger, and L. Stein. Benzodiazepines: anxiety-reducing activity by reduction of serotonin turnover in the brain. *Science*, 177(44):180–3, 1972.

[218] M. Wolff, N. Benhassine, P. Costet, R. Hen, L. Segu, and M. C. Buhot. Delay-dependent working memory impairment in young-adult and aged 5-ht1bko mice as assessed in a radial-arm water maze. *Learn Mem*, 10(5):401–9, 2003.

[219] V. K. Yadav, F. Oury, N. Suda, Z. W. Liu, X. B. Gao, C. Confavreux, K. C. Klemenhagen, K. F. Tanaka, J. A. Gingrich, X. E. Guo, L. H. Tecott, J. J. Mann, R. Hen, T. L. Horvath, and G. Karsenty. A serotonin-dependent mechanism explains the leptin regulation of bone mass, appetite, and energy expenditure. *Cell*, 138(5):976–89, 2009.

[220] T Yamaguchi, M Sawada, T Kato, and T Nagatsu. Demonstration of tryptophan 5-monooxygenase activity in human brain by high sensitive high-performance liquid chromatography with fluorometric detection. *Biochem. Int.*, 2:295–303, 1981.

[221] K. B. Zajicek, C. S. Price, S. E. Shoaf, P. T. Mehlman, S. J. Suomi, M. Linnoila, and J. D. Higley. Seasonal variation in csf 5-hiaa concentrations in male rhesus macaques. *Neuropsychopharmacology*, 22(3):240–50, 2000.

[222] F. D. Zepf, C. Stadler, L. Demisch, M. Schmitt, M. Landgraf, and F. Poustka. Serotonergic functioning and trait-impulsivity in attention-deficit/hyperactivity-disordered boys (adhd): influence of rapid tryptophan depletion. *Hum Psychopharmacol*, 23(1):43–51, 2008.

[223] X. Zhang, J. M. Beaulieu, R. R. Gainetdinov, and M. G. Caron. Functional polymorphisms of the brain serotonin synthesizing enzyme tryptophan hydroxylase-2. *Cell Mol Life Sci*, 63(1):6–11, 2006.

[224] X. Zhang, J. M. Beaulieu, T. D. Sotnikova, R. R. Gainetdinov, and M. G. Caron. Tryptophan hydroxylase-2 controls brain serotonin synthesis. *Science*, 305(5681):217, 2004.

[225] X. Zhang, R. R. Gainetdinov, J. M. Beaulieu, T. D. Sotnikova, L. H. Burch, R. B. Williams, D. A. Schwartz, K. R. Krishnan, and M. G. Caron. Loss-of-function mutation in tryptophan hydroxylase-2 identified in unipolar major depression. *Neuron*, 45(1):11–6, 2005.

[226] S. Zhao, J. Edwards, J. Carroll, L. Wiedholz, R. A. Millstein, C. Jaing, D. L. Murphy, T. H. Lanthorn, and A. Holmes. Insertion mutation at the c-terminus of the serotonin transporter dis-

rupts brain serotonin function and emotion-related behaviors in mice. *Neuroscience*, 140(1):321–34, 2006.

[227] Z. Q. Zhao, M. Scott, S. Chiechio, J. S. Wang, K. J. Renner, R. W. th Gereau, R. L. Johnson, E. S. Deneris, and Z. F. Chen. Lmx1b is required for maintenance of central serotonergic neurons and mice lacking central serotonergic system exhibit normal locomotor activity. *J Neurosci*, 26(49):12781–8, 2006.

[228] Z. Zhou, E. J. Peters, S. P. Hamilton, F. McMahon, C. Thomas, P. J. McGrath, J. Rush, M. H. Trivedi, D. S. Charney, A. Roy, S. Wisniewski, R. Lipsky, and D. Goldman. Response to zhang et al. (2005): loss-of-function mutation in tryptophan hydroxylase-2 identified in unipolar major depression. neuron 45, 11-16. *Neuron*, 48(5):702–3; author reply 705–6, 2005.

[229] Z. Zhou, A. Roy, R. Lipsky, K. Kuchipudi, G. Zhu, J. Taubman, M. A. Enoch, M. Virkkunen, and D. Goldman. Haplotype-based linkage of tryptophan hydroxylase 2 to suicide attempt, major depression, and cerebrospinal fluid 5-hydroxyindoleacetic acid in 4 populations. *Arch Gen Psychiatry*, 62(10):1109–18, 2005.

[230] P. Zill, T. C. Baghai, P. Zwanzger, C. Schule, D. Eser, R. Rupprecht, H. J. Moller, B. Bondy, and M. Ackenheil. Snp and haplotype analysis of a novel tryptophan hydroxylase isoform (tph2) gene provide evidence for association with major depression. *Mol Psychiatry*, 9(11):1030–6, 2004.

[231] P. Zill, A. Buttner, W. Eisenmenger, B. Bondy, and M. Ackenheil. Regional mrna expression of a second tryptophan hydroxylase isoform in postmortem tissue samples of two human brains. *Eur Neuropsychopharmacol*, 14(4):282–4, 2004.

[232] P. Zill, A. Buttner, W. Eisenmenger, H. J. Moller, B. Bondy, and M. Ackenheil. Single nucleotide polymorphism and haplotype analysis of a novel tryptophan hydroxylase isoform (tph2) gene in suicide victims. *Biol Psychiatry*, 56(8):581–6, 2004.

PUBLICATIONS

1. Klempin F, Beis D, **Mosienko V**, Kempermann G, Bader M, and Alenina N. Serotonin is required for exercise-induced adult hippocampal neurogenesis. In revision by J Neurosci.

2. **Mosienko V**, Matthes S, Beis D, Bader M, Alenina N. Normal behavior in mice with decreased TPH2 activity is associated with reduced serotonin turnover and 5-HT1A receptor sensitization. Submitted to J Neuropsychopharm.

3. **Mosienko V**, Narboux-Neme N, Angenard G, Klempin F, Pitychoutis PM, Deneris E, Bader M, Giros B, Alenina N, Gaspar P. Postnatal growth defects in mice with constitutive depletion of central serotonin. ACS Chem Neurosci. 2013 Jan 16;4(1):171-81.

4. **Mosienko V**, Bert B, Beis D, Fink H, Bader M, Alenina N. Exaggerated aggression and decreased anxiety in mice deficient in brain serotonin. TranslPsychiatry 2012, 2, e122.

5. Matthes S, **Mosienko V**, Bashammakh S, Alenina N, Bader M. Tryptophan hydroxylase as novel target for the treatment of depressive disorders. Pharmacology 2010;85(2):95-109.

6. Alenina N, Kikic D, Todiras M, **Mosienko V**, Qadri F, Plehm R, Boye Ph, Sohr R, Hoertnagl H, Tenner K, Bader M. Growth retardation and altered autonomic control in mice lacking brain serotonin. PNAS, 2009;106(25):10332-7.

7. Liskovykh M, Chuykin I, Ranjan A, Safina D, Popova E, Tolkunova E, **Mosienko V**, Minina JM, Zhdanova NS, Mullins JJ, Bader M, Alenina N, Tomilin A. Derivation, characterization, and stable transfection of induced pluripotent stem cells from Fischer344 rats. PloS One, 2011; 6(11):e27345.

8. Chuykin I, Lapidus I, Popova E, Vilianovich L, **Mosienko V**, Alenina N, Binas B, Krivokharchenko A, Bader M. Characterization of trophoblast and extraembryonic endoderm cell lineages derived from rat preimplantation embryos. PLoS One, 2010; 5(3) e9794

PUBLICATIONS

1. Klempin F, Beis D, **Mosienko V**, Kempermann G, Bader M, and Alenina N. Serotonin is required for exercise-induced adult hippocampal neurogenesis. excepted to J Neurosci.

2. Narboux-Neme N*, Angenard G*, **Mosienko V***, Klempin F, Pitychoutis PM, Deneris E, Bader M, Giros B, Alenina N, Gaspar P. Postnatal growth defects in mice with constitutive depletion of central serotonin. ACS Chem Neurosci. 2013 Jan 16;4(1):171-81. (*equal contribution)

3. **Mosienko V**, Bert B, Beis D, Fink H, Bader M, Alenina N. Exaggerated aggression and decreased anxiety in mice deficient in brain serotonin. TranslPsychiatry 2012, 2, e122.

4. Matthes S, **Mosienko V**, Bashammakh S, Alenina N, Bader M. Tryptophan hydroxylase as novel target for the treatment of depressive disorders. Pharmacology 2010;85(2):95-109.

5. Alenina N, Kikic D, Todiras M, **Mosienko V**, Qadri F, Plehm R, Boye Ph, Sohr R, Hoertnagl H, Tenner K, Bader M. Growth retardation and altered autonomic control in mice lacking brain serotonin. PNAS, 2009;106(25):10332-7.

6. Liskovykh M, Chuykin I, Ranjan A, Safina D, Popova E, Tolkunova E, **Mosienko V**, Minina JM, Zhdanova NS, Mullins JJ, Bader M, Alenina N, Tomilin A. Derivation, characterization, and stable transfection of induced pluripotent stem cells from Fischer344 rats. PloS One, 2011; 6(11):e27345.

7. Chuykin I, Lapidus I, Popova E, Vilianovich L, **Mosienko V**, Alenina N, Binas B, Krivokharchenko A, Bader M. Characterization of trophoblast and extraembryonic endoderm cell lineages derived from rat preimplantation embryos. PLoS One, 2010; 5(3) e9794

EIDESSTATTLICHE ERKLÄRUNG

Hiermit erkläre ich, dass ich die vorgelegte Dissertation mit dem Titel 'Behavioral, neuronal, and developmental consequences of genetically decreased Tryptophan hydroxylase 2 activity' selbständig und nur unter Verwendung der angegebenen Hilfsmittel angefertigt habe.

Das Weiteren erkläre ich, dass ich mich nicht anderweitig um einen Doktorgrad beworben habe und auch keinen entsprechenden Doktorgrad besitze.

Ferner erkläre ich, dass ich von der dem angestrebten Verfahren zugrunde liegenden Promotionsordnung der Mathematisch/Naturwissenschaftlichen Fakultät I vom 06.07.2009 Kenntnis genommen habe.

Valentina Mosienko
Berlin, den 15.04.2013