Spatio-temporal Expression Patterns of the Serotonin Synthesis Enzymes TPH1 and TPH2 and Effects of Acute Stress

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Claudia Kriegebaum

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Dekan:	Prof. Dr. Martin Müller Lehrstuhl für Pharmazeutische Biologie der Julius-Maximilians-Universität Würzburg Julius-von-Sachs-Platz 2, 97082 Würzburg
Erstgutachter:	Prof. Dr. Klaus-Peter Lesch Klinik für Psychiatrie, Psychosomatik und Psychotherapie der Julius-Maximilians-Universität Würzburg Füchsleinstr. 15, 97080 Würzburg
Zweitgutachter:	PD Dr. Bertram Gerber Lehrstuhl für Genetik und Neurobiologie der Julius-Maximilians-Universität Würzburg Biozentrum, Am Hubland, 97074 Würzburg
Kooperationspartner:	Dr. Yu-Qiang Ding Institute of Neuroscience (ION) 320 Yue Yang Road Shanghai, 200031 P.R.China

A INDEX

B. LIST OF SCIENTIFIC PUBLICATIONS

Original articles:

1. Gutknecht L., Jacob C., Strobel A., Kriegebaum C., Muller J., Zeng Y., Markert C., Escher A., Wendland J., Reif A., Mossner R., Gross C., Brocke B., Lesch K. P. (2007). **Tryptophan hydroxylase-2 gene variation influences personality traits and disorders related to emotional dysregulation.** Int J Neuropsychopharmacol 10: 309-320.

2. Gutknecht L., Waider J., Kraft S., Kriegebaum C., Holtmann B., Reif A., Schmitt A., Lesch K. P. (2008). **Deficiency of brain 5-HT synthesis but serotonergic neuron formation in Tph2 knockout mice.** J Neural Transm 115: 1127-1132.

3. Gutknecht L.*, Kriegebaum C.*, Waider J., Schmitt A., Lesch K. P. (2009). **Spatio-temporal expression of tryptophan hydroxylase isoforms in murine and human brain: convergent data from Tph2 knockout mice.** Eur Neuropsychopharmacol 19: 266-282 (*equal contribution).

4. Kriegebaum C., Gutknecht L., Bartke L., Reif A., Lesch K.P., Schmitt A. (2009) **The expression of the transcription factor FEV in adult human brain and its association with affective disorders.** Submitted to J Neural Transm August 27st 2009.

Review articles:

5. Kriegebaum C., Gutknecht L., Schmitt A., Lesch K.P., Reif A. (2009). **Serotonin kompakt - Neurobiologie, Genetik und Psychopathologie.** Submitted to Fortschritte der Neurologie • Psychiatrie July 18st 2009.

C. LECTURES

1. Kriegebaum C. (15 Jun 2009) **Spatio-temporal Expression Patterns of the Serotonin Synthesis Enzymes TPH1 and TPH2 and Effects of Acute Stress.** Neurobiological colloquium, Department of Psychiatry, Psychosomatics and Psychotherapy, University of Würzburg.

2. Kriegebaum C. (01 Dez 2008) **The Happiness Hormone: Serotonin or Oxytocin?** Neurobiological colloquium, Department of Psychiatry, Psychosomatics and Psychotherapy, University of Würzburg.

3. Kriegebaum C. (24. Jul 2008) **Die induzierbare Knockout-Maus.** Presentation for F1 practical course for biological students, Department of Psychiatry, Psychosomatics and Psychotherapy, University of Würzburg.

4. Kriegebaum C. (10. Apr 2008) **The Two Isoforms of Tryptophan Hydroxylase: Relative Expression Studies II.** Fifth Würzburg Brain and Behaviour Days: Comparative research on emotion processing. A joint venture of Graduate College (GRK) 1156: *"From Synaptic Plasticity to Behavioural Modulation in Genetic Model Organisms"* and GRK 1253: "Emotions" within the International Graduate School of Life Sciences.

5. Kriegebaum C. (16. Jan 2008) **TPH2 - Das relevante Enzym der neuronalen Serotoninsynthese in Maus und Mensch.** Scientific meeting, Department of Psychiatry, Psychosomatics and Psychotherapy, University of Würzburg.

6. Kriegebaum C. (27. Apr 2007) **The Two Isoforms of Tryptophan Hydroxylase: Relative Expression Studies.** Fourth Würzburg Brain and Behaviour Days: *Presentation of the latest results,* meeting of the Graduate College (GRK) 1156: *"From Synaptic Plasticity to Behavioural Modulation in Genetic Model Organisms"* within the International Graduate School of Life Sciences.

7. Kriegebaum C. (07. Apr 2006) **Relative Expression of Tryptophan Hydroxylase 1 and 2: Spatial and Temporal Analysis.** Third Würzburg Brain and Behaviour Days: *Presentation of the first results,* meeting of the Graduate College (GRK) 1156: *"From Synaptic Plasticity to Behavioural Modulation in Genetic Model Organisms"* within the International Graduate School of Life Sciences.

8. Kriegebaum C. (03. Dez 2005) **Expression Studies of Serotonin System Specific Genes in Mice and Humans.** Second Würzburg Brain and Behaviour Days: *A critical evaluation of available methods,* meeting of the Graduate College (GRK) 1156: *"From Synaptic Plasticity to Behavioural Modulation in Genetic Model Organisms"* within the International Graduate School of Life Sciences.

9. Kriegebaum C. (30. Nov 2005) **Untersuchungen zur Expression Serotoninsystem-spezifischer Gene in Mensch und Maus - FEV/Pet1 und TPH1/2.** Scientific meeting, Department of Psychiatry, Psychosomatics and Psychotherapy, University of Würzburg.

D. PRESENTATIONS AT CONFERENCES

1. Kriegebaum C., Schmitt A., Nietzer S., Lesch K.P., Gutknecht L. (Poster) **Spatio-temporal expression pattern of the two isoforms of tryptophan hydroxylase.** Newmood AGM, 2008 in Stockholm, Sweden.

2. Kriegebaum C., Schmitt A., Nietzer S., Lesch K.P., Gutknecht L. (Poster) **The two isoforms of tryptophan hydroxylase: relative expression studies.** ECNP (*European college of neuropsychopharmacology*) workshop in neuropsychopharmacology for young scientists, 2007 in Nice, France.

3. Kriegebaum C., Schmitt A., Nietzer S., Lesch K.P., Gutknecht L. (Poster) **The two isoforms of tryptophan hydroxylase: TPH1 and TPH2.** 9th congress of DGBP (*Deutsche Gesellschaft für biologische Psychiatrie*) 2006 in Munich, Germany.

4. Schmitt A., Nietzer S., Meier M., Ortega G., Kriegebaum C., Gutknecht L., Bogusch L., Riederer P., Deckert J., Lesch K.P. (Poster) **Differential regulation of synaptic vesicle proteins in serootonin transporter deficient mice - a mouse model for anxiety disorders.** 9th congress of DGBP (*Deutsche Gesellschaft für biologische Psychiatrie*) 2006 in Munich, Germany.

5. Gutknecht L., Kriegebaum C., Lesch K.P., Schmitt A. (Poster) **Relative expression of Tryptophan hydroxylase 1 and 2: spatial and temporal analysis.** 5th Forum of European Neuroscience (FENS, Federation of European Neuroscience Societies) 2006 in Vienna, Austria.

E. CURRICULUM VITAE

Personal data

Name	Claudia Brigitte Kriegebaum
Date of birth	11.12.1980 in
City of birth	Schweinfurt, Germany
Marital status	unmarried, no children

Professional career

since 09/2005	**PhD programme** at the Molecular Psychiatry, Department of Psychiatry, Psychosomatics and Psychotherapy, Julius-Maximilians University of Würzburg (Supervisor: Prof. Dr. K.-P. Lesch). ***PhD thesis***: *„Spatio-temporal Expression Patterns of the Serotonin Synthesis Enzymes TPH1 and TPH2 and Effects of Acute Stress."*
09/2007 – 11/2007	**Research fellowship** at the ION (*Institute of Neurosciences*) within the framework of GRK 1156 with **Shanghai, China**
09/2005 – 08/2008	**PhD student fellowship** of the DFG Graduiertenkolleg (GRK 1156): *"From Synaptic Plasticity to Behavioural Modulation in Genetic Model Organisms"* within the International Graduate School of Life Sciences

University Education

07/2005	Diploma in Biology with the main subject of **Neurobiology**
10/2004 - 6/2005	**Diploma thesis**: *"Expression studies of specific genes of the serotonergic system, FEV/Pet1 and Tph1/Tph2 in mice and humans"* at the Department of Psychiatry, Psychosomatics and Psychotherapy, University of Würzburg
10/2000 - 07/2005	University of Würzburg, Course of study: **Biology**

School Education

07/2000	University entrance diploma ("Abitur")
09/1991- 07/2000	Olympia-Morata-Gymnasium of Schweinfurt, Germany

F. ABSTRACT GERMAN

Durch zahlreiche Untersuchungen ist belegt, dass eine gestörte Tryptophan-Hydroxylase (TPH)-abhängige Serotonin (5-HT)-Synthese an einer veränderten emotionalen Reaktion sowie einer veränderten Stress-Antwort beteiligt ist und damit auch in der Ätiologie und Pathogenese psychischer Erkrankungen eine Rolle spielt. Dennoch werden nach wie vor die unterschiedlichen Expressionsmuster der beiden Isoformen *TPH1* und *TPH2*, die für zwei Formen des Schrittmacherenzyms der 5-HT-Synthese kodieren, kontrovers diskutiert. Zentrales Anliegen dieser Arbeit ist daher eine Klärung der TPH1- und TPH2-Expression während der prä- und postnatalen Entwicklung des murinen Gehirns, sowie im adulten humanen Gehirn und in einigen peripheren Organen und der Zirbeldrüse. Durch die Verwendung von vier verschiedenen Methoden (Real time-PCR, *In situ*-Hybridisierung, Immunhistochemie und Westernblot-Analysen) wurde systematisch die Gewebs- und Isoform-spezifische Expression in Maus und Mensch auf prä- und posttranslationaler Ebene nachgewiesen. TPH2-Expression wurde Spezies-übergreifend in den Raphe-Kernen des Hirnstamms wie auch in Fasern zur Zirbeldrüse und im Gastrointestinaltrakt detektiert. Auch TPH1 konnte in diesen peripheren Organen (die Zirbeldrüse eingeschlossen) nachgewiesen werden, jedoch fand sich keine signifikante TPH1-Expression im Gehirn, weder während der Entwicklung des Maus-Gehirns noch im humanen und murinen adulten Gehirn. Auch durch veränderte Bedingungen wie der Entfernung von Blutzellen aus dem Gewebe oder der Anwendung von akutem Immobilisierungsstress konnte keine Änderung der Expression gemessen werden.

Die aktive Wiederaufnahme von 5-HT in das präsynaptische Neuron durch den Serotonintransporter (SERT) ist ein wichtiger Mechanismus um die serotonerge Neurotransmission zu beenden. Mäuse, deren Sert-Gen deletiert wurde (Sert Knockout-Mäuse), stellen ein geeignetes Tiermodell für affektive Erkrankungen dar, insbesondere um eine lebenslang veränderte 5-HT-Homöostase in Verbindung mit belastenden Lebensereignissen zu untersuchen. Um die Bedeutung der TPH-Isoformen und deren korrekte Expression weiter zu untersuchen, wurde die Tph1- und Tph2-Expression in den Raphe-Kernen von Sert Knockout (KO)-Mäusen unter normalen Bedingungen und nach akutem Stress getestet. Interessanterweise konnten keine statistisch signifikanten Expressionsänderungen entdeckt werden. Mehr noch, relativ zu Tph2 konnte unabhängig von Behandlung, Geschlecht oder Genotyp keine relevante Tph1-Expression im Gehirn gemessen werden, was wiederum die Expressionsdaten aus nativen Tieren unterstützt.

Die Raphe-Neurone eines Gehirn-spezifischen konditionalen Tph2 KO-Modells zeigten weder Tph2-positive Zellen noch 5-HT, wiesen aber auch keine kompensatorische Aktivierung der Tph1-Expression im Gehirn auf. Zusätzlich repräsentiert eine zeit-spezifische, induzierbare KO-Maus ein Gehirn-spezifisches Tph2 Knockdown-Modell ab dem Erwachsenenalter, das eine stark reduzierte Anzahl an Tph2-positiven Zellen und 5-HT im Gehirn aufweist. Expressionsuntersuchungen zeigten interessanterweise, dass diese Gehirn-spezifischen Tph2 Knockout- und Knockdown-Modelle keine sichtliche Änderung in der Expression von 5-HT-System-assoziierten Genen aufweisen. Diese Ergebnisse bestätigen zum einen, dass die 5-HT-Synthese im murinen Gehirn während der kompletten Lebensspanne ausschließlich durch Tph2 katalysiert wird und weisen außerdem darauf hin, dass eine Tph2-abhängige 5-HT-Synthese weder während der Entwicklung noch im Erwachsenalter für die Ausbildung eines normalen serotonergen Systems benötigt wird, obwohl Tph1 den Verlust des 5-HT-Vorkommens im Gehirn der Tph2 KO-Mäuse nicht kompensiert.

Weiterhin beschäftigt sich diese Arbeit mit der Expression des Neuropeptids Oxytocin, das hauptsächlich im Hypothalamus produziert und z.B. als Antwort auf die Anregung von 5-HT und selektiven Serotonin Wiederaufnahme-Inhibitoren (SSRIs) ausgeschüttet wird. Auch wirkt Oxytocin als Neuromodulator im zentralen Nervensystem (ZNS) und ist maßgeblich bei der Schmerzmodulation, bei Angst-lösenden Effekten sowie einer verringerten Stressantwort beteiligt, wodurch das Risiko für affektive Erkrankungen vermindert werden könnte. In dieser Studie wurde die Expression von Oxytocin in verschiedenen Gehirnregionen (Cortex, Hippocampus, Amygdala, Hypothalamus und Raphe Nuclei) von weiblichen und männlichen Wildtyp- (WT) und Sert KO-Mäusen getestet, die entweder unter normalen Bedingungen gehalten wurden oder eine Stunde lang akutem Immobilisierungsstress ausgesetzt waren. Die Ergebnisse zeigten eine signifikant höhere Oxytocin-Expression in Gehirnregionen, die für die emotionale Reizverarbeitung zuständig sind (Amygdala und Hippocampus) in gestressten männlichen WT-Mäusen, während männliche Sert KO-Mäuse sowie weibliche WT- und Sert KO-Mäuse diese Stress-bedingten Unterschiede nicht aufwiesen. Diese Befunde sind im Einklang mit der Hypothese, dass Oxytocin eine schützende Rolle bei Stress, depressiver Stimmung und Angst übernimmt, weisen jedoch auf einen Geschlechterunterschied hin. Ferner legt das Fehlen einer veränderten Oxytocin-Expression in Sert KO-Mäusen eine Modulation der Oxytocin-Expression durch das serotonerge System nahe, was neue Forschungsperspektiven über eine veränderte Reaktion auf Stress und Angst-auslösende Reize in Sert KO-Mäusen eröffnet.

G. ABSTRACT ENGLISH

Several lines of evidence implicate a dysregulation of tryptophan hydroxylase (TPH)-dependent serotonin (5-HT) synthesis in emotions and stress and point to their potential relevance to the etiology and pathogenesis of various neuropsychiatric disorders. However, the differential expression pattern of the two isoforms *TPH1* and *TPH2* which encode two forms of the rate-limiting enzyme of 5-HT synthesis is controversial. Here, a comprehensive spatio-temporal analysis clarifies TPH1 and TPH2 expression during pre- and postnatal development of the mouse brain and in adult human brain as well as in peripheral organs including the pineal gland. Four different methods (real time PCR, *in situ* hybridization, immunohistochemistry and Western blot analysis) were performed to systematically control for tissue-, species- and isoform-specific expression on both the pre- and posttranslational level. TPH2 expression was consistently detected in the raphe nuclei, as well as in fibres in the deep pineal gland and in the gastrointestinal tract. Although TPH1 expression was found in these peripheral tissues, no significant TPH1 expression was detected in the brain, neither during murine development, nor in mouse and human adult brain. Also under conditions like stress and clearing the tissue from blood cells, no changes in expression levels were detectable.

Furthermore, the reuptake of 5-HT into the presynaptic neuron by the serotonin transporter (SERT) is the major mechanism terminating the neurotransmitter signal. Thus, mice with a deletion in the *Sert* gene (Sert KO mice) provide an adequate model for human affective disorders to study lifelong modified 5-HT homeostasis in interaction with stressful life events. To further explore the role of TPH isoforms, Tph1 and Tph2 expression was studied in the raphe nuclei of Sert deficient mice under normal conditions as well as following exposure to acute immobilization stress. Interestingly, no statistically significant changes in expression were detected. Moreover, in comparison to Tph2, no relevant Tph1 expression was detected in the brain independent from genotype, gender and treatment confirming expression in data from native animals.

Raphe neurons of a brain-specific Tph2 conditional knockout (cKO) model were completely devoid of Tph2-positive neurons and consequently 5-HT in the brain, with no compensatory activation of Tph1 expression. In addition, a time-specific Tph2 inducible (i) KO mouse provides a brain-specific knockdown model during adult life, resulting in a highly reduced number of Tph2-positive cells and 5-HT in the brain. Intriguingly, expression studies detected no obvious alteration in expression of 5-HT system-associated genes in these brain-specific Tph2 knockout and knockdown models. The findings on the one hand confirm the specificity

of Tph2 in brain 5-HT synthesis across the lifespan and on the other hand indicate that neither developmental nor adult Tph2-dependent 5-HT synthesis is required for normal formation of the serotonergic system, although Tph1 does not compensate for the lack of 5-HT in the brain of Tph2 KO models.

A further aim of this thesis was to investigate the expression of the neuropeptide oxytocin, which is primarily produced in the hypothalamus and released for instance in response to stimulation of 5-HT and selective serotonin reuptake inhibitors (SSRIs). Oxytocin acts as a neuromodulator within the central nervous system (CNS) and is critically involved in mediating pain modulation, anxiolytic-like effects and decrease of stress response, thereby reducing the risk for emotional disorders. In this study, the expression levels of oxytocin in different brain regions of interest (cortex, hippocampus, amygdala, hypothalamus and raphe nuclei) from female and male wildtype (WT) and Sert KO mice with or without exposure to acute immobilization stress were investigated. Results showed significantly higher expression levels of oxytocin in brain regions which are involved in the regulation of emotional stimuli (amygdala and hippocampus) of stressed male WT mice, whereas male Sert KO as well as female WT and Sert KO mice lack these stress-induced changes. These findings are in accordance with the hypothesis of oxytocin being necessary for protection against stress, depressive mood and anxiety but suggest gender-dependent differences. The lack of altered oxytocin expression in Sert KO mice also indicates a modulation of the oxytocin response by the serotonergic system and provides novel research perspectives with respect to altered response of Sert KO mice to stress and anxiety inducing stimuli.

In the following, abbreviations of rodent genes/proteins are given in lower case, while human genes/proteins are given in upper case (the latter is also used for the general term or when the term refers to both species), for example TPH2 for the human (or in general) and Tph2 for the murine tryptophan hydroxylase 2.

1. INTRODUCTION

1.1 Serotonin - sources, effects and metabolism

The discovery of serotonin (5-hydroxytryptamine, 5-HT) was one of the most important discoveries in neuroscience. First described as a vaso-constricting substance in enterochromaffine cells of the gut, later studies (especially using LSD (lysergic acid diethyl amide)) demonstrated a function as a neurotransmitter and implicated 5-HT in the etiology and pathogenesis of various psychiatric disorders. Like the catecholamines (adrenaline, noradrenaline, dopamine) and histamine, the indolamine serotonin belongs to the class of the monoaminergic transmitters, whose components are generated by the decarboxylation of amino acids.

1.1.1 The biosynthesis and neurotransmission of serotonin

The biosynthesis of serotonin mainly takes place in the cell bodies (see Fig. 1-1) and in lower amounts in the dendrites and axons of neurons (see Results, Fig. 3-6 d). The first step of the 5-HT synthesis is the hydroxylation of the essential amino acid **tryptophan** to 5-hydroxy-tryptophan using molecular oxygen and tetrahydrobiopterin (BH_4) as co-substrates. The following step, the decarboxylation of 5-hydroxy-tryptophan to 5-hydroxy-tryptamine (5-HT, **serotonin**) then requires pyridoxal-5'-phosphate (vitamin B6) as co-substrate. The initial and rate-limiting step is catalysed by the pacemaker enzyme trypthophan hydroxylase (**TPH**) with Fe^{2+} as co-factor. The faster following step is catalysed by the ubiquitous enzyme aromatic L-amino acid decarboxylase (AAAD) which also decarboxylates the catecholamines and histamine (see Fig. 1-1). For many years, one single gene encoding TPH was thought to be responsible for 5-HT biosynthesis in vertebrates. Based on the generation of trypthophan hydroxylase knockout (KO) mice in the year 2003, a second isoform (**TPH2**) was discovered and characterized (Côté et al., 2003; Walther and Bader, 2003; Walther et al., 2003). Hence, studies up to the year 2003 did not distinguish between **TPH1** and **TPH2**. In the present thesis,

the term "TPH" is used if no specific isoform is referred to (for further information about the different isoforms of TPH, see chapter 1.1.2 and 1.2). Since tryptophan, oxygen, and BH_4 are usually available in subsaturating substrate levels for TPH1 and TPH2, changes in their concentration may modify the production rate of 5-HT. Contrary to amino acids like tryptophan, the monoamines like 5-HT are virtually not able to cross the blood brain barrier.

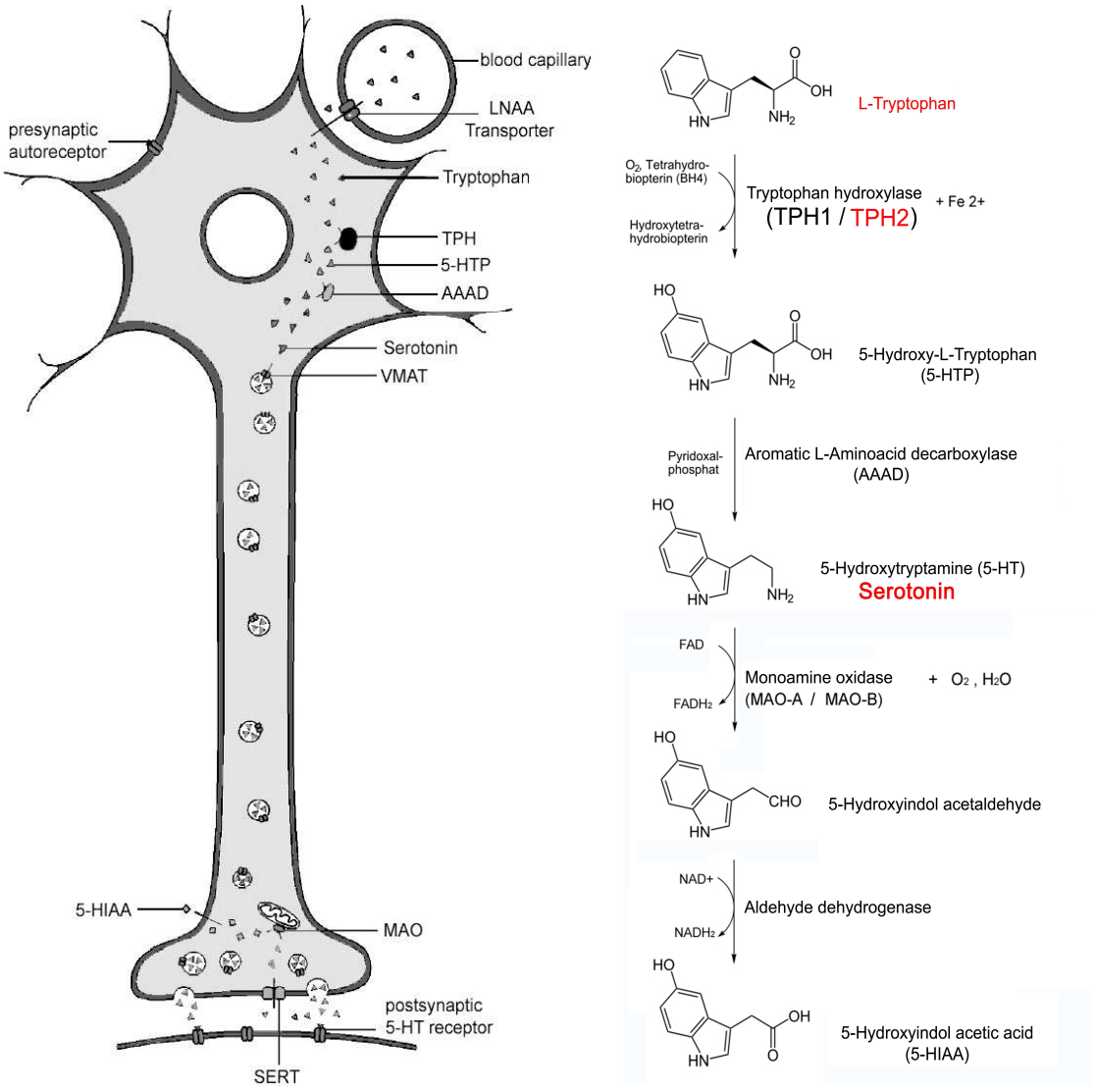


Fig. 1-1 Serotonin metabolism and neurotransmission.
Left: *Schematic illustration of a serotonergic neuron with synapse* ***Right:*** *Structural formulae of the serotonin metabolism. Tryptophan (Trp) is transported by the large neutral amino acid (LNAA) transporter through the blood brain barrier into the serotonergic neurons. In the cell body or the axon terminals Trp is converted into 5-hydroxy tryptophan (5-HTP). This reaction can be catalysed by two enzymes: tryptophan hydroxylase 1 or 2 (TPH1 or TPH2). However, TPH2 is the relevant isoform in the brain. 5-HTP is then converted into serotonin (5-HT) by the aromatic L-amino acid decarboxylase (AAAD) and stored in synaptic vesicles via the vesicular monoamine transporter (VMAT) 1 or VMAT2. Serotonin is released when an action potential reaches the terminals of a presynaptic serotonergic neuron by depolarizing the synaptic membrane and thereby inducing Ca^{2+} influx. The neurotransmitter molecules become signals, binding to receptor proteins in the membrane of postsynaptic or presynaptic cells (autoreceptors). After release of serotonin into the synaptic cleft, serotonin is actively re-transported into the presynaptic terminals by the serotonin transporter (SERT). Serotonin-molecules are stored again in vesicles or are deaminated by the mitochondrial membrane enzyme monoamine oxidase (MAO) A or MAO-B. Afterwards, the resulting intermediate 5-hydroxyindol-acetaldehyde is oxidized by the aldehyde dehydrogenase into 5-hydroxyindol acetic acid (5-HIAA) which can be measured from liquor and blood samples via HPLC (high performance liquid chromatography).*

With the aid of one of the two structurally very similar vesicular monoamine transporters (**VMAT**) VMAT1 or VMAT2, 5-HT is stored into neuronal vesicles or peripheral secretory granula (see Fig. 1-1 and Kriegebaum et al., 2009 submitted). When an action potential reaches the terminals of a presynaptic serotonergic neuron, it causes release of 5-HT by depolarizing the synaptic membrane thereby inducing Ca^{2+} influx. Calcium ions are intracellular messengers capable to activate a plethora of cellular functions (cf. Kriegebaum et al., 2009 submitted) causing neurotransmitter release into the synaptic cleft (see Fig. 1-1). The neurotransmitter molecules turn into signals when they bind to receptor proteins in the membrane of post- or presynaptic neurons. Once 5-HT is bound, the receptor generates either ion influx or metabolic signals in the cell. The co-release of several neuroactive substances onto appropriate postsynaptic receptors permits an extraordinary diversity of information to be transferred in a single synaptic action.

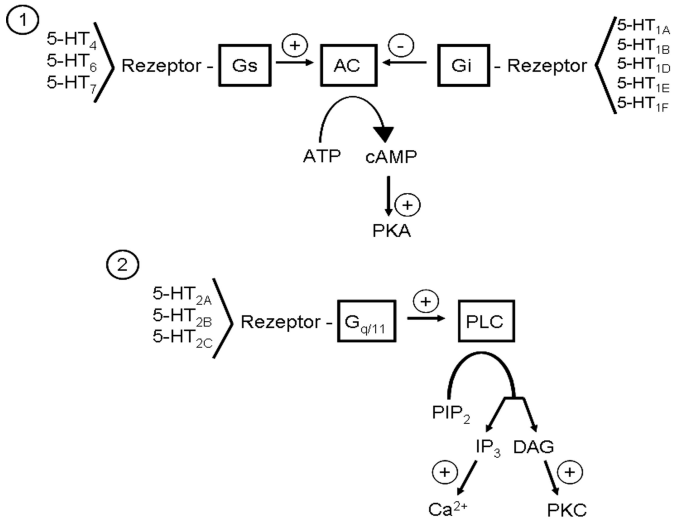


Fig. 1-2 5-HT receptors and G-protein coupled pathways. *5-HT_1 receptors typically inhibit adenylyl cyclase (AC) through G-proteins of the Gi family, whereas 5-HT_4, 5-HT_6, 5-HT_7 receptors typically stimulate AC through Gs family G-proteins. Activation of AC results in increased production of cAMP, leading to activation of protein kinase A (PKA). 5-HT_2 receptors activate phospholipase C through the $G_{q/11}$ family G-proteins, resulting in accumulation of phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositoltrisphosphat (IP_3) and diacylglycerol (DAG). Generation of IP_3 results in elevation of intracellular Ca^{2+} levels, whereas DAG activates the Ca^{2+} and phospholipid-dependent protein kinase C (PKC). Figure adapted from (Raymond et al., 2001).*

Molecular cloning studies have confirmed the existence of at least 14 different subtypes of 5-HT receptors which, according to their structural homology and effectors mechanisms, can be divided into seven families (**5-HT_1 - 5-HT_7**) with some families consisting of further subtypes (for instance 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_{1F}). Except for the 5-HT_3 receptor, which represents an ionotropic ligand-controlled ion channel, all other 5-HT receptors belong to the metabotropic superfamily of receptors which encode proteins that transduce signals through guanine nucleotide binding and regulatory proteins (G-proteins). The G-protein mediated signals can either inhibit (G_i-proteins, 5-HT_1 receptors) or stimulate (G_s-proteins, $5\text{-HT}_{4/6/7}$ receptors) adenylyl cyclase (**AC**) or activate the phospholipase C (**PLC**) pathway via $G_{q/11}$-proteins (5-HT_2 receptors) (see Fig. 1-2). After the release of the transmitter, presynaptically localized 5-HT_1 autoreceptors provide information about the concentration of 5-HT in the synaptic cleft (negative feedback) (see Fig. 1-1). For a detailed description of receptor mediated serotonergic action, the diversity and distribution of 5-HT receptors in the human brain and periphery as well as their physiological role see Kriegebaum et al., 2009 (submitted).

A key step that determines the intensity and duration of serotonergic neurotransmission is the active reuptake of 5-HT into the presynaptic terminals via the high-affinity membrane-bound serotonin transporter (**SERT**) (see Fig. 1-1). SERT is crucial in the control of both 5-HT homeostasis and extracellular concentration of 5-HT (for detailed information, see Kriegebaum et al., 2009 submitted). In recent years, multiple pharmacological, molecular and genetic approaches have implicated SERT in the regulation of complex behaviours associated with affect, emotion, reward and addiction.

After reuptake, 5-HT molecules are stored again in vesicles. However, a substantial amount of re-transported 5-HT is deaminated through the H_2O, O_2 and FAD (flavin adenine dinucleotid) dependent monoamine oxidase (**MAO**)-A or MAO-B which is localized in the membrane of mitochondria. Afterwards, the resulting intermediate 5-hydroxyindol acetaldehyde is oxidized into 5-hydroxyindol acetic acid (**5-HIAA**) which can be measured in liquor and blood samples via HPLC (high performance liquid chromatography). The following faster oxidation is catalyzed by aldehyde dehydrogenase, accompanied by the reduction of the oxidant NAD^+ (nicotine amide adenine dinucleotid) (see Fig. 1-3, more details in Kriegebaum et al., 2009 submitted).

1.1.2 Development and distribution of serotonin

The majority of 5-HT neurons, which feature the expression of TPH2 as the most prominent marker, are born in two main clusters in the anterior and posterior embryonic hindbrain between embryonic days (E)10.5 – 11.5 in the mouse and gestational weeks 5-6 in humans (Cordes, 2005). Later, these clusters are organized into nine cell groups (B1-B9) along the midline of the brainstem, referred to as the raphe nuclei (see Fig. 1-3). Serotonergic cells of the anterior group give rise to the raphe nuclei of the midbrain and upper pons (**rostral raphe complex**, B9-B5) and form widespread projections to the diencephalon, the forebrain and some cerebellar sites (Fig. 1-3). In the forebrain, serotonergic innervations arise mainly from the median raphe nucleus (MRN) and the dorsal raphe nucleus (DRN). The largest group of 5-HT neurons (B7) corresponds to the DRN. The DRN displays a complex internal morphology, with distinct subregions varying across the anterior-posterior (A-P) axis. Studies from Clark and co-workers revealed differences in the density of expression of TPH2 in the ventromedial, dorsomedial, and dorsolateral subnuclei of the DRN, as well as distinct variation in expression across the A-P axis. These findings provide additional evidence that subregions of the DRN are heterogeneous and need to be considered independently (Clark et al., 2006). The MRN corresponds to B8 and is localized at the junction between pons and midbrain. The rostral raphe complex is involved in control of memory, affective and emotional states, cognition,

thermoregulation, eating, sleeping and sexual as well as anxiety behaviour. 5-HT neurons of the **caudal raphe complex** (posterior group, B4-B1) develop during the second developmental wave, approximately 1-2 days after the anterior group has emerged, and build the principle descending projections to the dorsal horns (B3) and ventral horns (B1/B4, B2) of the spinal cord (Fig. 1-3). For a detailed version of the human serotonergic system see Kriegebaum et al., 2009 (submitted).

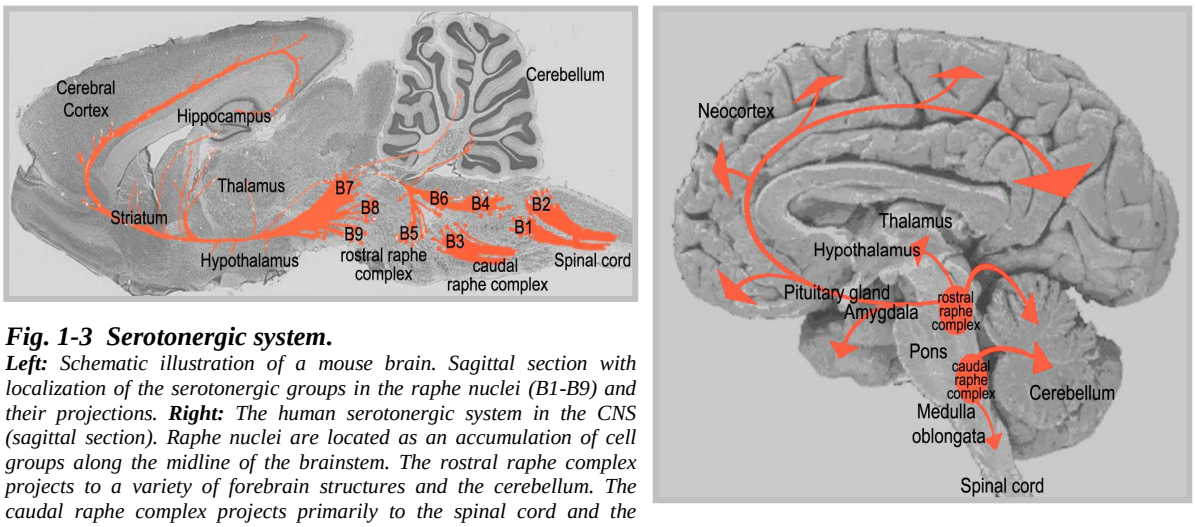


Fig. 1-3 Serotonergic system.
Left: *Schematic illustration of a mouse brain. Sagittal section with localization of the serotonergic groups in the raphe nuclei (B1-B9) and their projections.* ***Right:*** *The human serotonergic system in the CNS (sagittal section). Raphe nuclei are located as an accumulation of cell groups along the midline of the brainstem. The rostral raphe complex projects to a variety of forebrain structures and the cerebellum. The caudal raphe complex projects primarily to the spinal cord and the cerebellum.*

Rodent studies revealed that the serotonergic phenotype is determined by a series of transcription factors like Nkx2.2, Nkx6.1 and Mash1, that generate precursor cells of serotonergic neurons, and sonic hedgehog (Shh), FGF4, FGF8, Gata2, Gata3, Lmx1b and **Pet1,** that act in concert to orchestrate the activation of marker genes like **Tph2**, AAAD, Sert, Vmat2 and 5-HT receptors thereby defining the serotonergic cell type (Cordes, 2005). 5-HT itself shows a strong organizing function and might act as a developmental signal for receptive cells in the developing nervous system (Lauder, 1990). During brain development 5-HT regulates morphogenetic activities, such as neural differentiation, axon outgrowth, and configuration of synaptic connections. Diverse studies on the **inhibition of TPH** resulting in reduced 5-HT concentration have shown altered cell division of different populations of neuronal precursors as well as decreased density of synapses in the hippocampus causing learning deficits in adult rats (Mazer et al., 1997). Although in vivo studies generally underscore this notion (Vitalis et al., 2007), interestingly, conditional Lmx1b knockout mice (Ding et al., 2003; Zhao et al., 2006) are viable without apparent developmental abnormalities despite the fact that these animals completely lack 5-HT in the brain. Development and ontogenesis of the serotonergic system is described in detail in Kriegebaum et al., 2009 (submitted).

Certainly, the main amount (98 %) of 5-HT in the body can be found in the periphery, predominantly in the enterochromaffine cells of the gastrointestinal tract (90 %) where the number of 5-HT-positive cells is higher in the duodenum as compared with the stomach and TPH1 expression is lower in the duodenum as compared with the antrum and fundus (van Lelyveld et al., 2007). In minor amounts, 5-HT exists as well in some blood cells like thrombocytes, lymphocytes, granulocytes and mast cells. In the periphery, 5-HT activates intrinsic and extrinsic primary afferent neurons to initiate peristaltic and secretory reflexes and to transmit information to the central nervous system, respectively (Gershon and Tack, 2007). 5-HT which is released from the gut into the lumen effects the contraction of smooth vascular muscles. In the blood, 5-HT coordinates platelet aggregation (primary haemostasis) and the inflammation of allergic reactions as well as macrophage phagocytosis.

As a substrate of the melatonin synthesis in the **pineal gland**, 5-HT is involved in the regulation of circadian rhythm (especially sleep-wake cycles, see Fig. 1-4). For more information about 5-HT in the periphery and about the context of 5-HT, melatonin and circadian rhythm, see Kriegebaum et al., 2009 (submitted).

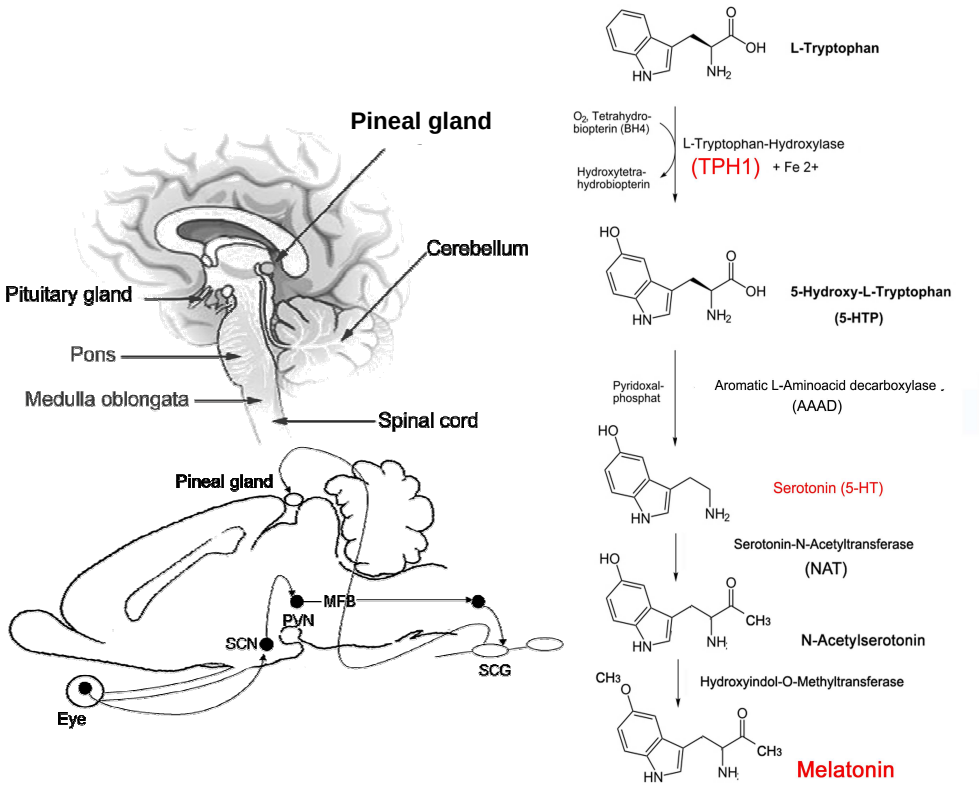


Fig. 1-4 Pineal gland and melatonin synthesis.
Left: *Schematic illustration of the localization of human (above) and mice (below) pineal gland.* ***Right:*** *Structural formula of the melatonin synthesis. Melatonin is synthesized within the pinealocytes of the pineal gland from tryptophan by Tryptophan hydroxylase (TPH)1. Synthetic activity occurs during the dark phase, with a major increase in the activity of serotonin-N-acetyltransferase (arylalkylamine N-acetyl transferase, AA-NAT). AA-NAT is usually rate limiting in melatonin production, but serotonin availability may also play a role. The rhythm of production is endogenous, being generated by interacting networks of clock genes in the suprachiasmatic nuclei (SCN) of the hypothalamus. The SCN rhythm is synchronized to 24 hours primarily by the light-dark cycle acting via the retina and the retinohypothalamic projection to the SCN. MFB = medial forebrain bundle, PVN = paraventricular nucleus, SCG = superior cervical ganglion, SCN = suprachiasmatic nucleus.*

1.1.3 Distribution of the serotonin synthesis enzymes TPH1 and TPH2

Over the last years, there was considerable inconsistency with respect to the different characteristics of TPH originating from brain cells and that originating from the periphery, e. g blood cells. In the year 2003, two working groups, Walther et al. and Côté et al., simultaneously succeeded to generate Tph1 KO mice. The resulting animals, although being deficient for 5-HT in the periphery and in the pineal gland, exhibited close to normal levels of 5-HT in the brainstem. Walther et al. concluded that a second isoform, termed Tph2, is predominantly expressed in the brainstem but not in peripheral tissues, while the classical *Tph1* gene is expressed in the gut, pineal gland, spleen, and thymus (Walther and Bader, 2003). Côté et al. established as well that Tph2 is expressed in neurons of the raphe nuclei but additionally in neurons of the myenteric plexus, whereas they also found Tph1 located in the pineal gland and the enterochromaffin cells of the gastrointestinal tract (Côté et al., 2003). By measuring the daily profiles of Tph-mRNA levels in the rat raphe nuclei, Malek et al. demonstrate that Tph2 is exclusively expressed in the rat raphe nuclei, whereas Tph1 is expressed in the pineal gland. Furthermore, both under normal light-dark cycle (LD) and under constant darkness, Tph2 mRNA levels vary within both median and dorsal raphe nuclei (Malek et al., 2005). Studies of Sugden et al. demonstrated that Tph1 mRNA expression is 105-fold more abundant in the rat pineal gland than Tph2 and shows an approximately 4-fold nocturnal increase, whereas Tph2 expression within the pineal gland shows no significant variation within time of day, which indicates that Tph2 expression occurs in the small proportion of "non-pinealocyte" cells (Sugden, 2003). In the retina, Tph1 is the prevalent isoform and shows robust diurnal rhythms, with highest levels at night (Liang et al., 2004). Hence, Tph1 might not only be involved in melatonin synthesis in the pineal gland but also in the retinal photoreceptor cells. Contrary to Tph2, whose transcripts were seen in oocytes and two-cell stages, transcripts of Tph1 were not detected at any of these stages (Basu et al., 2008). Tph1 expression was also found in murine osteoblasts and osteoclasts, indicating their ability to produce 5-HT. Both Tph1 and Tph2 are expressed in gustatory fibres and differentiated taste receptor cells of the developing mouse taste papillae (Ortiz-Alvarado et al., 2006).

Gundlah et al. pointed out that 17ß-estradiol increased Tph1 mRNA expression in the DRN of ovariectomized mice and could as well detect Tph1 mRNA in the brainstem of native and untreated animals (Gundlah et al., 2005). Interestingly, the group of Nakamura found that Tph1 is expressed preferentially in the brain during the late stage (P21) of development of the mouse CNS, whereas Tph1 mRNA was not detected at P7 and only at very weak signals in 2 months old mice. Thus, they suggest that Tph1 may act on the development of 5-HT neurons specifically during late developmental stages thereby exerting an

influence on later-life behaviour and, possibly, drug response (Nakamura et al., 2006; Nakamura and Hasegawa, 2007). A recent study claimed to detect Tph1 mRNA in the dorsal raphe nucleus (DRN) of the rat, so that the authors concluded that Tph1 in the serotonergic neurons of the DRN might be relevant for stress-induced psychopathologies (Abumaria et al., 2008). A study using human post-mortem tissue demonstrates that TPH2 mRNA is expressed at the highest level in the raphe nuclei but TPH1 mRNA levels were higher than that of TPH2 in cortex, thalamus, hypothalamus, hippocampus, amygdala and cerebellum (Zill et al., 2007). In contrast, TPH2 mRNA was not present in peripheral tissues like heart, liver, lung, duodenum, adrenal gland and kidney (Zill et al., 2004). Given these discrepant data, no firm conclusions on the differential TPH1 and TPH2 expression in central and peripheral nervous systems of mice, rats, monkeys and humans during pre- and postnatal development and in adulthood can be made at present.

1.2 Structural and functional properties of TPH1 and TPH2

The existence of two different TPH isoforms, encoded by different genes, meanwhile is undisputed and a growing body of evidence suggests a tissue-specific pattern of expression of both isoforms. This suggests the possibility that their catalytic function is regulated differentially, which might reflect a physiological adaptation to different needs for regulation of 5-HT production in the brain and in peripheral organs. The genes encoding TPH1 and TPH2 are located on chromosome 7B4 and 10D1 in the mouse and on chromosome 11p15.5 and 12q21.1, respectively, in humans. The two isoforms of TPH belong to the superfamily of pterin-dependent aromatic amino acid hydroxylases, which also includes tyrosine hydroxylase (TH) and phenylalanine hydroxylase (PAH). All members of this family share considerable structural similarities and require the same co-factor (Fe^{2+}) and co-substrate (BH_4) for functioning. The aromatic amino acid hydroxylases consist of three functional regions: a regulatory N-terminal domain, a catalytic domain and a C-terminal tetramerization domain.

The two TPH isoforms are substantial homologous in their sequence and almost identical in their catalytic domains, but different in their N-terminal regulatory domains and some kinetic properties. TPH1 and TPH2 exhibit an overall sequence identity of 71% (Walther and Bader, 2003; Walther et al., 2003) compared to a sequence identity of 52% between TPH1 and PAH which also has tryptophan hydroxylase activity (Kappock and Caradonna, 1996). TPH2 has an additional 41 amino acid N-terminal regulatory region, not present in TPH1 and unique for this enzyme (Tenner et al., 2007; Murphy et al., 2008). Therefore, the N-terminal domain of TPH2 is much larger than that of TPH1 and bears some similarities to the TH and the PAH

sequence. This region contains sites that markedly reduce enzyme expression levels by altering both enzyme synthesis and stability. TPH1 has a 2.5-fold greater maximum expression and a 25% faster rate of synthesis as compared to TPH2 (Murphy et al., 2008). The extended N-terminus of TPH2 (more specifically, amino acids corresponding to the residues 10-20) is responsible for reducing TPH2 protein expression without altering mRNA levels.

Both TPH1 as well as the TPH2 exist as tetramers *in vivo*. The tetramerization is mediated by the last 17 amino acids of the C-terminus (Tenner et al., 2007). Both isoforms exhibit similar levels of solubility but TPH2 is degraded more rapidly than TPH1. A phosphorylation site for protein kinase A (PKA), serine at position 19, is only present in TPH2 but not in TPH1 (McKinney et al., 2005; Kuhn et al., 2007). PKA phosphorylation regulates TPH2 protein expression and increases 5-HTP production, ergo increases 5-HT synthesis (Murphy et al., 2008). TPH1 is phosphorylated at Ser58 by PKA and both Ser58 and Ser260 by CaMKII, phosphorylation sites that are also conserved in TPH2. *TPH2* gene transcription and mRNA expression are also induced by calcium mobilization through a minimal calcium-responsive segment within 88 base pairs (bp) of the transcription start site (Lenicov et al., 2007). The molecular mass of TPH2 (57 kDa) is slightly higher than that of TPH1 (51 kDa) and the V_{max} value of TPH1 is higher than for TPH2, which may be a result of inherent differences in catalytic efficiency or enzyme stability (McKinney et al., 2005). K_m values for BH_4 appeared to be lower for TPH2 than for TPH1, whereas the K_m for tryptophan (Trp) is higher for TPH2 than for TPH1 (see Fig. 1-5). Both isoforms are able to hydroxylate tyrosine (Tyr) and phenylalanine (Phe) as well. However, the K_m for both Tyr and Phe are higher for TPH2 than for TPH1 and the V/K value for Trp relative to Phe is much higher for TPH2 than for TPH1, suggesting a higher substrate preference for Trp for TPH2 than for TPH1 (McKinney et al., 2005). Thus, TPH2 shows increased substrate selectivity in comparison to TPH1, but has to pay this benefit with reduction in the affinity for Trp and a decreased catalytic turnover.

Proper serotonergic function requires adequate production of 5-HT. TPH2 is mainly thought to be the relevant isoform in brain raphe neurons. Thus, alterations in the activity of TPH2 may yield an increase or decrease of 5-HT available for release and thereby influence the function of serotonergic nerve terminals. Taken together, TPH1 seems to be the "*quick-and-dirty*" worker, which might be unfavourable for the tightly regulated 5-HT synthesis in the brain. For example, the rate of 5-HT is directly related to the blood levels of Trp. Hence, abnormally high blood levels of Phe like in phenylketonuria patients would lead to competitive inhibition of TPH and a reduced production of 5-HT, from which TPH2 could be protected by its high substrate specifity.

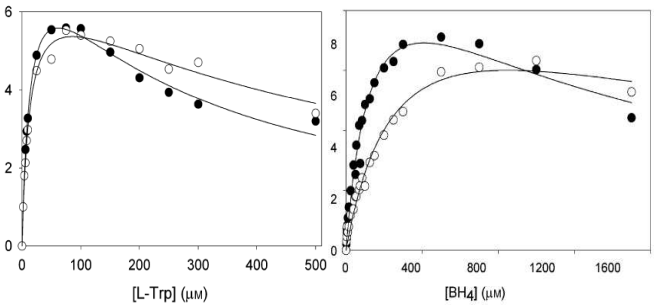


Fig. 1-5 Kinetic properties of TPH1 and TPH2
Effects of L-tryptophan (L-Trp) and (6R-L-erythro-5,6,7,8-tetrahydrobiopterin (BH_4) on tryptophan hydroxylase activity of TPH1 (●) and TPH2 (○) expressed using the in vitro transcription and translation (ITT) system. Figure taken from (McKinney et al., 2005).

***Table1-1** Differences between TPH1 and TPH2*

	TPH1	**TPH2**
Chromosome (human)	11p15.5	12q 21.1
Chromosome (mouse)	B4	10 D1
Exons (human)	10	11
Exons (mouse)	11	11
mRNA (human)	1335 nuc.	2992 nuc.
mRNA (mouse)	2042 nuc.	2655 nuc.
Protein (human)	444 aa	250 aa
Protein (mouse)	447 aa	497 aa

1.3 Biological consequences of serotonin and TPH1/2

At early stages of development, when the blood brain barrier is not yet fully formed, increased and decreased levels of central and/or peripheral 5-HT may cause abnormalities in the brain at the later developmental stages. For example, high blood 5-HT would enter the brain of the developing foetus through the developing blood brain barrier and cause loss of 5-HT terminals in the brain through a negative feedback mechanism (Whitaker-Azmitia, 2005). The loss of serotonergic innervations which seems to be present e. g. in some dysfunctions like autism is probably caused by this effect. Treatment of rats with a 5-HT agonist during development creates loss of serotonergic innervations mainly in the amygdala and in the paraventricular nucleus (PVN) of the hypothalamus and causes a phenotype considered to relate to autism. In addition, mice with reduced 5-HT synthesis during development - like Mao-a knockout mice - also show pronounced abnormalities in late developmental stages like altered axon branching, deformation of dendrites and apoptosis during development (Gaspar et al., 2003). Furthermore, peripheral maternal 5-HT and therefore functional maternal Tph1 (rather than embryonal 5-HT from functional embryonic Tph2) is crucial for murine development, as demonstrated in heterozygous (Tph1+/–) embryos from a null (Tph1-/-) mother and a wildtype father. These pups displayed dramatic abnormalities in the development of the brain and other tissues (Côté et al., 2007). Moreover, disruption of the *Tph1* gene demonstrates the importance of peripheral 5-HT in cardiac function, because anatomical examination of Tph1 KO mice revealed a larger heart size and abnormal cardiac activity, which ultimately leads to heart failure (Côté et al., 2004).

1.3.1 The role of serotonin and TPH1/2 in stress and the HPA axis

Various types of physical and/or mental stressors are able to stimulate CRH (corticotropin releasing hormone) release from the PVN of the hypothalamus, which leads to release of ACTH (adrenocorticotropic hormone) from the pituitary gland and thereby activates the adrenal gland to synthesize glucocorticoids: cortisol in humans and corticosterone in rodents. This cascade is referred to as the hypothalamic-pituitary-adrenal (HPA) axis. High blood levels of cortisol as found during stress inhibit the CRH and ACTH release, thereby constituting a natural feed-back control system to terminate stress response and protect the body from hormonal overreaction. Ascending serotonergic projections of the raphe nuclei as well as noradrenergic projections of the locus coeruleus modulate the stress response. In some conditions, such as chronic stress exposure or in patients with altered stress behaviour like in affective disorders and panic disorder patients, an inability to terminate the stress response can be found and the adaptive mechanisms are turning into pathological maladaptation (see also Fig.1-6 and Kriegebaum et al., 2009 submitted).

According to several studies, altered levels of 5-HT due to changes of the 5-HT synthesis are hypothesized to be associated with altered stress response. For instance, a dose-dependent decrease in raphe TPH2 protein levels could be detected in response to a four day treatment with a synthetic glucocorticoid (Clark et al., 2005; Clark et al., 2008). Chen and co-workers, described polymorphisms in the 3'-UTR (untranslated region) of rhesus monkey *TPH2* which modulate the HPA axis function, presumably by affecting the levels of *TPH2* expression (Chen et al., 2006). However, the results of Gizatullin et al. suggest that in a particular group of stress-induced depressed patients TPH1 appears to be more relevant to depression pathogenesis than TPH2, because unlike as in *TPH1* they could not detect genetic variation within the *TPH2* gene variation to be associated with stress-induced depression (Gizatullin et al., 2008). In addition, Abumaria et al. showed a 2.5-fold upregulation of Tph1 but not Tph2 mRNA in the rat DRN after chronic stress treatment and hypothesized that Tph1 in the serotonergic neurons of the DRN might be relevant for stress-induced psychopathologies (Abumaria et al., 2008).

1.3.2 Serotonergic dysfunction in psychiatric disorders

5-HT has been repeatedly implicated in almost all psychiatric disorders like depression, obsessive compulsive disorder (OCD), anxiety, panic, autism, personality disorders and impulsivity and aggression. These hypotheses basically derive from pharmacological data, but the underlying physiological mechanisms remain still unknown to a large extent.

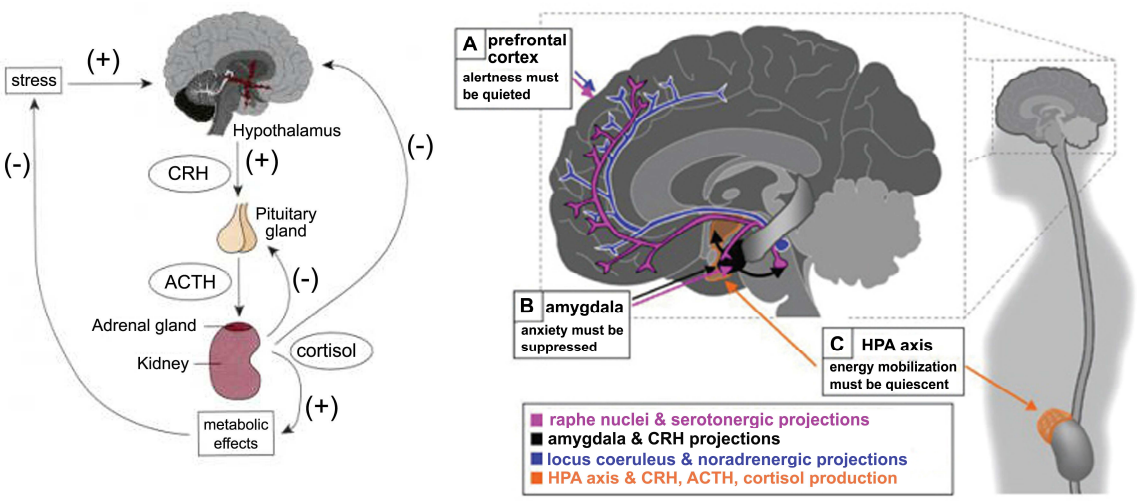


Fig. 1-6 Stress and the HPA axis.
Stressors lead to CRH (corticotropin releasing hormone) release from the paraventricular nucleus (PVN) of the hypothalamus which activates the release of ACTH (adrenocorticotropic hormone) from the pituitary gland. ACTH directs the adrenal glands to synthesize and release cortisol leading to metabolic effects and termination of stress response via a negative feedback system. Serotonergic projections of the raphe nuclei as well as noradrenergic projections of the locus coeruleus modulate the bodily stress response. CRH release also suppresses the expression of anxiety by inhibiting the amygdala and mobilizes the body to act in a "flight or fight" reaction. Figure modified from (Stahl and Wise, 2008)

Central serotonergic deficiency leads to altered motivational behaviour and neuroadaptive processes resulting in emotional disturbance and appears to underlie the pathophysiology of addictive behaviours like pathological gambling, sexual addiction, bulimia nervosa and the abuse of psychoactive substances or other drugs including alcohol (for more information about the effects of psychoactive substances and drugs on the serotonergic system see Kriegebaum et al., 2009; submitted). According to genetic variation and environmental factors, 5-HT mediated behaviours may vary from minor personality abnormalities to major psychiatric disturbances like suicidal behaviour. 5-HT also appears to be associated with sleep, eating, aggressive, as well as impulsive traits. Furthermore, although therapeutic interventions in ADHD (attention-deficit/hyperactivity disorder) still focus on the dopaminergic system, recent studies indicate a serotonergic dysfunction as well (Oades, 2007). However, molecular genetic studies mainly demonstrate an association of genes encoding key proteins involved in 5-HT neurotransmission with affective and anxiety disorders. In addition, studies on gene deficient animals suggest a role for the 5-HT system in behavioural and affective dysfunctions.

Decreased 5-HT and 5-HIAA levels in the cerebrospinal fluid (CSF) and blood samples of depressed patients (Delgado et al., 1990; Meltzer, 1990) and in the brain of suicide victims (Stanley et al., 1986; Arranz et al., 1997) were repeatedly demonstrated. Additionally, decreased levels of 5-HIAA in the CSF of adolescents with ADHD were detected, correlating

more with aggression than impulsivity (Miczek et al., 2002). In patients with affective disorders, the administration of 5-HT precursors like tryptophan and 5-HTP may induce attenuation of depressive symptoms (Mendels et al., 1975) and a tryptophan depleting diet may cause pathological aggravation of symptoms in depressive patients (Delgado et al., 1990; Merens and van der Does, 2007) and gloomy mood in healthy volunteers (Young et al., 1985). Major evidences for the implication of 5-HT in psychiatric disorders arise furthermore from the effectiveness of antidepressant drugs like MAO inhibitors, tricyclics and selective serotonin reuptake inhibitors (SSRIs) which all target the 5-HT system (Fig. 1-7). Currently, the 5-HT-selective SSRIs are the most popular antidepressants, applied not only for treatment of affective disorders like depression but also for eating disorders like anorexia and bulimia nervosa as well as for treatment of anxiety, panic disorder and OCD. Moreover, although there is no convincing effect of SSRIs in treatment of ADHD, sometimes an improvement in other impulse control disorders like pathological gambling and some personality disorders like borderline and antisocial personality disorder can be observed. SSRIs possess a latency of action from one to two weeks in depression and even longer in OCD, whereas in anxiety and panic disorders time of onset is relatively fast with a potential for initial aggravation of symptoms. The latency of action of SSRIs might result from changes in 5-HT receptor expression which in turn may cause altered density of postsynaptic 5-HT receptors or, alternatively, from a desensitization of 5-HT_{1A} autoreceptors.

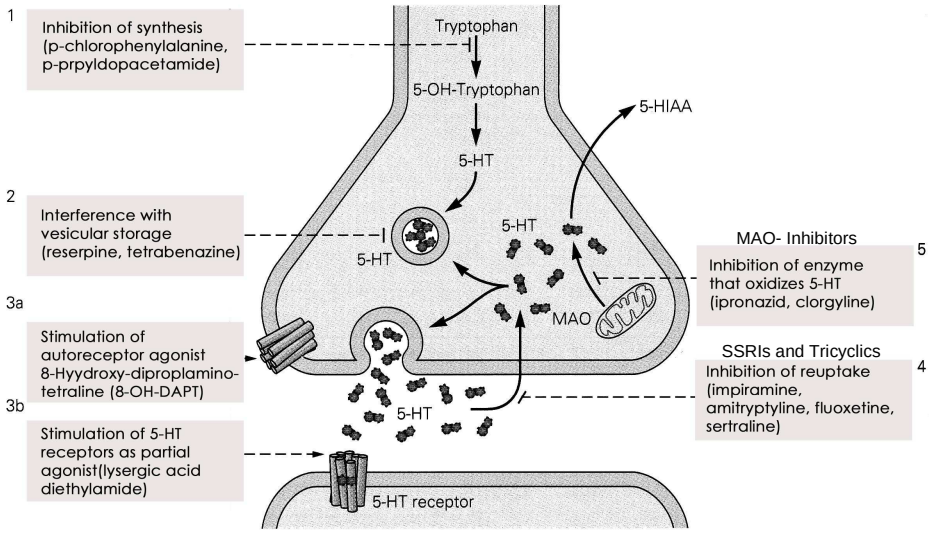


Fig.1-7 The effect of antidepressants and other drugs on the serotonergic synapse.
Antidepressants and other psychotropic compounds have five possible effect sites at the serotonergic synapse: 1. the synthesis (inhibition of tryptophan hydroxylase (TPH) by p-chlorophenylalanin or p-propyldopacetamid), 2. the storage (reserpin and tetrabenazin inhibit the reuptake of amine transmitters by binding at the vesicular monoamine transporter (VMAT)), 3. receptor interactions a) by the 5-HT_{1A} receptor agonist 8-hydroxy diprolamino tetralin (8-OH-DPAT) or b) by lysergic acid diethyl amide (LSD), which acts as 5-HT_{2A} receptor agonist, 4. the reuptake of serotonin (5-HT) by tricyclics (like imipramine and amitryptyline) or by selective serotonin reuptake inhibitors (SSRIs) (like fluoxetine and sertraline) and 5. the catabolism of 5-HT to 5-hydroxyindol acetic acid (5-HIAA) by inhibition of the monoamine oxidase (MAO) by MAO inhibitors like tranylcypramine. Figure adapted from (Kandel et al., 2000)

Local injection of neurotoxins into the upper raphe nuclei of rats leads to a disruption of ascending serotonergic projections resulting in reduction of repression of impulsive behaviour and, consequently, increased aggressive behaviour. Administration of SSRIs is able to restore these neurotoxin-mediated effects. Mao-a knockout mice, which exhibit higher 5-HT levels paralleled by decreased 5-HIAA levels, display an intense enhanced aggression and the spontaneous firing of serotonergic neurons is significant reduced, whereas Tph2 activity is increased (Evrard et al., 2002; Popova, 2006). In addition, a length variation of the human *MAO-A* gene, which confers vulnerability to antisocial behaviour in alcohol-dependent males (Brunner et al., 1993; Samochowiec et al., 1999), is linked to impulsivity, hostility and lifetime aggression history in men (Manuck et al., 2000; Caspi et al., 2002; Reif et al., 2007). Furthermore, it appears to be a risk factor for panic disorder and unipolar depression in female patients (Deckert et al., 1999; Schulze et al., 2000). Sert KO mice (see chapter 1.5) exhibit elevated 5-HT levels in the synaptic cleft and diminished 5-HT levels in the cell; on the behavioural level, they show increased anxiety-like behaviour (Li et al., 2000), increased sensitivity to stress (Lanfumey et al., 2000), and decreases in aggressive behaviour (Li et al., 1999). In line with this, the short variant of a human length polymorphism in the promoter region of SERT (5-HTTLPR) leading to altered functional SERT expression (Lesch, 2001) is associated with emotional lability and significantly higher scores of neuroticism, which is related to anxiety, stress reactivity and depression (Canli and Lesch, 2007). Additionally, studies on the brains of depressed suicides showed elevated expression of TPH2 mRNA and increased amounts of TPH2 protein in neurons of the dorsal and median raphe nuclei which may reflect a homeostatic response to deficient 5-HT levels in the brains of depressed suicides (Bach-Mizrachi et al., 2006; Bach-Mizrachi et al., 2008). Given these examples, a functioning central serotonergic system seems to be central for normal behaviour. For a detailed description of the role of 5-HT in psychiatric disorders see Kriegebaum et al., 2009 (submitted).

1.3.3 The influence of TPH2 on the effect of antidepressant drugs

Comparing basal extracellular 5-HT and the response to the SSRI citalopram in different strains of mice with functionally different allelic forms of *Tph2* showed that mice strains carrying a mutated Tph2 enzyme (a proline to arginine mutation at position 447, see chapter 1.5) had less 5-HT in the medial prefrontal cortex and dorsal hippocampus dialysate (Calcagno et al., 2007). Using the paradigm of the forced swim test to assess a potential antidepressant effect of drugs in rodents (Porsolt et al., 1977), no antidepressant effect of citalopram in mouse strains carrying the mutated arginine-form of Tph2 (non-responders to SSRIs) was found. Administration of tryptophan however could restore the antidepressant effect of SSRIs in mice

not responding to the SSRI alone (Cervo et al., 2005; Invernizzi, 2007). Further studies on the functional effects of the Pro206Ser variant in the human TPH2 enzyme, which might correspond to the murine Pro447Arg mutation, provided evidence for a reduced thermal stability and solubility of the mutated TPH2 enzyme, suggesting reduced 5-HT production in the brain as a pathophysiological mechanism in affective disorders (Cichon et al., 2008). Interestingly, long-term but not short-term SSRI (fluoxetine) treatment selectively modulates mRNA levels of TPH2 only in differentiated cells of a cell line (Di Lieto et al., 2007). Two-week fluoxetine treatment significantly reduced the expression of Tph2- and Sert-mRNA in the brainstem of rats, which is consistent with decreased 5-HT levels and 5-HT turnover in the brain, and might contribute to the anxiogenic effect of this drug (Dygalo et al., 2006). However, fluoxetine treatment for four and eight weeks but not for two weeks, significantly increased basal Tph2 mRNA levels and Sert-mRNA levels in the midbrain of rats and 5-HIAA content in the amygdala, an effect that was correlated with the appearance of antidepressant-like effects in the forced swim test (Shishkina et al., 2007). Studies on fluoxetine non-response demonstrated that this condition is associated with three single nucleotide polymorphisms (SNPs) in the human *TPH2* gene (Peters et al., 2004). Thus, TPH2 seems to play an important role in the responsiveness to antidepressant drugs and/or the effectivity of their action.

1.4 The impact of oxytocin on serotonin, stress, anxiety and depression

The neuropeptide-hormones vasopressin and oxytocin are known for their role in social encounters, pair bonding and parental care and are mainly produced in the paraventricular nucleus (PVN) and supraoptic nucleus (SOV) of the hypothalamus (Fig. 1-8). Magnocellular cells of the hypothalamus, containing oxytocin or vasopressin, project to the pituitary and release the neuropeptides into the systemic circulation where primarily vasopressin is known to control blood pressure. Besides the hypothalamus, oxytocin and vasopressin can be found in other brain regions like the limbic system and the brainstem. In the rat brain, oxytocinergic fibres and terminals are described in different brain regions like hypothalamus, thalamus, hippocampus, amygdala, entorhinal cortex, olfactory bulb, substantia nigra, locus coeruleus, raphe nuclei, cerebellum and the spinal cord (Gimpl and Fahrenholz, 2001). In the periphery, oxytocin is known to occur in the thymus, the mammary gland, the ovaries, the uterus, the adrenal gland and the pancreas (Jezova et al., 1996). Additionally, there are oxytocinergic fibres innervating the pineal gland directly through the pineal stalk (Moller and Baeres, 2002). However, plasma oxytocin can not cross the blood brain barrier.

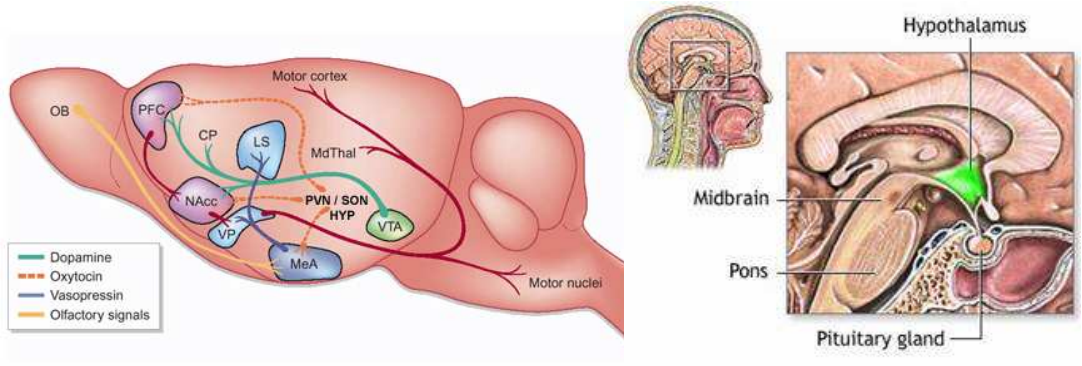


Fig. 1-8 The oxytocinergic system.
Left: *Sagittal section through a prairie vole brain, figure taken from (Young and Wang, 2004).* ***Right:*** *Sagittal section through a human brain with detailed view on the hypothalamus and the pituitary gland. Oxytocin and vasopressin are mainly produced in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus (HYP). Magnocellular cells of the hypothalamus containing oxytocin and vasopressin project to the pituitary gland and release the neuropeptides into the systemic circulation. Besides the hypothalamus, oxytocinergic fibres and terminals are described in different brain regions like thalamus, hippocampus, amygdala, entorhinal cortex, olfactory bulb, substantia nigra, locus coeruleus, raphe nuclei, cerebellum and the spinal cord (Gimpl and Fahrenholz, 2001). CP = caudate-putamen, LS = lateral septum, MeA = medial nucleus of the amygdala, MdThal = mediodorsal thalamus, NAcc = nucleus accumbens, OB = olfactory bulb, PFC = prefrontal cortex, VP = ventral pallidum, VTA = ventral tegmental area.*

The prime role of oxytocin is the regulation and induction of labour and milk-ejection from the mammary gland, which is triggered when the infant begins to suck on the nipple. Oxytocin release during labour and lactation gives rise to the development of mother-child bonding and maternal care. Furthermore, oxytocin- and vasopressin-secretion from the neurohypophyse takes place during sexual activity and is highest during orgasm in both sexes. Thus, oxytocin seems not only be responsible for the early development of mother-child bonding but also for trust and tenderness between sexual partners thereby facilitating the formation of pair bonding. Although both vasopressin and oxytocin facilitate the formation of pair bonding in both sexes, oxytocin appears to play a more important role in females, whereas vasopressin appears to play a more important role in males. Interestingly, oxytocin KO mice display normal maternal behaviour, but female oxytocin KO mice are unable to use olfactory signals to choose male partners and react to their behaviour (Kavaliers et al., 2006). Additionally, male oxytocin KO mice display decreased social recognition which seems to be associated with loss of oxytocin in the central and medial nucleus of the amygdala (Ferguson et al., 2001). Evidence from neuroeconomic experiments demonstrate increased trust in humans after intranasal application of oxytocin in both sexes, accompanied by reduced activation of the amygdala as evidenced by functional magnetic resonance imaging (fMRI) (Kosfeld et al., 2005; Baumgartner et al., 2008). Likewise, (Keri et al., 2008) could associate interactions based on trust in humans with increased plasma oxytocin levels in healthy subjects, an effect which completely disappears in patients suffering from schizophrenia.

Activation of serotonergic neurotransmission can influence oxytocinergic neurotransmission and visa versa. Moreover, the distribution of SERT-labelled fibres follows the distribution of oxytocin-labelled cells and an overlap of SERT-positive and oxytocin-positive cells was seen in the PVN of the hypothalamus (Emiliano et al., 2007). Thus, projections from serotonergic cells of the DRN innervating both the hypothalamus and the amygdala may present a part of neurocircuit involved in emotional response to stress in association with the oxytocinergic system. Treatment of rats with a 5-HT agonist during development leads to later-life autistic-like behaviour (see above) and cellular changes in two relevant brain regions, the central nucleus of the amygdala (CeA) which displays a reduction of incoming oxytocinergic fibres and the PVN of the hypothalamus where a decrease in oxytocinergic cells was found (Whitaker-Azmitia, 2005). In line with these findings, decreased oxytocin serum levels were observed in autism (Green et al., 2001) and oxytocin infusions could reverse some behaviours of autistic children (Hollander et al., 2003). An increased oxytocin secretion in the brain probably facilitates social encounters by reducing the associated anxiety. Thus, oxytocin seems to have positive effects in the regulation of anxiety and fear behaviour. Women suffering from panic attacks report about improvement of symptoms during lactation. Pregnancy appears to be a protective period for some anxiety disorders, including panic disorder, which might be due to increased oxytocin levels occurring during this period (*Proceedings of a conference, Chantilly, Virginia, USA. CNS Spectr* (2004)). In female as well as male rats exposed to an anxiety paradigm, both centrally and peripherally injected oxytocin shows a positive effect against anxiety behaviour (Ring et al., 2006). By inhibition of oxytocin receptors with a central oxytocin receptor antagonist on the one hand and a peripheral oxytocin receptor antagonist on the other hand, this study also provides evidence that, in the regulation of fear response, oxytocin acts in the CNS.

Oxytocin and vasopressin seem to modulate the autonomic response to fear in opposing manners. Vasopressin enhances aggressiveness, anxiety, and stress levels and the consolidation of fear memories, whereas oxytocin decreases anxiety and stress and facilitates social encounters, maternal care, pair bonding and trust into social interactions. Different neurons in the CeA are involved in the autonomic, innate fear response, however acquired anxiety and fear memory proceeds in the lateral and basolateral nucleus of the amygdala (LA and BLA). The CeA receives inputs from sensory-processing areas (like the olfactory bulb and the prefrontal cortex) as well as from the BLA and LA and in turn projects to the hypothalamus and the brainstem, thus mediating the autonomic response to fear. At the CeA, a widespread appearance of oxytocin and vasopressin receptors occurs. Huber et al. could show that vasopressin acts excitatory on neurons of the medial part of the CeA, whereas oxytocin acts inhibitory on neurons of the medial part of the CeA via activation of

GABAergic projections (Huber et al., 2005). These results could provide an explanation for the opposing effects of oxytocin and vasopressin in modulating anxiety behaviour.

Oxytocin not only modulates fear learning and the retrieval of learned fear by reducing the fear response to learned cues, but also the expression of fear during stress. Different kinds of stressors like social isolation, swim stress, shaker stress, immobilization, and dehydration cause an increase of oxytocin release mediated in large part by 5-HT_{1A}-, 5-HT_{2A}- und 5-HT_{2C}-receptors (Jorgensen et al., 2002). In lactating women which show an increase in oxytocin levels a suppression of response of the HPA axis was found. The women furthermore had decreased plasma ACTH, cortisol, glucose and norepinephrine levels (*Proceedings of a conference, Chantilly, Virginia, USA. CNS Spectr* (2004)). Oxytocin infusions in human males also decrease plasma cortisol and ACTH levels by affecting the adrenal gland to induce reduced cortisol release and/or synthesis (Legros et al., 1988). In social isolated steers an increased cortisol release was measured which could be reversed by intracerebroventricular injection of oxytocin (Yayou et al., 2008). Presumably, oxytocin neurons are stimulated by CRH-neurons, the hormone which is released from the hypothalamus in response to stress (see chapter 1.3.1.). Intraventricularly injected CRH causes increased oxytocin secretion in both female and male rats (Bruhn et al., 1986). In addition, a co-localisation of oxytocin and CRH could be detected in the PVN of the hypothalamus (Jezova et al., 1995) and a co-localisation of 5-HT_{1A} and 5-HT_{2A} receptors was found in oxytocinergic and CRH-neurons (Zhang et al., 2004). Stimulation of 5-HT_{1A} receptors in the PVN with a selective 5-HT_{1A} receptor agonist results in release of ACTH and oxytocin (Osei-Owusu et al., 2005).

Besides the oxytocin-mediated response to stress and anxiety, oxytocin seems to play an important role in pain modulation and depressive behaviour. Pain stimulation in rats decreases oxytocin concentration in the SON of the hypothalamus and increases it in the locus coeruleus, the raphe nuclei, the caudate nucleus and the spinal cord (Yang et al., 2007). Furthermore, intravenously injected oxytocin in high doses (300µg) was reported to evoke strong analgesic effects lasting more than 70 min in patients with intractable cancer pain at a time when opiates were no longer effective (Madrazo et al., 1987). Oxytocin also seems to be involved in therapeutic effects of SSRIs in association with social encounters and anxiety. Injection of a 5-HT_{2A} receptor agonist results in increased plasma oxytocin levels. Fluoxetine injections reduce significantly the oxytocin response to 5-HT_{2A} receptor stimulation (Damjanoska et al., 2003; Landry et al., 2005). Chronic treatment with fluoxetine and paroxetine causes desensitization of 5-HT_{1A} receptors in oxytocinergic neurons of the PVN (de Jong et al., 2007). Intriguingly, oxytocin by itself was able to exert antidepressant effects in two animal models of depression. An intraperitoneal injection of oxytocin significantly reduces the duration of immobility in the behavioural despair test, a known behavioural

paradigm for depression-like behaviour, indicating reduced depression-like behaviour of the treated animals. Likewise, in the learned helplessness test, oxytocin significantly reduced the escape failures and the latency to escape, the effect being even more intense than that of imipramine, a tricyclic antidepressant (Arletti and Bertolini, 1987). Thus, there seems to be a link between the serotonergic and oxytocinergic system to modulate neuronal functionality for social, emotional and sexual behaviour in association with trust and social encounters. Dysfunctions of the oxytocinergic and/or the serotonergic system might lead to the etiology of psychiatric disorders like depression, anxiety and panic disorders, social phobia, as well as altered social behaviour or autism.

1.5 The mouse model organism for human disorders

Since the mouse genome shows a 95% homology to the human genome, this species appears to be an adequate model organism studying brain functions as well as to establish and develop novel drugs and potential treatment strategies for neuropsychiatric disorders. In the last years techniques for generating knockout (KO) mice with specific gene deletions have improved and undoubtedly revolutionized science as also reflected by the Nobel price for Physiology or Medicine in 2007. However, despite of the undoubted advantages of the manifold applications for gene knockout in pharmacology, limitations have to be accepted just as well. To give some examples: Targeted inactivation of one gene *ab initio* in every cell as accomplished in classical constitutive KO mice might induce embryonic or postnatal lethality. Furthermore, a missing gene might affect many developmental processes throughout ontogeny and compensatory mechanisms may be activated in constitutive KOs. Thus, behavioural data of constitutive KOs should be interpreted with caution. However, the development of conditional (c) KO mice, in which a specific gene can be inactivated in a tissue- or time-specific manner, might circumvent these disadvantages. The use of transgenic techniques and knockout strategies in mice resulted in KO mice with inactivated genes of the serotonergic system like Sert, Tph1, Tph2, Mao-a, Mao-b, different 5-HT receptors, Pet1, Lmx1b and Vmat2. For a detailed overview about different types of KO mice, see chapter 2.2.4 and Kriegebaum et al., 2009 (submitted).

Besides genetic engineering, nature itself generates genetically different mouse strains featuring Tph2 forms with a mutation in the C-terminal region, which results in disturbed behaviour. Some mouse strains like C57BL/6J, C57BL/6N and 129X1/Sv express a form of Tph2 with a proline in position 447, whereas DBA/2 substrains including DBA/2J, DBA/2N, DBA/2Ola, BALB/c and A/J mice have a C-to-G mutation at nucleotide 1473 encoding for arginine at

this position (see also above). Zhang et al. could detect a two-fold higher activity of Tph2 in mouse strains homozygous for the 1473C allele (proline form) as compared to the arginine carriers and show substantially lower levels of 5-HT in the brain of mouse strains with the arginine Tph2 form (Zhang et al., 2004). Additionally, the V_{max} for both Trp and BH_4 is about 50% lower in an engineered P447R mutant enzyme (arginine form) than that of the wildtype enzyme (proline form) (Sakowski et al., 2006). Kulikov et al. found a close association of mouse strains carrying the proline Tph2 form and enhanced aggressive behaviour showing an increased number of attacks of males towards other males (Kulikov et al., 2005). Thus, for studies on the serotonergic system it is crucial to use mice on the same genetic background especially when investigating Tph2 KO mice.

In the present study, conditional (c) and inducible (i) Tph2 KO mice as well as constitutive Sert KO mice were investigated. The Tph2 iKO and cKO mice were produced in our laboratory and in collaboration with the working group of Dr. Ding at the Institute of Neuroscience, Shanghai, China. Cyclization recombinase (cre)-transgenic mice were crossed with *Tph2* floxed mice (see chapter 2.2.4.2) possessing a floxed deletion in the exon 5 of the *Tph2* gene. Three different types of cre transgenic mice were used for the crossing: 1. Cre under control of the promoter of the intermediate filament nestin which is mostly expressed in nerve cells and implicated in the radial growth of the axon resulting in Nestin-cre x *Tph2* floxed (Tph2 cKO^{Nes}). 2. Cre under control of the promoter of the ETS domain transcription factor Pet1, which is highly important during development (from E11 on) and together with other transcription factors defines the serotonergic cell type resulting in Pet1-cre x *Tph2* floxed (Tph2 cKO^{Pet1}). 3. Cre both under the control of the promoter of Pet1 and coupled to a ligand-binding domain of the human estrogen receptor (ER) for tamoxifen-induction resulting in Pet-creER x *Tph2* floxed (Tph2 iKO) (for the method see chapter 2.2.4).

The Sert KO mice possess an inactivated *Sert* gene in the exon 2 (Bengel et al., 1998) and feature elevated extracellular 5-HT level in the striatum (Mathews et al., 2004) and in the substantia nigra (Fabre et al., 2000), but exhibit 20-30 % reduced 5-HT level in the whole brain (Bengel et al., 1998). In addition, the amount of serotonergic neurons is reduced in the dorsal raphe nucleus (Lira et al., 2003). Generally, in the Sert KOs, 5-HT levels are elevated in the extracellular synaptic cleft and diminished within serotonergic cells, which also results in altered expression of 5-HT receptors like $5\text{-}HT_{1a}$, $5\text{-}HT_{1b}$ and $5\text{-}HT_3$ receptors (Fabre et al., 2000; Schmitt et al., 2003). For example, the density of $5\text{-}HT_{1a}$-receptors is reduced in the hypothalamus and increased in the hippocampus. As outlined above, Sert KO mice feature several behavioural changes including increased anxiety- and depression-like behaviour (Li et al., 2000). Exposure to the odour of a predator is selectively anxiogenic in the plus maze and light/dark box tests in Sert KO mice (Adamec et al., 2006). Furthermore, Sert KO mice

show increased sensitivity to stress, and decreases in aggressive behaviour. The corticosterone response to chronic stress (Lanfumey et al., 2000) as well as the ACTH response to mild acute stress (Li et al., 1999) is enhanced in Sert KO mice. Some of these behavioural changes are similar to phenotypes found in humans with short alleles of a repeat length variation polymorphism in the SERT promoter region (see chapter 1.3.2). Hence, due to their molecular and behavioural profile, Sert KO mice might well serve as a model for disorders of emotional dysregulation and affective behaviours.

2 MATERIAL AND METHODS

2.1 Material

2.1.1 Human tissue

For *in situ* hybridization (ISH) and immunohistochemistry (IHC) native, unfixed human brainstem blocks of a 33 year old male were used. Tissue from small and large intestine (jejunum and colon) was removed by surgery from a human male who donated this tissue for research, and immediately frozen and stored at -80 °C. 16 µm sections were produced at -20 °C and stored at -80 °C. For quantitative real-time PCR (qRT PCR) human total RNA of different brain regions (cerebral cortex, hippocampus, putamen, caudate nucleus, cerebellum and the two raphe-containing regions: pons and medulla oblongata) was obtained from BD Biosciences (Erembodegem, Belgium). The RNA was pooled from 10-35 male and female Caucasians (16-70 years) which have died from sudden death. Additionally, total RNA was extracted by our laboratory from human jejunum and colon tissue which was removed by surgery.

2.1.2 Murine tissue

Wildtype (WT) mice, serotonin transporter constitutive knockout (Sert KO) mice as well as two different tryptophan hydroxylase 2 conditional knockout (Tph2 cKO) mice and a Tph2 inducible knockout (iKO) mouse were used for this work. Most of the mice had a C57/BL6 strain background the others were crossbreeds of C57/BL6, 129/SvEMS and FVB/N strains (table 2-1). All mice were kept and generated under normal light-dark-cycles and can be classified according to their treatment into four groups:

1. **No treatment**; after CO_2 anesthetization, instantaneously euthanizing by cervical dislocation; removal of brains and/or peripheral tissues including pineal gland immediately after euthanasia.

2. No treatment; after ether anesthetization with still intact cardiovascular system, 10 minutes **perfusion of tissue** with either 1x tissue-osmotic, isotonic phosphate buffered saline (PBS) to clear the tissue from blood cells or perfusion with 4x paraformaldehyde (PFA) to fixate the tissue; immediate removal of brains.

3. 1 hour immobilization **stress treatment** by inserting the mice in 50 ml tubes (Sarstedt, Nümbrecht, Germany); after CO_2 anesthetization, instantaneously euthanizing by cervical dislocation; immediate removal of blood and brains.

4. 4-5 intraperitoneal **injections of tamoxifen** with a one week interval between first and second injection and a one day interval between the following injections to induce a specific gene deletion in Tph2 iKO mice; euthanasia of mice three weeks after the last injection.

Native and PBS-perfused tissue was immediately frozen in dry ice-cooled isopentane (temporary storage on dry ice, longer storage at -80 °C). Fixated tissue was shaken over night in 20% sucrose solution and stored at -80 °C afterwards. Preparation of brain regions for qRT-PCR was done on a cooling plate (-20 °C). For ISH and IHC, native or fixated tissues were cut into 16 µm sections at -20 °C using a cryostat (HM 500 O, Microm). Slices were mounted on cooled *superfrost plus* slides (Menzel, Braunschweig, Germany) and stored at -80 °C for later application. For qRT-PCR, total RNA was extracted from different brain regions and from peripheral organs including pineal gland (see chapter 2.2.1).

2.1.3 Oligodesoxynucleotids

Oligodesoxynucleotids (primer, see table 2-2) were designed by our laboratory and obtained from MWG-Biotech (Ebersberg, Germany). QuantiTect primer assays were obtained from QIAGEN (Hilden, Germany).

2.2 Methods

2.2.1 Quantitative real time polymerase chain reaction (qRT-PCR)

Like the conventional PCR, the qRT-PCR gives rise to exponential duplication of DNA double-strands by the use of specific primers and a heat-resistant *Taq*-polymerase. In contrast however, the exponential increase of PCR products can be graphically illustrated in qRT-PCR by the use of fluorescence dyes (in the present work, SYBR green I) and the measurement of the fluorescence once per PCR cycle. SYBR green I intercalates into double-stranded DNA and produces green fluorescence of λmax = 521 nm wavelength which can be measured in the presence of blue light (λmax = 494 nm). The use of standard dilution series with known initial concentrations provides reference values to calculate both specific PCR kinetics and the initial mRNA concentration which was present in the investigated samples. The cycle which presents the transition into the exponential phase defines the so called threshold cycle (C_T). Since C_T-values are dependent on the initial amount of template cDNA (related to

Table 2-1 *Used mice and murine tissues*

Group	Genotype	Strain background	Amount	Treatement	Sex	Age	Dissected regions	Method
Periphery	WT	C57/BL6	4	non	M	6 months	**peripheral organs:** liver, heart, lung, thymus,kidney, adrenal gland, spleen, stomach, small intestine, large intestine, testis;**pineal gland** **brain:** raphe region (control)	qRT-PCR
	WT	C57/BL6	2	non	M	4 months	small intestine, large intestine	ISH, IHC
Develop-ment	WT	C57/BL6	2 per stage	non	U	E9.5, E11.25, E13, E15.5, E18.5, P0.5, P1.5, P2.5, P4, P5, P7, P9, P10, P11, P13, P14, P15, P17, P19, P23, 2 months, 4 months	whole brain **without** pineal gland	qRT-PCR
	WT	C57/BL6	2 per stage	non	U	E9.5, E11.25, E13,E15.5, E18.5, P0.5, P2.5, P5, P10, P16, P21, 2 months	whole brain **with** pineal gland (in serial sections)	ISH, IHC
Blood	WT	C57/BL6	3	non	M	adult	cortex, hippocampus, striatum, cerebellum, raphe region; pineal gland	qRT-PCR
			3		F	adult		
	WT	C57/BL6	3	PBS perfusion	M	adult		
			3		F	adult		
Stress	WT	C57/BL6	8	non (control)	M	2-6 months	cortex, hippocampus, hypothalamus, amygdala, raphe region	qRT-PCR
			8		F	4-9 months		
	WT	C57/BL6	8	stress	M	2-6 months		
			6		F	4-9 months		
	Sert KO	C57/BL6	10	non (control)	M	2-6 months		
			8		F	4-9 months		
	Sert KO	C57/BL6	9	stress	M	2-6 months		
			9		F	4-9 months		

E = embryonic day, F = female, KO = knockout, M = male, P = postnatal day, Sert = serotonin transporter, U = unknown, WT = wildtype

Table 2-1 *Used mice and murine tissues*

Group	Genotype	Strain background	Amount	Treatment	Sex	Age	Dissected regions	Method
Tph2 cKO	Tph2 cKO^{Nes}	C57/BL6	5	non	M	5 months	frontal/ prefrontal cortex, striatum, hippocampus, hypothalamus amygdala, rostral and caudal raphe region, entorhinal cortex	qRT-PCR
			5		F	5 months		
	Tph2 cKO^{Pet1}	C57/BL6 x 129/SvEMS x FVB/N	6	non	M	2-4 months		
			5		F	2-4 months		
	Tph2 iKO	C57/BL6 x 129/SvEMS x FVB/N	4	tamoxifen injections	M	3-4 months		
			2		F	3-4 months		
Tph2 cKO controls	Tph2 ^{floxed}	C57/BL6	1	non	M	4 months		
			1		F	3 months		
	WT	C57/BL6	4		M	1-2 months		
			3		F	2-4 months		
	Pet1-cre	C57/BL6 x 129/SvEMS x FVB/N	1		F	3 months		
Tph2 iKO controls	Pet1-creER	C57/BL6 x 129/SvEMS x FVB/N	2	tamoxifen injections (control)	M	3-4 months		
			2		F	3-4 months		
	Tph2^{floxed}	C57/BL6	2		M	3-4 months		
			2		F	3-4 months		
Tph2 cKO	Tph2 cKO^{Nes}	C57/BL6	2	non	U	P21, 2 months	whole brain (in serial sections)	IHC
	Tph2 cKO^{Pet1}	C57/BL6 x 129/SvEMS x FVB/N	1	non	U	2 months		
	Tph2 iKO	C57/BL6 x 129/SvEMS x FVB/N	3	tamoxifen injections	U	E15.5, 3 months, 5 months		
controls	WT	C57/BL6	2	non	U	2 months		
	Pet1-creER	C57/BL6 x 129/SvEMS x FVB/N	3	tamoxifen injections (control)	U	E15.5, 3 months, 5 months		
Test for antibodies	WT	C57/BL6	10	non	U	adult	frontal brain, raphe region, pineal gl.	western blot

cKO = conditional knockout, cre = cyclization recombinase, E = embryonic day, ER = ligand-binding domain of human estrogene receptor, F = female, iKO = inducible knockout, M = male, P = postnatal day, Tph2 = Tryptophan hydroxylase 2, U = unknown, WT = wildtype

the mRNA, which was present originally in the tissue), they can be used as quantitative units. That means, the more mRNA was present in the tissue, the earlier occurs the transition into the exponential phase corresponding to a low C_T-value.

Table 2-2 *Sequences of nucleotide primer pairs*

Description		Sequence, 5´ -> 3´	Complementary to nucleotides	Genetic base accession	Annealing temperature
For quantitative real time PCR					
hACTB	forward reverse	CATGGAGAAAATCTGGCACCACA GAGACCTTCAACACCCCAGC	316-338 465-446	NM_001101	60 °C
hGAPDH	forward reverse	ATGGGGAAGGTGAAGGTCGG TCATATTGGAACATGTAAACCATG	103-122 251-228	NM_002046	60 °C
hPPIA	forward reverse	CCCACCGTGTTCTTCGACAT CCAGTGCTCAGAGCACGAAA	93-112 208-189	NM_021130	60 °C
hRPL13A	forward reverse	CCTGGAGGAGAAGAGGAAAGAGA TTGAGGACCTCTGTGTATTTGTCAA	487-509 612-588	NM_012423	60 °C
mActb	forward reverse	AGGTGACAGCATTGCTTCTG GTTGAGGTGTTGAGGCAGCCAG	1641-1650 1831-1810	NM_007393	60 °C
mGapdh	forward reverse	AACGACCCCTTCATTGAC GTGCTGAGTATGTCGTGGA	144-161 334-316	NM_008084	60 °C
mArbp	forward reverse	CGACCTGGAAGTCCAACTAC ATCTGCTGCATCTGCTTG	93-112 201-184	NM_007475	60 °C
mRn18s	forward reverse	GAAACTGCGAATGGCTCATTAAA CCACAGTTATCCAAGTAGGAGAGGA	82-104 156-132	NR_003278	60 °C
TPH1	forward reverse	ACATGCACTTTCTGGACATGC CAGTGCCATGAATGAGCTGCA	1026-1046 1274-1254	NM_004179	59.1 °C
TPH2	forward reverse	GCAGCAGTTCAGTCAATGTCAT GTCTCATTTATGCCCT TTTCTTTTT	1659-1684 1939-1915	NM_173353	61.5 °C
Tph1	forward reverse	GCGCCCGTTTGGACTGAAGTA GAGCATCAGTCTAGAGCCAGGGC	1624-1644 1830-1808	NM_009414	61.5 °C
Tph2	forward reverse	TGGGGATTTGATGCCTAGAACC ACACAAAAATGCTCTAAAGAACCCA	1590-1611 1773-1749	NM_173391	63.5 °C
Mm_Actb_2_SG	*housekeeping gene*		QuantiTect primer assay (QIAGEN)		60 °C
Mm_Gapdh_3_SG	*housekeeping gene*		QuantiTect primer assay (QIAGEN)		60 °C
Mm_Ubc_1_SG	*housekeeping gene*		QuantiTect primer assay (QIAGEN)		60 °C
Mm_Rn18s_2_SG	*housekeeping gene*		QuantiTect primer assay (QIAGEN)		60 °C
Mm_Htr1a_1_SG	*5-HT_{1a} receptor gene*		QuantiTect primer assay (QIAGEN)		60 °C
Mm_Htr1b_1_SG	*5-HT_{1b} receptor gene*		QuantiTect primer assay (QIAGEN)		60 °C
Mm_Htr2a_1_SG	*5-HT_{2a} receptor gene*		QuantiTect primer assay (QIAGEN)		60 °C
Mm_Htr2c_1_SG	*5-HT_{2c} receptor gene*		QuantiTect primer assay (QIAGEN)		60 °C
Mm_Slc6a4_1_SG	*serotonintransporter gene*		QuantiTect primer assay (QIAGEN)		60 °C
Mm_Oxt_1_SG	*oxytocin gene*		QuantiTect primer assay (QIAGEN)		60 °C

Table 2-2 *Sequences of nucleotide primer pairs*

Description		Sequence, 5´ -> 3´	Complementary to nucleotides	Genetic base accession	Annealing temperature
For generation of ISH-probes					
TPH1	forward reverse	ACATGCACTTTCTGGACATGC CTTCTACTCTTTTTGCCATCTCTTCA	1026-1046 1400-1375	NM_004179	61.5 °C
TPH2	forward reverse	TGGGGATTTGATGCCTGGAACTAT TCTTGGGACTATGAATTTTATGCTGG	1604-1627 2328-2303	NM_173353	63.0 °C
Tph1	forward reverse	GCGCCCGTTTGGACTGAAGTA ACTCTCTCTGAAAATGCACACCTGG	1624-1644 1946-1922	NM_009414	60.4 °C
Tph2	forward reverse	TGGGGATTTGATGCCTAGAACC GGAGTCGTAGAGAAACAAAGGGATT	1590-1611 2457-2433	NM_173391	62.5 °C
For sequencing					
M13	forward reverse	GTAAAA CGACGGCCAG CAGGAAACAGCTATGAC	205-221 446-431	Dual promoter plasmid pCRII (Invitrogen, Karlsruhe, Germany)	51,6 °C 50,3 °C

2.2.1.1 Preparation of qRT-PCR

Total-RNA was isolated from tissue of human small and large intestine (jejunum and colon) using the trizol procedure (Invitrogen, Karlsruhe, Germany) and purified and removed from potential remaining genomic DNA with the QIAGEN RNeasy kit in combination with DNase I treatment by the DNeasy tissue kit (QIAGEN, Hilden, Germany). The same procedure was performed with all investigated murine tissues (total brain of different developmental stages, different brain regions and peripheral organs). Relative quality and purity as well as concentration of RNA was controlled by gel electrophoresis and measured by photometry using the Nanodrop system (Peqlab, Erlangen, Germany). cDNA first-strand was synthesized applying 500 ng total RNA to the iScript™ cDNA synthesis kit (Bio-Rad, Munich, Germany) and diluted (1:5) with RNase-free water (Merck, Darmstadt, Germany). PCR parameters for specific hybridization of primer pairs (see table 2-2) were established by test runs and the resulting PCR product was identified by gel electrophoresis and isolated via the *quantum prep freeze´n squeeze* DNA gel extraction kit (BioRad, München, Germany). A logarithmic dilution series (standard series) of six consecutive dilutions (from 10^{-1}ng/µl to 10^{-6}ng/µl) was produced by diluting the isolated PCR product to calculate diverse kinetic PCR factors. This procedure was not required for the QuantiTect primer assays from QIAGEN, since particular parameters like annealing temperature for primers and PCR efficiency are provided by the company.

2.2.1.2 qRT-PCR method

qRT-PCR using a 96-well plate or a 384-well plate was performed on the iCycler iQ™ or the CFX384 real-time PCR detection system, respectively, from Bio-Rad (Munich, Germany) with the reaction components of table 2-3 a) or 2-3 b). Both reactions were performed with the reaction protocol of table 2-4. Triplicates (96-well) or duplicates (384-well) were done for every sample and the mean which was determined by the proprietary software of the particular detection system was used for further analysis. Melting curve analyses were done immediately after the PC reaction controlling amplification specifity. For this, every 10th sec temperature was increased in 0.5 °C-steps from 55 °C to 95 °C. At the respective melting temperature of the double-stranded products in the solution, denaturation produces DNA single-strands leading to a change of fluorescence signal (ΔF) which can be measured and graphically illustrated. Multiple products in the reaction mixture would result in multiple melting curves. For *non-QIAGEN* primers, every run of qRT-PCR was performed with the particular standard series of the amplifying targeting gene to calculate PCR efficiency.

Table 2-3 a) *Reaction components for qRT-PCR using 96-well plates.*

Reagent	**Amount**	
iQ™ 2x SYBR green supermix (BioRad, Munich, Germany)	12.5	µl
forward primer (5-10 pmol/µl)	1.0	µl
reverse primer (5-10 pmol/µl)	1.0	µl
template DNA (500 ng/µl)	1.0	µl
(cDNA from the tissues or standard dilution)		
H_2O (Merck, Darmstadt, Germany)	9.5	µl
Volume	25.0	µl

Table 2-3 b) *Reaction components for qRT-PCR using 384-well plates.*

Reagent	**Amount**	
QuantiTect fast SYBR green mix (QIAGEN, Hilden, Germany)	4.0	µl
QuantiTect primer assay (QIAGEN, Hilden, Germany)	1.0	µl
template cDNA (500 ng/µl)	1.0	µl
H_2O (Merck, Darmstadt, Germany)	4.0	µl
Volume	10.0	µl

Table 2-4 *qRT-PCR protocol.*

Cycle	Temperature	Time	Relevance	Number of cycles
1	95 °C	3 min	Initiale hotstart to activate the hotstart-polymerase	1x
2	95 °C	30 sec	DNA denaturation	40x
	Annealing temperature (see table 2-2)	45 sec	Hybridization of primer and duplication of DNA; measurement of fluorescence variation (ΔF)	
3	95°C	1 min	DNA denaturation	1x
4	55 - 95°C in 0,5 °C-steps	10 sec per step	Melting curve analysis	80x
5	15 °C	∞	Stop	∞

2.2.1.3 Data analysis

To correct inaccuracies of the RNA quality and between the cDNA samples, normalization of the expression data was performed. To this end, C_T-values of every investigated housekeeping gene were converted into relative quantity values (Q-values) with the algorithm described by Vandesompele et al. (2002) considering respective minimal C_T and PCR efficiency values. The stability of gene expression (M-value) was determined via the GeNorm software (Vandesompele et al., 2002). As a consequence of the algorithmic calculation by GeNorm, normalization factors (NF) were generated from the two most stable (smallest M-value) of the four investigated housekeeping genes and used to normalize values of the respective gene of interest (GOI) by calculating the ratio (GOI/NF). For the 96-well RT-PCR, the determined concentrations of the standard series were used to calculate mRNA copy numbers per µl, which have been present in the investigated tissues. For the 384-well RT-PCR, calculated Q-values of the gene of interest were normalized and used as relative quantity values. Both mRNA copy numbers and Q-values were normalized with the respective established normalization factors and illustrated as relative expression levels.

2.2.2 *In situ* hybridization (ISH)

ISH is a method to visualize the expression of specific genes on the transcriptional level in different tissues. In this work gene-specific digoxigenin-labelled cRNA probes for *TPH1* and *TPH2* were applied to specifically detect TPH1- or TPH2 mRNA in the investigated human and murine tissues, respectively.

2.2.2.1 Preparation of ISH

Human (TPH1/2) and murine (Tph1/2) cRNA probes in the 3'-untranslated region (3'-UTR) were generated to maintain isoform-specific probes and to avoid cross-reactions between the detection of TPH1- and TPH2 mRNA (used primers see table 2-2). cDNA probes were produced by conventional PCR, controlled by gel electrophoresis and cloned into the dual promotor pCRII vector using the TA cloning® kit (Invitrogen, Karlsruhe, Germany) which applies the T-vector cloning method developed by (Marchuk et al., 1991). The vector was transformed by heat shock (30 sec at 42 °C) in *E. coli* bacteria. Positive clones were selected by ampicillin resistance, identified by blue-white selection, and proliferated. Plasmid DNA was isolated via the *QIAprep Spin Miniprep* kit (QIAGEN, Hilden, Germany) and differentiated with restriction enzyme analysis (with EcoRI) and gel electrophoresis. The orientation of the insert as well as the correct sequence of the probe was controlled by sequencing using the *Big Dye ® terminator v 1.1 cycle sequencing kit* and the ABI Prism™ 310 genetic analyzer (Applied Biosystems, Foster City, USA). After sequencing the pCRII vector was linearised with restriction enzymes. Depending on the orientation of the embedded probe (table 2-5), specific digoxigenin-labled cRNA-sense and -antisense probes were produced using *in vitro* transcription on the dual promoter vector which possesses a promoter sequence for both the T7-RNA-polymerase (downstream of the insert) and the Sp6-RNA-polymerase (upstream of the insert). The *in vitro* transcription was performed on phenol-chloroform cleared and ethanol precipitated, linearised plasmids by the use of the *DIG RNA labeling kit (Sp6/T7)* (Roche, Mannheim, Germany). Afterwards, cRNA probes were again cleaned from remaining proteins via phenol/chloroform precipitation, concentrated with ethanol precipitation and analyzed on a 1 % agarose gel.

Table 2-5 *ISH probes.*

Gene	Probe	Sequence of linearised vector	Restriction enzyme for linearization	Orientation in the vector	Size of probe
TPH1	Antisense	Sp6-TPH1- pCRII	XhoI	5'→ 3'	375 bp
	Sense	pCRII-TPH1-T7	HindIII		
TPH2	Antisense	Sp6-TPH2- pCRII	XhoI	5'→ 3'	280 bp
	Sense	pCRII-TPH2-T7	KpnI		
Tph1	Antisense	pCRII-Tph1-T7	HindIII	3'→ 5'	323 bp
	Sense	Sp6-Tph1- pCRII	XhoI		
Tph2	Antisense	Sp6-Tph2- pCRII	XhoI	5'→ 3'	414 bp
	Sense	pCRII-Tph2-T7	SpeI		

Table 2-6 *ISH reaction protocol.*

Step	Solution	Number	Time	Temperature	Relevance
1	Fresh prepared 4% PFA (para-formaldehyde) in 0,1 M PBS (phosphate buffered saline)	1x	5 min	RT (room temperature)	Fixation of tissue
2	Decreasing ethanol series (100%, 95%, 80%, 70%)	1x per dilution	2 min per step	RT	Rehydration
3	2x SSC (saline-sodium citrate)	2x	10 min per step	RT	Washing
4	0,02 N HCl	1x	5 min	RT	Denaturation of cell backing proteins like collagen and γ-actin to get a high permeable tissue.
5	2x SSC	2x	5 min per step	RT	Washing
6	0,25 % acetic anhydrid in 0,1 M triethanolamin	1x	at least 20 min	RT	Acetylation to neutralize positive-charged amino groups from proteins which might provide unspecific binding sites for the negative charged cRNA probe.
7	2x SSC	2x	5 min per step	RT	Washing
8	**Hybridization buffer:** 50% deionized formamid, 4xSSC, 1x denhardt's solution, 10% dextransulfat and 250 µg/ml denatured salmon sperm-DNA	1x	1 h	60 °C	The salmon sperm-DNA blocks unspecific binding sites which might be accepted as targeting sequences for cRNA probes.
9	Hybridization buffer + **10 ng/µl** of the respective sense or antisense **probe**	1x	over night	60 °C	Hybridization of the cRNA probe to the mRNA sequence existing in the investigated tissue (only for the antisense probe).
10	2x SSC	2x	10 min per step	RT	Washing
11	50% formamid in 2x SSC	1x	30 min	60 °C	Formamid binds to hydrogen bonds leading to linearization of cRNA. It keeps the cRNA probe denaturized at low temperatures and facilitates the hybridization.
12	2x SSC	2x	10 min per step	RT	Washing
13	40 µg/ml RNaseA in RNase buffer	1x	30 min	37 °C	RNase A cuts unspecifically bound cRNA probes into small fragments.
14	**RNase buffer:** 500 mM NaCl, 10 mM Tris-HCl (pH 7,5) and 1mM EDTA	3x	2 min per step	RT	Washing
15	RNase buffer	1x	30 min	60 °C	Removal of non-hybridized RNA pieces.
16	1x TBS (Tris buffered saline)	1x	5 min	RT	Equilibration for blocking buffer.
17	0,5% blocking reagent in 1x TBS	1x	30 min	RT	The blocking buffer masks unspecific binding sites for the Anti-DIG-aP antibody.
18	1,5 U/ml anti-DIG-aP antibody in blocking buffer	1x	1 h	RT	Binding of the antibody to the digoxigenin (DIG)-labelled cRNA probes.
19	1x TBS	2x	5 min per step	RT	Washing
20	**DIG3 buffer:** 100 mM NaCl, 100 mM Tris-HCl (pH 9,5) and 50 mM $MgCl_2$	1x	5 min	RT	Equilibration for alkaline phosphatase (aP) which uses magnesium as a co-factor.
21	Nucleic Acid Detection kit (Roche, Mannheim, Germany) with BCIP and NBT for **aP-reaction dilution**	1x	over night	RT	The aP separates a phosphate residue from the compound BCIP. The dephosphorylated product reacts in keto-enol-tautomerie. Intermediate-oxidation emerges blue indigo. NBT is used as oxidant and additional colour booster. The product diformazan shows blue-violet colour.
22	1x PBS	3x	5 min	RT	Terminating of reaction and washing of slides.

2.2.2.2 ISH reaction

Hybridization was performed on 16 µm human and murine cryostat tissue sections. Hybridized DIG-cRNA probes were detected via alkaline phosphatase (aP) coupled anti-DIG antibody. The aP can produce an indigo blue precipitate after addition of BCIP (5-Bromo-4-chloro-3-indolyl-phosphate) and NBT (4-Nitro-blue-tetrazolium-chloride) and thereby visualize the probe. To ensure specific binding of probes, both negative controls with sense probes and RNase treatment after the hybridization step were done. In contrast to the antisense probe, which possesses a sequence complementary to the mRNA sequence, the sense probe exhibits the identical sequence of the mRNA sequence and is not able to hybridize with the existing mRNA in the investigated tissue. The ISH reaction was carried out according to the reaction protocol of table 2-6. After ISH reaction, slides were covered with aquatex (Merck, Darmstadt, Germany) and stored in the dark.

2.2.3 Immunohistochemistry (IHC)

The IHC is a method to detect proteins in tissue sections (i.e., it detects gene expression on the translational level) via the use of monoclonal or polyclonal primary antibodies specifically binding to the respective targeting protein in the investigated tissue. In this work specific polyclonal primary antibodies against Tph1 and Tph2 were used detection of human and murine protein.

2.2.3.1 Preparation of IHC - Western blotting

For the generation of murine Tph1 and Tph2 specific antibodies, two peptides were selected for each Tph protein isoform considering their internal characteristics and an high specificity for the respective isoform. The peptides comprising the murine amino acids (aa) 458-472 (TRSIENVVQDLRSDL) and aa 476-488 (CDALNKMNQYLGI) corresponding to the C-terminal part were chosen for the generation of antibodies against Tph2 protein. The peptides aa 6-20 (KENKENKDHSSERGR+C) located in the N-terminal domain and aa 433-447 (VISDALARVTRWPSV) corresponding to the C-terminal part of Tph1 were used as immunogenes for antibodies against Tph1. Peptide synthesis, coupling to keyhole limpet haemocyanin (KLH) and immunization of rabbits were carried out at Eurogentec (Seraing, Belgium) following their AS-DOUB-LX protocol. After collection of pre-immune serum, samples were injected into rabbits, followed by three boosts over a period of three months.

Hyperimmune serum samples were collected on average one month after each boost and sent to our laboratory. The polyclonal antibodies were tested for reactivity and specificity against Tph1 and Tph2 on Western blots presenting proteins extracted from pineal glands and raphe region preparations as well as frontally brain slices which were free from known serotonergic neuron cell bodies obtained from adult C57BL/6J mice. Homogenization was performed by sonication on ice in a buffer containing 10 mM Tris (pH 7.4), 100 mM NaCl, 10 mM EDTA, 0.5% reducing agent and a protease inhibitor cocktail. Homogenates were centrifuged at 1000 x g for 10 min at 4 °C to remove cell debris and protein content in the supernatant and quantified by the Bradford assay. After a short heat-induced denaturation, proteins (20 µg per lane) were resolved on 10% SDS polyacrylamide gels and transferred electrophoretically to nitrocellulose membranes. After membrane strips were pre-incubated for 2 h with blocking buffer (LSB: 10 mM Tris, pH 7.5, 500 mM NaCl, 0.1% Tween 20; containing 5% non-fat dry milk), incubation with the primary antibodies (anti-Tph1 or anti-Tph2 antibodies containing serums) diluted 1:400 in blocking buffer was performed overnight at 4 °C. Following several washes, the blots were incubated for 2 h at RT with horseradish peroxidase-conjugated goat anti-rabbit antibodies (1:1000; Cell Signaling Technology, Danvers, USA) and bound immunoglobulins were visualized using monitored chemiluminescence.

2.2.3.2 The IHC reaction

The IHC was carried out on 16 µm human and murine cryostat tissue sections. Bound primary antibodies were detected via the avidin-biotin-complex with conjugated horseradish peroxidase. The peroxidase can oxidate the chromogen DAB (3,3'-diaminobenzidin) to a brownish product which leads to detection of the bound antibodies. The IHC reaction was carried out according to the reaction protocol of table 2-7. After the IHC reaction, slides were covered with auquatex (Merck, Darmstadt, Germany) and stored in the dark.

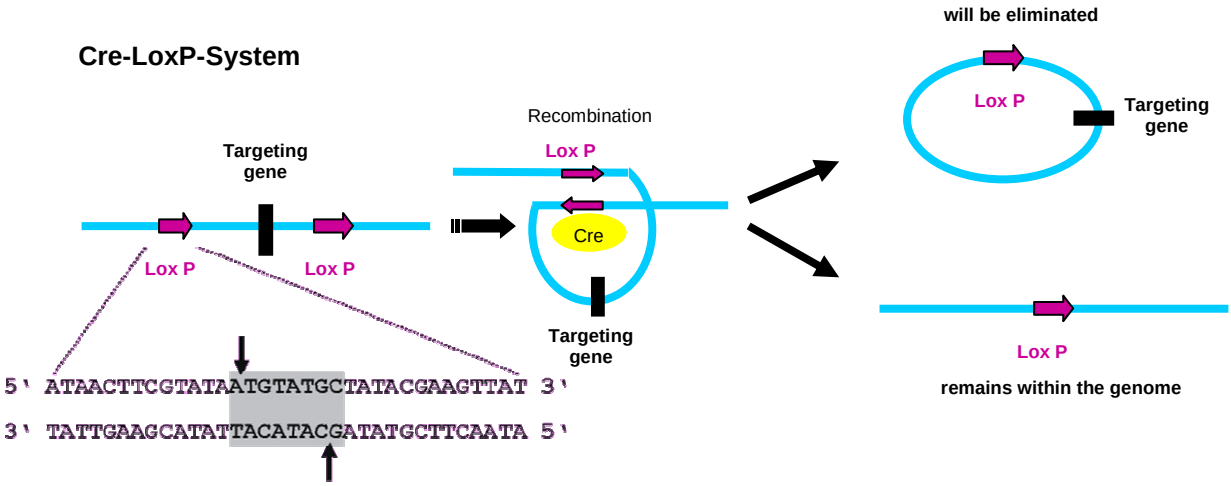
2.2.4 Generation of Tph2 conditional knockout (cKO) mice

Independent from other co-factors, cre-recombinase (cyclization recombination) which naturally occurs in the bacteriophage P1 catalyses the recombination between two loxP recognition sites (locus of crossover of P1). The loxP sequence consists of eight base pairs which are flanked by two palindromic sequences of 13 base pairs. A DNA segment flanked by two loxP sites (floxed DNA-segment) is cut out from genomic DNA by cre in form of a circular product which afterwards degrades in the cell (see Fig. 2-1). A single loxP sequence remains at the genomic DNA. For the generation of a mouse with a tissue-specific cre/loxP mediated gene deletion (cKO mouse), two distinct genetically modified

Table 2-7 *IHC reaction protocol*

Step	Solution	Number	Time	Temperature	Relevance
1	**Citrate buffer:** 10 mM Citrate buffer (pH 8.0), 10 mM EDTA, 0.05% Tween 20 (**for Tph1**); 10 mM citrate buffer (pH 6.0), 0.05 % Tween 20 (**for Tph2**)	1x	14 min	96 °C	Demasking of antigenes by heat-induced epitope retrieval.
2	non	1x	40 min	RT	Cooling down
3	1x TBS (Tris buffered saline)	3x	5 min	RT	Washing
4	0,6% H_2O_2 in TBS	1x	30 min	RT	Blocking of endogenous peroxidase activities.
5	1x TBS	3x	5 min	RT	Washing
6	**Blocking buffer:** 5 % NGS (normal goat serum), 2% BSA and 0.25% Triton X-100	1x	1 h	RT	Masking of unspecific binding sites.
7	**Primary antibody** (1:900 in blocking buffer)	1x	over night	4 °C	Binding of the primary antibody.
8	1x TBS	3x	5 min	RT	Washing
9	Biotinylated **secondary antibody**: goat anti-rabbit IgG (Vector Laboratories, Wiesbaden, Germany) 1:900 diluted in blocking buffer	1x	1.5 h	RT	Formation of a primary-secondary antibody complex.
10	1x TBS	3x	5 min	RT	Washing
11	Streptavidin-biotin peroxidise complex (**ABC**) (Vector Laboratories, Wiesbaden, Germany)	1x	1 h	RT	**Mixing of reaction components 30 min before**. Three molecules of horseradish peroxidase-conjugated biotin can bind one molecule avidin. The bound avidin carries additionally a binding site for the biotinylated secondary antibody. The ABC then binds the primary-secondary antibody complex.
12	1x TBS	3x	5 min	RT	Washing
13	DAB substrat	1x	ca. 5 min	RT	Visualizing of peroxidase activity.
14	1xTBS	3x	1 min	RT	Stopping of reaction and washing of slides.

mouse lines have to be crossed. One line carrying the loxP-flanked targeting gene (floxed mouse line) and the other expressing cre under the control of a tissue-specific promotor (cre transgenic mouse line). Crossbreding of the floxed line with the cre line manifests in the excision of the targeting gene (gene knockout) in all cre-expressing cells of the offspring. Tph2 cKO mice were generated by crossing a *Tph2* floxed line with Nes-cre, Pet1-cre and Pet1-creER lines (see below).



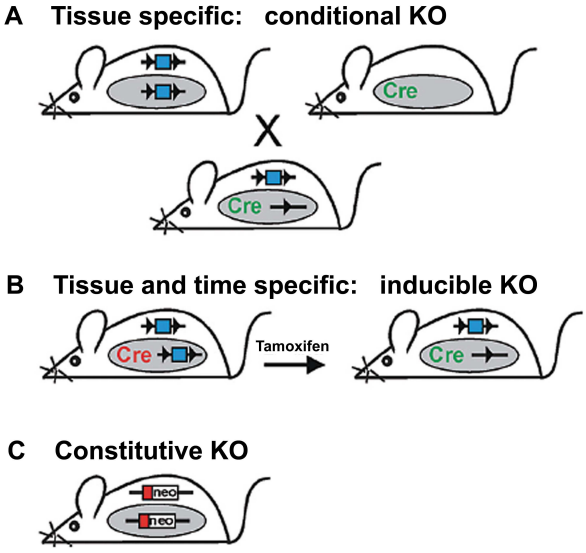


Fig.2-1 Cre/loxP mediated excision and elimination of DNA.

Above: *The base pair sequence of a loxP site (purple arrows) is shown and the central region where the recombination takes place is highlighted. The cre (cyclization recombinase) catalyses the recombination between two loxP recognition sites (floxed segment) leading to a circular product containing the floxed targeting segment which then degrades in the cell.* ***Below:*** *Comparison between conditional and conventional (constitutive) deletion of mouse genes.* ***A)*** *For generation of tissue-specific gene deletion a floxed targeting mouse is crossed with a tissue-specific cre mouse.* ***B)*** *A time-specific control of the recombination is carried out on ligand-activated cre-recombinases. First, cre being inactive (red), but after injection of a ligand (tamoxifen) cre is activated (green).* ***C)*** *In contrast, in constitutive KOs a gene is deleted ab initio in every cell of the body, for example by a neomycin resistance gene. Figure adapted from (Lukowski et al., 2005).*

2.2.4.1 Cyclization recombinase (cre) transgenic mouse line

Transgenic mice expressing cre recombinase can be generated by injecting the male pronucleus of oocytes with a cloned cre construct. Recombinant oocytes were then transferred into the uterus of a pseudo-pregnant female which gives rise to the birth of transgenic founder animals, since the construct is robustly integrated into the genome by recombination. The cre site integrates randomly in the genome. Thus, expression features of every founder mouse for a cre mouse line have to be established by genotyping using PCR on genomic DNA obtained e.g. from mouse tail biopsies.

Mice expressing cre-recombinase in neuronal and glia cell precursors according to Tronche et al. (1999) were used. Cre expression was under the control of the rat *nestin* (Nes) promoter and enhancer present in the second intron of the rat *nestin* gene. Nestin is an intermediate

filament which is mostly expressed in nerve cells and implicated in the radial growth of the axon. Nes-cre transgenic mice were generated by injecting BL6/SJF2 oocytes with the nestin-cre DNA construct (Tronche et al., 1999). Furthermore, mice expressing cre in serotonergic precursors by expressing cre under the control of the ETS domain transcription factor Pet1 were used. Pet1 is very important during development (from E11 on) and together with other transcription factors defines the serotonergic cell type. Pet1 was shown to be expressed specifically in central serotonergic cells (Pfaar et al., 2002). Pet1-cre mice were generated at the ION, Shanghai, China by using BAC-based transgenesis. The cre coding sequence and 3'-splice/transcription termination signals was inserted into the Pet1 locus at the initiation codon of the first exon by homologous recombination (Dai et al., 2008).

Finally, for generation of a iKO mouse, a Pet1-cre mouse line was used with cre fused to a mutated ligand-binding domain of human estrogen receptor (ER), which mediates tamoxifen-dependent homologous recombination at LoxP sites (Feil et al., 1997) driven by the Pet1-promotor resulting in Pet1-creER mice. CreER is unable to pass the membrane of the cell nucleus until the human estrogen receptor agonist tamoxifen is injected. After tamoxifen has bound to ER, creER is able to enter the membrane of the cell nucleus and to catalyse the cyclization recombination. Both the Pet1-cre and the Pet1-creER transgenic mouse line was provided by the group of Dr. Ding at the ION, Shanghai, China.

Cyclization recombination *in situ* can initially be analysed by using crossbreds of a cre mouse line with a ROSA26 reporter strain. ROSA26 cre reporter mice possess a lacZ transgene encoding for the enzyme ß-galactosidase inserted into the rosa locus (expressed in every body cell) whose expression is dependent on cre-mediated recombination (Soriano, 1999). Histochemical analysis (X-Gal staining) in newborn mice reveal ß-galactosidase activity by extensive blue staining in the case of effective cre recombination (see Fig. 2-2). For illustration, to determine whether Pet1-CreER is able to excise DNA specifically in 5-HT neurons, a founder line was crossed to the ROSA26 cre reporter strain to generate $R26R^{Pet1\text{-}CreER}$ mice. Brains obtained from E15.5 old $R26R^{Pet1\text{-}CreER}$ mice were examined for LacZ expression after tamoxifen treatment at E13.5 (2-4 mg per 40 g of body weight). By using X-Gal (5-Brom-4-chlor-3-indoxyl-β-D-galactopyranosid) as substrate for the enzyme ß-galactosidase, cells with active ß-galactosidase are histochemically detectable. β-galactosidase hydrolyses X-Gal to galactose und 5-bromo-4-chlor-indoxyl, the latter reacts in combination with air-oxygen to a deep blue-coloured product. Strong blue staining was detected in the midbrain, which corresponds to the localisation of the dorsal, median and caudal raphe nuclei (Fig. 2-2) which verifies effective cre recombination.

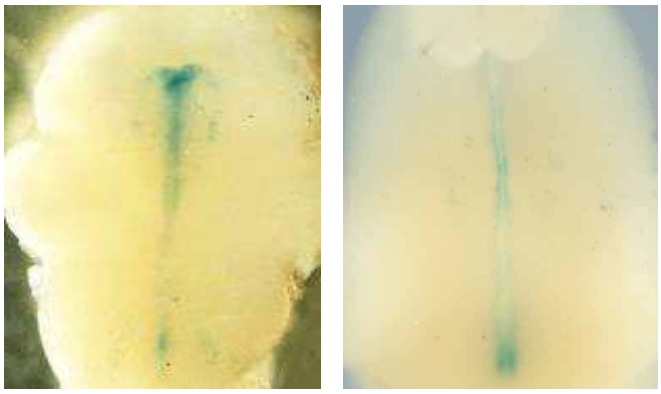


Fig. 2-2 X-Gal staining of an E15.5 old R26R$^{Pet1\text{-}CreER}$ embryo.
After injection of tamoxifen at E13.5, brains were dissected at E15.5 and dissolved in X-Gal (5-Brom-4-chlor-3-indoxyl-β-D-galactopyranosid) a substrate for the enzyme ß-galatosidase. R26R$^{Pet1\text{-}CreER}$ mice are able to express the inserted LacZ transgene which encodes for the enzyme ß-glactosidase in a cre-mediated recombination dependent manner. β-galactosidase hydrolyses X-Gal to galactose und 5-bromo-4-chlor-indoxyl, the latter results in combination with oxygen in a blue product. Strong blue staining was detected in the midbrain, which corresponds to the localisation of the dorsal, median and caudal raphe nuclei. E = embryonic day.

2.2.4.2 *Tph2* floxed mouse line

Elimination of exon 5 of *Tph2* resulting in a truncated non-functional Tph2 protein by creating a shift in the reading frame was used to generate *Tph2* floxed mice. Exon 5 is coding for an amino acid sequence at the start of the catalytic domain and ends with a partial codon. Therefore, a floxed *Tph2* gene construct leading to a *Tph2* allele with exon 5 flanked by LoxP sites (floxed *Tph2* allele) was generated by cloning (Fig. 2-3) (for a detailed description see (Gutknecht et al., 2008)). The targeting construct was linearised and electroporated into 129 R1 embryonic stem (ES) cells which were subjected to G418 selection. After verification by PCR, Southern blots and sequencing, an ES clone was injected into C57/BL6 blastocysts and implanted into pseudo-pregnant mice. A chimeric M displaying germ-line transmission was then used to propagate the floxed *Tph2* allele on a C57/BL6 background for several generations.

2.2.4.3 Recombination by the cre-loxP system – different brain-specific Tph2 cKO models

Tph2 cKOs with an inactivated *Tph2* gene in *nes* expressing cells (primarily neuronal cells) were obtained by breeding *Tph2* +/floxed mice with nestin-cre transgenics (Tronche et al., 1999) maintained on a C57/BL6 background. To inactivate *Tph2* in the raphe nuclei, the Pet1-cre mice were crossed with heterozygous *Tph2* floxed mice and their Pet1-cre/Tph2+/-

offspring were mated with *Tph2* floxed/floxed mice. In the same way, Tph2 iKO mice were generated by crossing *Tph2* floxed mice with the transgenic Pet1-creER mouse line. Tph2 iKO mice were injected 4-5 times with tamoxifen (6-8 mg per 40 g of body weight) in adulthood (age ≥ 2 months). Crossing and induction results in three adult brain-specific Tph2 cKO mice:

1. (Nes-cre x *Tph2* floxed) conditional KO mouse → **Tph2 cKO^{Nes}**
2. (Pet1-cre x *Tph2* floxed) conditional KO mouse → **Tph2 cKO^{Pet1}**
3. (Pet1-creER x *Tph2* floxed) inducible KO mouse → **Tph2 iKO**

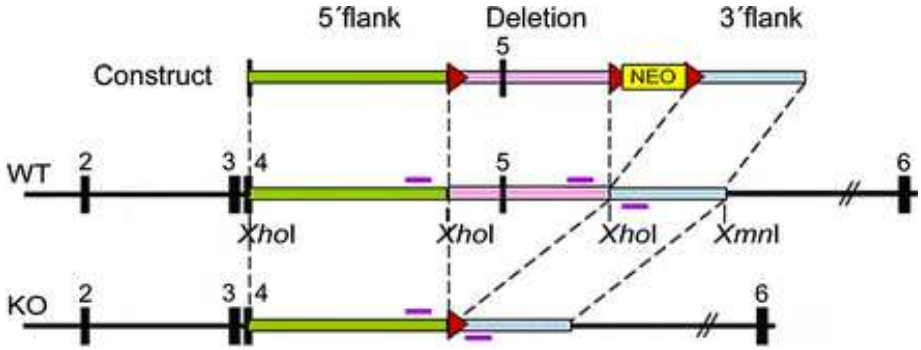


Fig. 2-3 Generation of Tph2 floxed mouse lines.
Targeting strategy. ***Above:*** *Targeting vector designed to remove exon 5 and flanking sequences (XhoI-XhoI deletion, pink) by homologous recombination in wildtype (WT) genomic DNA (middle). Exons 2–6 are depicted by vertical black boxes. XhoI-XhoI 5′ and XhoI-XmnI 3′ flanks are indicated in green and blue, respectively.* ***Below:*** *Predicted structure of the Tph2 null allele (KO) after excision by cre recombinase of the sequence flanked by LoxP sites (red triangles). Horizontal lines illustrate the position of primers used for genotyping. Figure taken from Gutknecht et al., 2008.*

3 RESULTS

3.1 Clarification of the spatio-temporal expression of TPH1/TPH2

To investigate the expression of the two different isoforms of tryptophan hydroxylase, TPH1 and TPH2, two methods at the transcriptional level (quantitative real time (qRT)-PCR and *in situ* hybridization (ISH)) and two methods at the translational level (immunohistochemistry (IHC) and Western blotting) were used with different developing and adult mouse tissues (brain and peripheral organs) as well as with human brain and gut regions. For both murine and human amplicons (Tph1/2 and TPH1/2) efficiency of qRT-PCR amplification was equal or close to 100%. ISH and IHC were performed on 16 µm cryo-sections and the reactivity and specificity of both the riboprobes and the antibodies for Tph1- and TPH1-detection were proven with positive controls like pineal gland in mice and intestine in humans. Negative controls using sense probe for ISH and preimmune serum for IHC validated the specific staining of antisense probes and antibodies, respectively, by the absence of any signal in the tissues examined.

3.1.1 Tph1 and Tph2 expression in adult and developing mouse tissues

3.1.1.1 Tph1/2 expression in brain regions related to gender and the absence of blood

To study the influence of gender on the expression of murine Tph1 and Tph2 in different brain regions, qRT-PCR was performed on twelve adult wildtype (WT) C57/BL6 mice of both sexes (6 females, 6 males). To investigate a possible confounding effect of circulating blood cell traces, which might serve as a source of Tph1- or Tph2 mRNA, half of the litter (3 females, 3 males) were PBS-perfused to eliminate any residual blood cells from the tissue. Brains were dissected in different brain regions like cortex, striatum, hippocampus, cerebellum and the raphe nuclei containing regions. Moreover, pineal gland was dissected and used as a positive control for Tph1 expression. No differences in the expression of Tph1 or Tph2 were detected between female and male mice (data not shown), thus in figure 3-1, means of females and males are displayed. Likewise, no differences in Tph1- or Tph2 mRNA levels were identified between perfused blood-free brains and untreated controls indicating no influence of blood cells on the expression of Tph1/2 in brain tissue. Furthermore, Tph2 mRNA was distributed in the whole brain, but about 30-fold lower than in the raphe region specifically expressing Tph2. In comparison, Tph1 mRNA levels were intense in the pineal gland, where

Tph2 was expressed in an approximately 40-fold lower amount. In contrast to Tph2, Tph1 mRNA was virtually undetectable in all brain regions including the raphe nuclei (Fig. 3-1, please note the different scales).

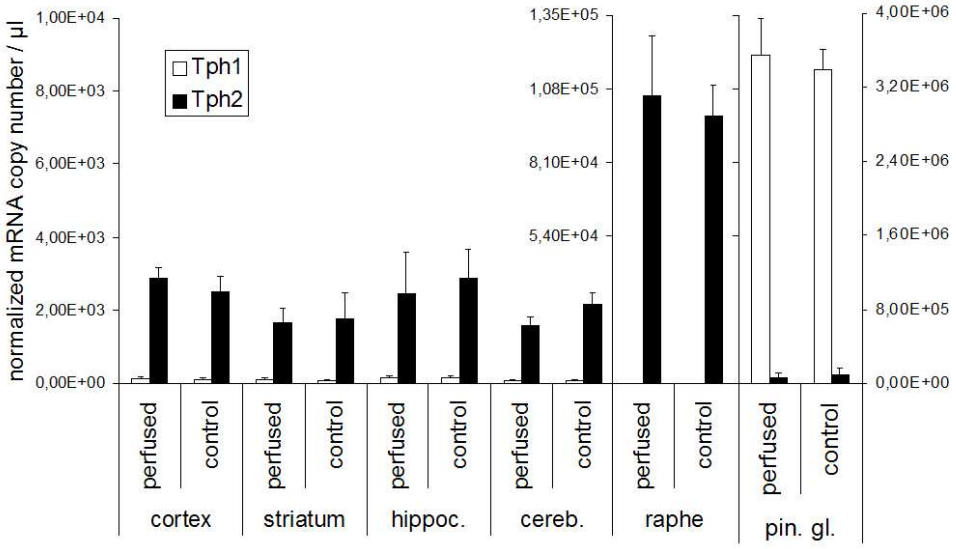


Fig. 3-1 Quantitative real time PCR of different murine brain regions and pineal gland.
Comparison of relative expression of murine Tph1 and Tph2 expressed as normalized mRNA copy numbers per µl existing in the investigated tissues. Calculated copy numbers were normalized by GeNorm (Vandesompele et al., 2002) with the two most stable housekeeping genes of four. Scales represent ***left:*** *values for cortex, striatum, hippocampus and cerebellum,* ***middle:*** *values for raphe region,* ***right:*** *values for pineal gland. qRT-PCR could not detect a difference between perfused tissues and controls. Highest expression of Tph2 was found in raphe region, whereas Tph1 expression only appears in pineal gland. Data are means ± SEM of six wildtype PBS-perfused brains or native controls (males (n = 3) and females (n = 3)), respectively.*

3.1.1.2 Tph1/2 expression in adult murine peripheral organs

To investigate the differential expression of Tph1 and Tph2 in other parts of the body, qRT-PCR was performed using different peripheral organs like liver, heart, lung, thymus, kidney, adrenal gland, spleen, stomach, small intestine, large intestine, and testis of four adult male WT C57/BL6 mice. For Tph1 or Tph2 mRNA, pineal gland or raphe containing region (brainstem), respectively, was used as a positive control (Fig. 3-2 A). ISH and IHC were performed on 16 µm sections of different peripheral organs, but unfortunately failed to result in a clear staining except for IHC on murine gut regions (Fig. 3-2 B). Fig. 3-2 A shows qRT-PCR results with, as expected, the highest expression level of Tph2 in the brainstem and the highest expression level of Tph1 in the pineal gland (right scale). Besides the positive controls, the highest expression of Tph2 was found in small intestine with lower levels detected in the other peripheral organs, whereas high Tph1 levels were seen in stomach and small and large

intestine, whereas low levels were observed in the thymus (left scale). In contrast to Tph2, Tph1 mRNA was not detectable in the other peripheral organs.

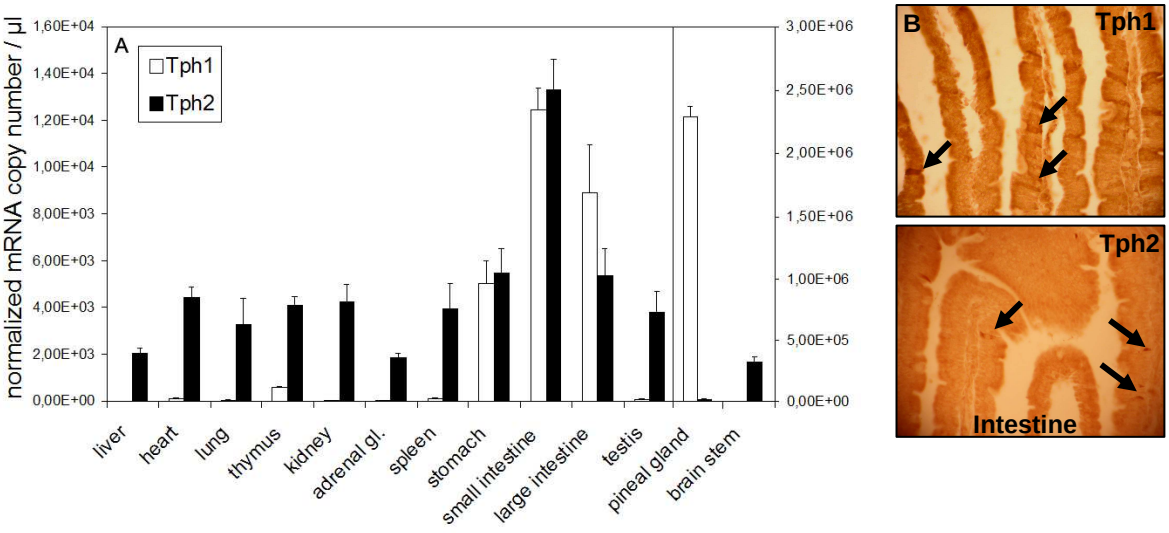


Fig. 3-2 Expression of Tph1 and Tph2 in different murine peripheral organs.
A: *Comparison of relative expression of murine Tph1 and Tph2 detected by qRT-PCR. Values are normalized mRNA copy numbers per µl. Calculated copy numbers were normalized by GeNorm (Vandesompele et al., 2002) using the two most stable housekeeping genes of four. Scales represent* ***left:*** *values of liver, heart, lung, thymus, kidney, adrenal gland, spleen, stomach, small intestine, large intestine and testis,* ***right:*** *values of pineal gland and brain stem as positive controls for Tph1 or Tph2, respectively. Besides the positive controls, highest expression of Tph2 was found in small intestine and at lower levels in the other peripheral organs, whereas high Tph1 levels were seen in stomach and small and large intestine, while low level were observed in thymus. Tph1 expression was not detectable in the other peripheral organs. Data are means ± SEM of four adult male wildtype mice.* ***B:*** *Immunohistochemistry with specific antibodies for the Tph1- or the Tph2 protein, respectively, on 16 µm murine intestine sections. Stained cells are marked by black arrows.*

3.1.1.3 Tph1/2 expression in developing mouse brain

qRT-PCR was used to investigate the expression of the two Tph isoforms in the CNS during development of the mouse brain. For this, whole brains of different developmental stages (two pups from different mothers per stage) were applied and carefully prepared so that pineal glands were removed. Twenty-one developmental stages from embryonic day (E)9.5 to postnatal day (P)23 and two adult stages (2 and 4 months) were investigated (Fig. 3-3). Figure 3-3 depicts whole brain Tph1 and Tph2 expression during development. The earliest detectable Tph2 expression appeared at E11.25, increased exponentially until the end of gestation and peaked at birth (P0.5). Intermediate to high Tph2 mRNA levels were maintained throughout postnatal development with the second highest level occurring around 3 weeks of age. qRT-PCR analyses of brains from young adults and during adulthood displayed slightly lower level of Tph2 expression. In comparison, qRT-PCR could not detect Tph1 expression during all stages of ontogeny and across adult life (Fig. 3-3).

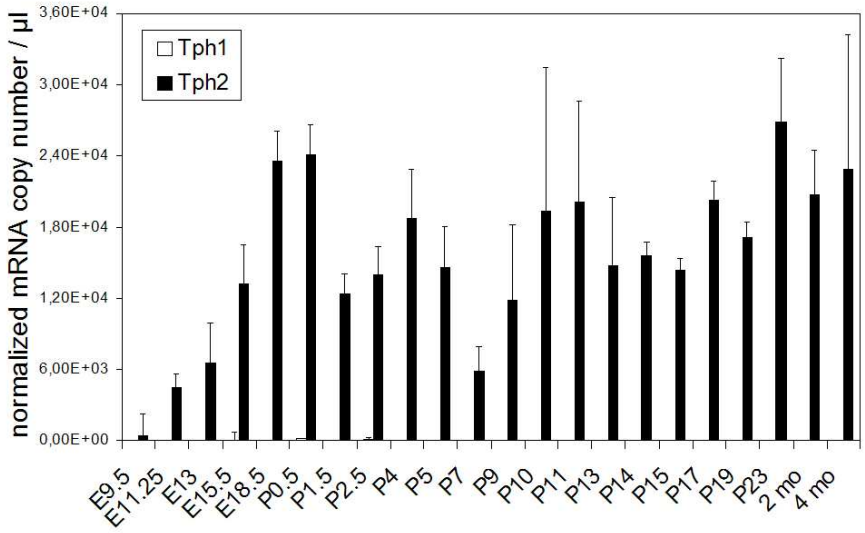


Fig. 3-3 Quantitative real time PCR of different developmental whole brains.
Comparison of relative expression of murine Tph1 and Tph2 shown by normalized mRNA copy numbers per µl existing in the investigated tissues. Calculated copy numbers were normalized by GeNorm (Vandesompele et al., 2002) with the two most stable housekeeping genes of four. Data are means ± SEM of whole brains of two investigated wildtype mouse pups from two different mothers. The earliest detectable Tph2 expression appeared at E11.25. During all stages of development and across adult life Tph1 expression levels are virtually not detectable in comparison to Tph2 levels. E = embryonic day, P = postnatal day, mo = months.

Furthermore, two different yet complementary histological methods, ISH and IHC, were used to detect Tph1 and Tph2 mRNA or protein, respectively, in the developing brain. Mouse brains were prepared so that the pineal gland remained attached to the brain serving as an internal positive control for Tph1 expression. Then relative expression of both Tph isoforms was cell-specifically visualized. Nine murine developmental stages, from E9.5 to P21, and adult mouse brains were analysed entirely. ISH and IHC were performed on whole brain slice series from different developmental stages using every seventh brain section. Since both pre- and post-translational levels of analysis yielded concordant results, they are presented in parallel in the following figures. Tph2 mRNA and protein were not detected in the CNS of E9.5 embryos (not shown) but for both a clear cellular signal was obtained beginning at E11.25 near the floor plate in the rostro-ventral part of the hindbrain (Fig. 3-4 A/a,c,d), where the first serotonergic neurons are known to differentiate, and were detected in the raphe nuclei at E13 and E15.5 (e.g. Fig. 3-4 B/a,d). Tph1 mRNA and protein were not detected in the brain at E9.5, E11.25 (Fig. 3-4 A/b,e), E13 or E15.5 but appeared for the first time in the periphery of the pineal gland in E15.5 embryos (Fig. 3-4 B/b,e), whereas Tph2 mRNA and -protein were not detected in the pineal body (e.g. E15.5, Fig. 3-4 B/c).

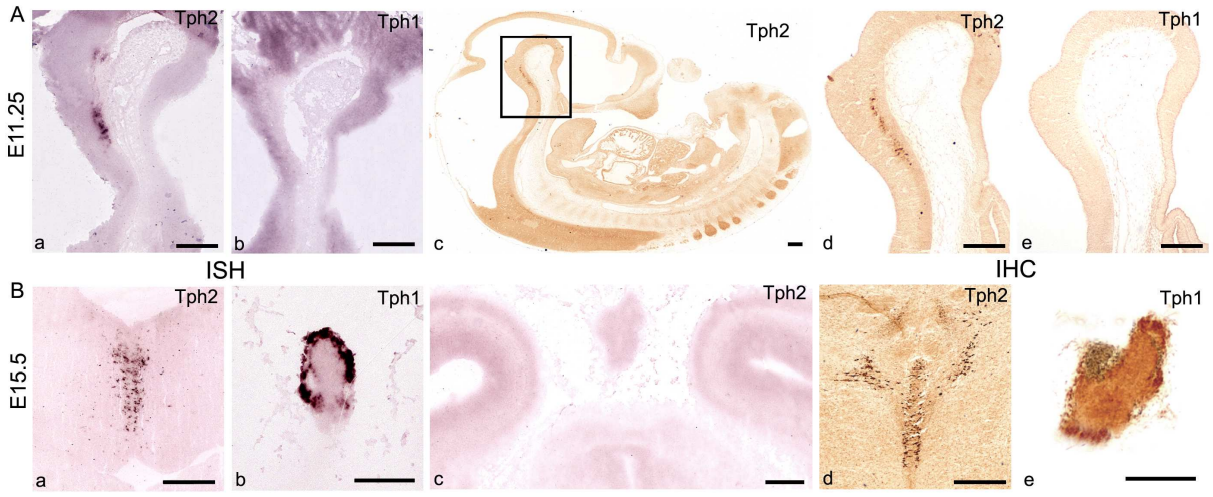


Fig. 3-4 In situ hybridization and immunohistochemistry of early developmental mouse slices.
Panel A: *Embryonic sections of an E11.25 old mouse embryo.* ***Panel B:*** *Embryonic sections of an E15.5 old mouse embryo. A/a,b and B/a,b,c representing ISH staining. A/c,d,e and B/d,e representing IHC staining. The first clear Tph2 signal of both ISH and IHC staining appeared at E11.25 near the floor plate in the hindbrain (A/a,c,d) whereas Tph1 ISH and IHC staining appeared first in the periphery of the pineal body at E15.5 (B/b,e) and was not detected in the early developmental brain (A/b,e). Scalebars are equivalent to 180 µm. E = embryonic day.*

In addition, Tph1 and Tph2 expression patterns obtained by ISH (panel A) and IHC (panel B) from embryonic stages to adulthood are shown in figures 3-5 and 3-6. Figure 3-5 displays the developmental stages E18.5, P0.5, P5, P10, P16 and one adult stage (2 months). Special attention was paid to the stage P21 (Fig. 3-6) to verify the unexpected results of Nakamura et al., i.e. that Tph1 is specifically expressed in the murine brain in late developmental stages like P21, and then disappearing again in adulthood (Nakamura et al., 2006). In none of the investigated developmental stages Tph1 mRNA or protein staining was observed, neither in the whole brains nor in the raphe nuclei (Fig. 3-5 A/a-f, B/a-f) in which was also true for P21 (Fig. 3-6 A/b, B/b). The reactivity and specificity of the Tph1 riboprobe and antibodies were proven by the strong signal obtained in the pineal gland, as shown in inserts in the respective figures (Fig. 3-5 A/a-f, B/a-f) and in P21 whole brain sections including the pineal gland (Fig. 3-6 A/b, B/b). Tph2 mRNA and -protein were found exclusively in the raphe region and positive cells showed the typical anatomic pattern consistent with raphe serotonergic cells' soma distribution from the most rostral central raphe nucleus to the most caudal obscurus raphe nucleus at all developmental stages and in adulthood (see Fig. 3-4 B/a,d; Fig. 3-5 A/g-l, B/g-l and Fig. 3-6 A/c, B/c). Tph2 protein positive fibres were observed along the floor of the aqueduct, above the corpus callosum, in the walls of the lateral ventricles, towards and within the olfactory bulb but also in isolated axons densely innervating different brain structures. Although Tph2 mRNA and protein were not detected in the cell soma belonging to the pineal gland complex, where in contrast Tph1 is strongly expressed, Tph2 protein positive

fibres were detected in the rostral part of the pineal complex and few fibres could also be seen in the very distal portion of the pineal stalk (data not shown). Tph2 protein localised in serotonergic axons presenting synaptic boutons and transversally cut axons can be seen at high magnification in figure 3-6 B/d. The specificity of the staining is underscored by the absence of signal in negative controls for both ISH and IHC when sense probe or preimmune serum were used, respectively (shown for P21, Fig. 3-6 A/a, B/a).

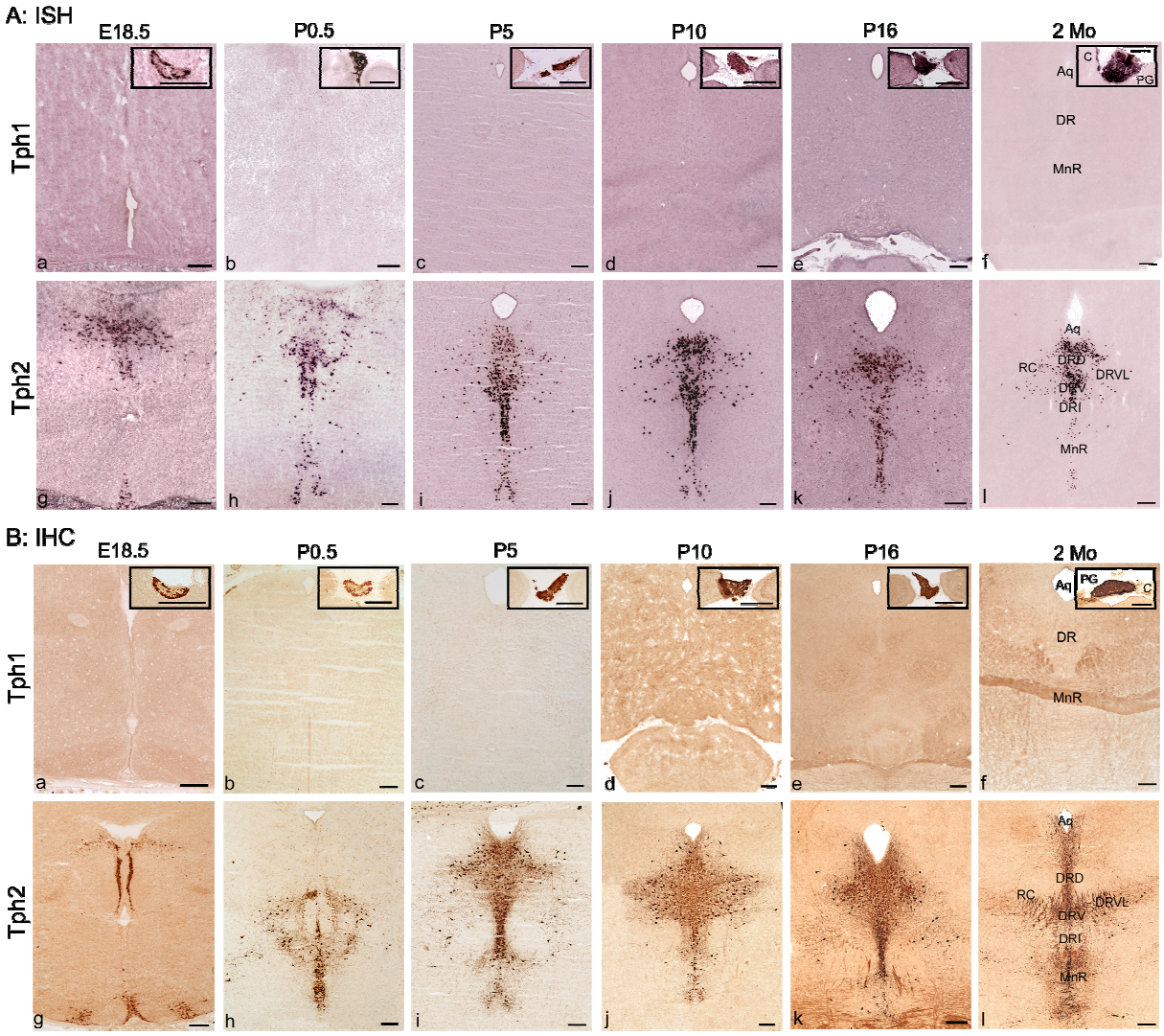


Fig. 3-5 In situ hybridization and immunohistochemistry of different developmental mouse brains.
Panel A: *In situ hybridization, the upper panel representing Tph1 staining, the lower panel Tph2 staining.* ***Panel B:*** *Immunohistochemistry, the upper panel represent Tph1 staining, the lower panel Tph2 staining. No staining of Tph1 mRNA (A/a-f) as well as Tph1 protein (B/a-f) was detected in all investigated brains, whereas the inserts of the respective pictures display a strong staining of Tph1 mRNA or -protein, respectively, in the pineal gland control region. However, Tph2 mRNA staining (A/g-j) as well as Tph2 protein staining (B/g-j) on comparable serial following brain slices exhibited the typical pattern of the raphe nuclei in the brain stem region. Aq = aqueduct, C = Cortex, DR = dorsal raphe nucleus, DRD = dorsal raphe nucleus dorsal, DRI = dorsal raphe nucleus inferior, DRV = dorsal raphe nucleus ventral, DRVL = dorsal raphe nucleus ventrolateral, MnR = median raphe nucleus, PG = pineal gland, RC = raphe cap. All scalebars represent 180 µm.*

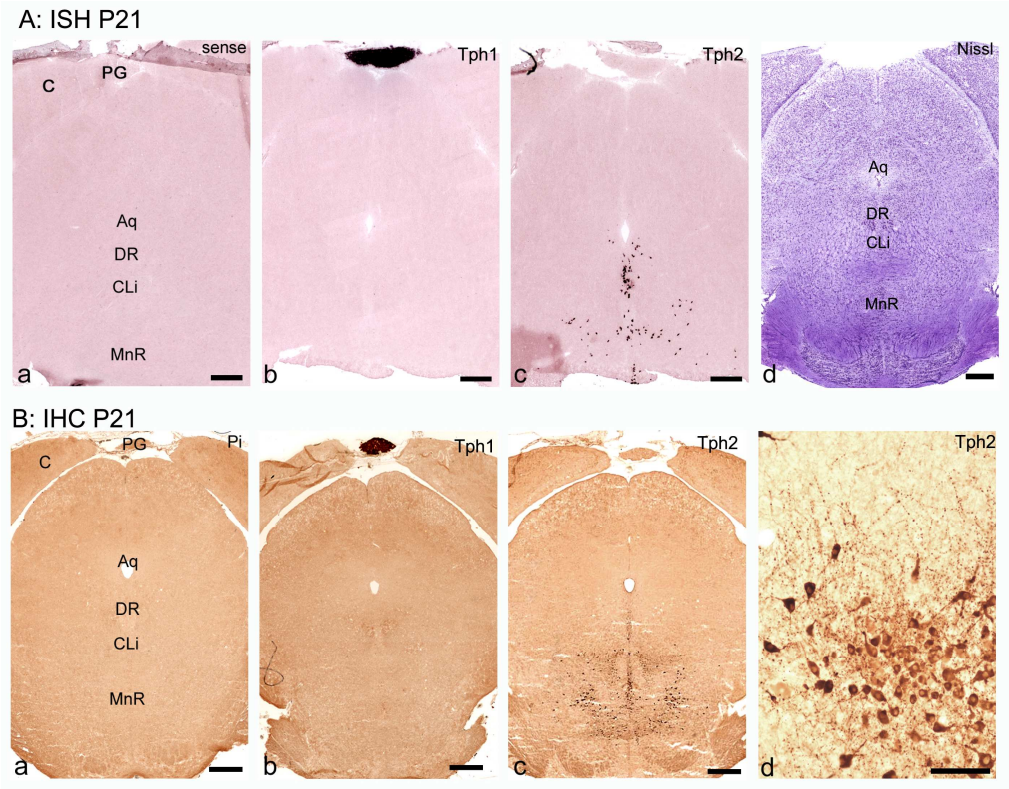


Fig. 3-6 In situ hybridization and immunohistochemistry of a P21 mouse brain
Panel A: *In situ hybridization.* ***Panel B:*** *Immunohistochemistry. Tph1 antisense-riboprobes as well as Tph1 antibodies show strong staining in pineal gland (A/b, B/b), where no Tph2 mRNA or -protein was detected (A/c, B/c). In contrast, Tph2 antisense-riboprobes as well as Tph2 antibodies stained cells in the typical pattern of the raphe nuclei (A/c, B/c), while Tph1-positive mRNA or protein cells are completely absent in this region (A/b, B/b). Negative controls for both in situ hybridization and immunohistochemistry validated the specific staining by the absence of signal when sense probe or preimmune serum (Pi) were used (A/a, B/a). Picture d of panel B shows Tph2 protein localised in serotonergic axons presenting synaptic boutons and transversally cut axons at high magnification (scalebar representing 50 µm). Picture d in panel A shows a nissl-stained coronal section of the brainstem including several different raphe nuclei. Aq = aqueduct, C = cortex, CLi = caudal linear raphe nucleus, DR = dorsal raphe nucleus, MnR = median raphe nucleus, PG = pineal gland. Scalebars are equivalent to 180 µm.*

3.1.1.4 Tissue-specific expression of Tph1/2 proteins investigated by Western blotting

Additionally to IHC in mouse tissues, Western blot analyses were performed in parallel providing further evidence for the reactivity and specificity of both the Tph1 and the Tph2 antibodies. Figure 3-7 shows Western blots of native proteins extracted from the frontal half of the brain, the raphe containing region, and the pineal gland after immunostaining with anti-Tph1 or anti-Tph2 antibodies. Anti-Tph1 antibodies did not bind to any protein isolated from the brain but strongly labelled a unique band in the pineal extracts, most likely representing Tph1. In contrast, the anti-Tph2 antibody labelled a unique band in the raphe region and in the frontal part of the brain yet less intensive in the latter, while no protein from the pineal glands was recognized. The band detected in the raphe nuclei likely represents the Tph2 isoform present in serotonergic neurons, whereas the less intensive band detected in the frontal part of the brain likely corresponds to Tph2 enzyme contained in serotonergic fibres spread all over the brain which were intensively stained in IHC

experiments. Based on the size marker ladder loaded in the first lane, Tph1- and Tph2 proteins were estimated to have a molecular weight of approximately 52 and 57 kDa, respectively.

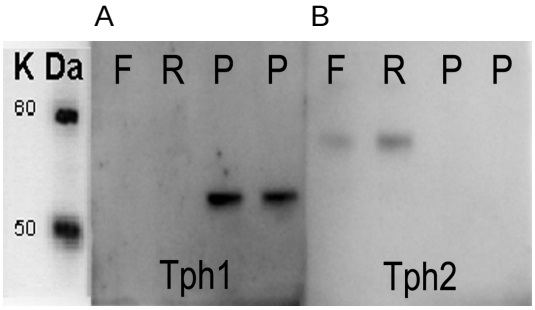


Fig. 3-7 Western blot analysis in murine tissues.
Panel A: *Tph1 antibodies.* ***Panel B:*** *Tph2 antibodies.*
F = frontal part of brain, R = raphe nuclei, P = pineal gland.

3.1.2 TPH1 and TPH2 expression in adult human tissues

qRT-PCR was performed on total RNA of different human brain regions like cortex, putamen, caudate nucleus, hippocampus, cerebellum, pons and medulla oblongata (total RNA obtained commercially) and one peripheral region (total RNA isolated from human jejunum) as positive control for human TPH1 expression. Highest expression levels of TPH2 mRNA were found in pons and medulla oblongata, which both contain several raphe nuclei (Fig. 3-8). In all other brain regions, considerably lower levels of TPH2 expression were detected. Besides the raphe nuclei, the highest TPH2 mRNA levels were found in cortex and hippocampus. All investigated brain regions, including raphe nuclei-containing regions, showed extremely low levels of TPH1 mRNA (Fig. 3-8) suggesting artifactual detection with no physiological relevance, whereas high levels were detected in jejunum. Interestingly, the latter also displayed quite high expression of TPH2. Furthermore, ISH and IHC were carried out on human brainstem sections as well as slices from human jejunum. Figure 3-9 displays representative tissue sections of human brainstem after ISH (Fig. 3-9 a, d) and IHC (Fig. 3-9 b, e). Similar results like in mice were obtained in the human raphe: TPH2 mRNA and protein were detected in cell bodies (Fig. 3-9 d, e) of the raphe nuclei, while no TPH1 mRNA or protein was detected in adjacent sections (Fig. 3-9 a, b) or when either TPH2 sense probe or preimmune serum were used (not shown). Control experiments performed in human jejunum revealed TPH1 protein in the epithelium (Fig. 3-9 c), consistent with enterochromaffin cells, while the use of preimmune serum as a negative control did not reveal any specific signal (Fig. 3-9 c'). Interestingly, TPH2 protein was as well detected in the jejunum epithelium (Fig. 3-9 f) corresponding most likely to enterochromaffin cells. These histological findings further confirm our results derived from qRT-PCR and demonstrate that both isoforms are expressed in human jejunum and strongly

support the notion that humans lack histologically detectable TPH1 expression in raphe serotonergic neurons.

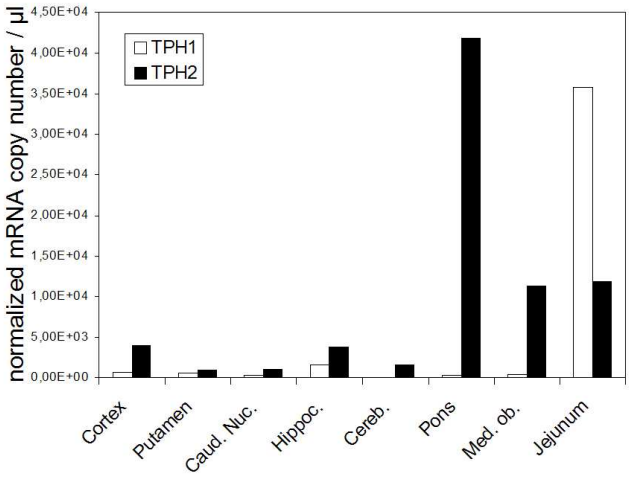


Fig. 3-8 Quantitative real time PCR of different human brain regions.
Comparison of relative expression of human TPH1 and TPH2 shown by the normalized mRNA copy number per µl. Calculated copy numbers were normalized by GeNorm (Vandesompele et al., 2002) using the two most stable housekeeping genes of four. TPH1 expression was detectable at high levels in the jejunum, whereas almost no expression was measurable in the brain except for the hippocampus in comparison to TPH2 expression levels. TPH2 expression was highest in pons and medulla oblongata, which include the raphe nuclei. Outside the raphe nuclei, the highest TPH2 mRNA levels were found in the cortex and hippocampus. Data are from one run with total RNA of different brain regions pooled from different human subjects and of total RNA of one human jejunum.

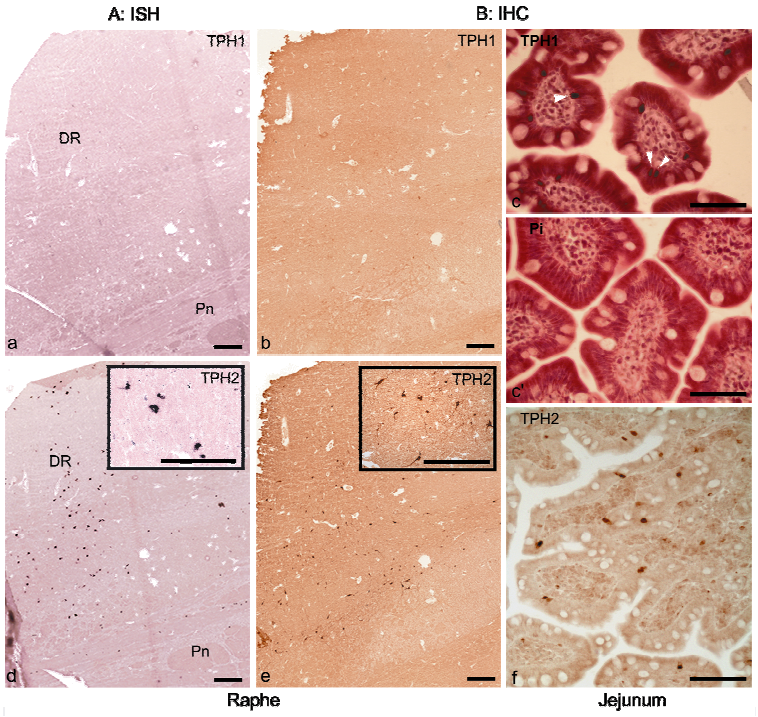


Fig. 3-9 TPH1 and TPH2 expression in the human CNS and in intestine.
Coronal sections of the human brainstem including raphe nuclei and of human jejunum. a, d illustrate in situ hybridization, b, c, e, f immunohistochemistry. No TPH1 mRNA or -protein positive cells were detected in the raphe nuclei (a, b), whereas TPH2 mRNA (d) and -protein (e) were detected along the midline of the human brainstem, where the raphe nuclei are located. Both TPH1 (c; white arrows) and TPH2 (f) antibodies detected positive cells in sections of the human jejunum. DR = dorsal raphe; Pn = pontine nuclei, Pi = preimmune serum. Scalebar is equivalent to 500 µm.

3.2 Phenotype of three different brain-specific Tph2 conditional knockout (cKO) models

Since our previous experiments confirmed that Tph2 is the relevant Tph-isoform in the murine brain, three different brain-specific Tph2 cKO models were further investigated. Tph2 cKO mice were generated by tissue-specific cre/loxP-mediated gene deletion in neuronal and glia cell precursors (Tph2 cKO^{Nes}) or only in serotonergic brain cells (Tph2 cKO^{Pet1} or Tph2 iKO). Moreover, Tph2 iKO embodies an inducible knockout (iKO) mouse providing time-specific Tph2 deletion, which allows to temporally switch off the *Tph2* gene, e.g. at the stage of adulthood. WT mice as well as mice expressing either cre-recombinase or the floxed Tph2 deletion (in no case both together) were used as controls. Controls were further differentiated in general controls (cKO controls) without any treatment and in controls for the inducible mice which likewise received tamoxifen treatment in order to compensate for the stress-induced changes by tamoxifen injection (iKO controls). To control for effective cre recombination in combination with *Tph2* gene deletion in the iKO, IHC studies were done with fluorescence double labelling for ß-galactosidase (green) and Tph2 (red) (Fig. 3-10). Therefore, controls (Pet1-creER line) and Tph2 iKO were crossbred with the ROSA26 cre reporter strain which possesses a lacZ transgene inserted into the rosa locus (expressed in every body cell) whose expression is dependent on cre-mediated recombination. Both controls and Tph2 iKO were treated five times with tamoxifen. Controls and Tph2 iKO showed positive cells in the typical pattern of serotonergic cells of the raphe nuclei (Fig. 3-10 a, b). Green labelled cells showed an effective double recombination of both ß-galactosidase and *Tph2* floxed gene. In contrast, double labelled cells demonstrated effective cre recombination for ß-galactosidase but not for *Tph2* floxed, in cells which then still express Tph2. Very low amounts of double labelled cells were detected in Tph2 iKO (Fig. 3-10 b, d). In comparison to controls, in the iKO mice most cells were either Tph2- or ß-gal-positive and the amount of Tph2-positive cells was reduced, demonstrating effective cre recombination in combination with *Tph2* gene deletion.

Furthermore, IHC studies were performed with specific Tph2 or Tph1 antibodies to verify the lack of Tph2 protein in the different cKOs in comparison to their respective controls and to investigate whether there are any compensatory effects leading to an increase of Tph1 expression in the brains of Tph2 cKO mice. Although each of the investigated Tph2 cKO models displayed a marked reduction of Tph2-positive cells in the raphe nuclei, the different models feature a variable decrease of Tph2 expression (Fig. 3-11 upper panel). Complete loss of Tph2 protein could only be shown in Tph2 cKO^{Pet1}. In contrast to Tph2 cKO^{Pet1}, which exhibited no Tph2-positive cells neither in the rostral nor the caudal raphe

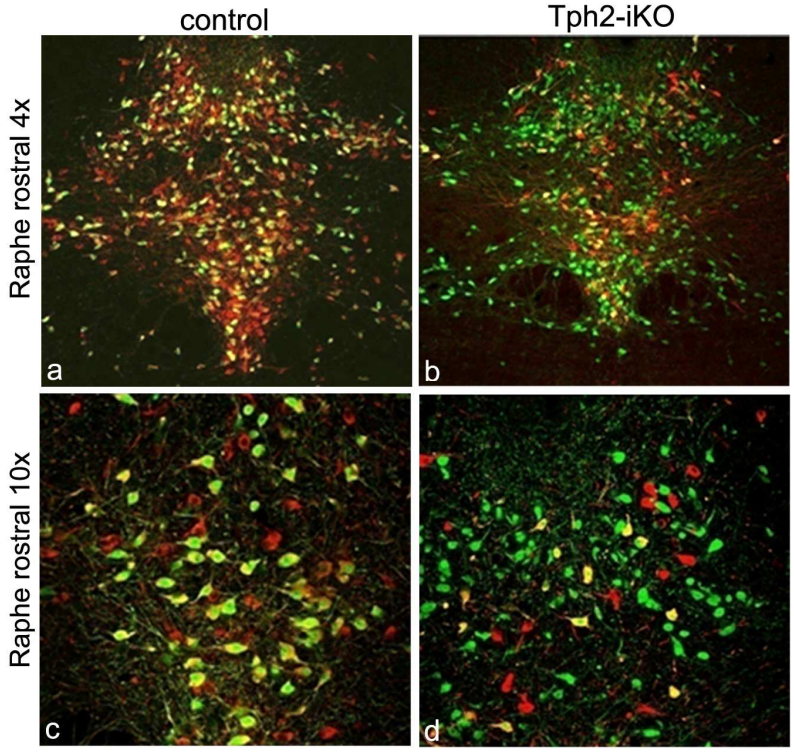


Fig. 3-10 ß-Galactosidase and Tph2 double-staining in the rostral raphe of Tph2 inducible knockout.
*Immunohistochemistry with specific ß-galactosidase (green) and Tph2 (red) antibodies after tamoxifen treatment. Tph2 inducible KOs (Tph2 iKO) and the Pet1-creER line (control) were crossbred with the ROSA26 cre reporter strain which possesses a lacZ transgene inserted into the rosa locus (expressed in every body cell) whose expression is dependent on cre-mediated recombination. Detection of green cells shows effective cre recombination of Tph2 floxed in the typical pattern of serotonergic cells of the raphe nuclei (**a, b**). Thus, double labelled cells demonstrate effective cre recombination for ß-galactosidase but not for Tph2-floxed in cells which still express Tph2 protein. Very low amount of double labelled cells was detected in Tph2 iKO (**b, d**) demonstrating the effective functioning of cre recombination in combination with Tph2 deletion. Pictures **c** and **d** illustrate cells in a higher magnification.*

nuclei (Fig. 3-11 f, l), Tph2 cKO^{Nes} presented remaining Tph2-positive cells mainly in the dorsal part of rostral raphe region (Fig. 3-11 e, k). A similar pattern of positive cells could be detected in Tph2 iKOs. Comparison of a five months old Tph2 iKO animal injected four times with a three months old animal injected five times showed significant differences of Tph2 expression in the rostral as well as the caudal raphe region (Fig. 3-11 c, i; d, j). The five times injected animal exhibited less Tph2-positive cells than the four times injected mouse in both the rostral and the caudal part of raphe region. Detection of remaining Tph2 expression in all Tph2 KOs, except for Tph2 cKO^{Pet1}, indicated that Tph2 cKO^{Nes} and Tph2 iKO strains represent more a knockdown model than a knockout model for Tph2, whereas Tph2 cKO^{Pet1} can be considered a brain-specific Tph2 absolute knockout. In addition, investigation of Tph1 expression in the different Tph2 KOs failed to detect any Tph1-positive cells in the rostral and the caudal (not shown) raphe region (Fig. 3-11 lower panel). Neither controls (Fig. 3-11 a, c, e) nor any of the investigated Tph2 cKOs (Fig. 3-11 b, d, f) exhibited Tph1 staining in the murine brain. However, the specificity of Tph1 antibodies was proven by strong staining in pineal gland (Fig. 3-11, small inserts of a, b).

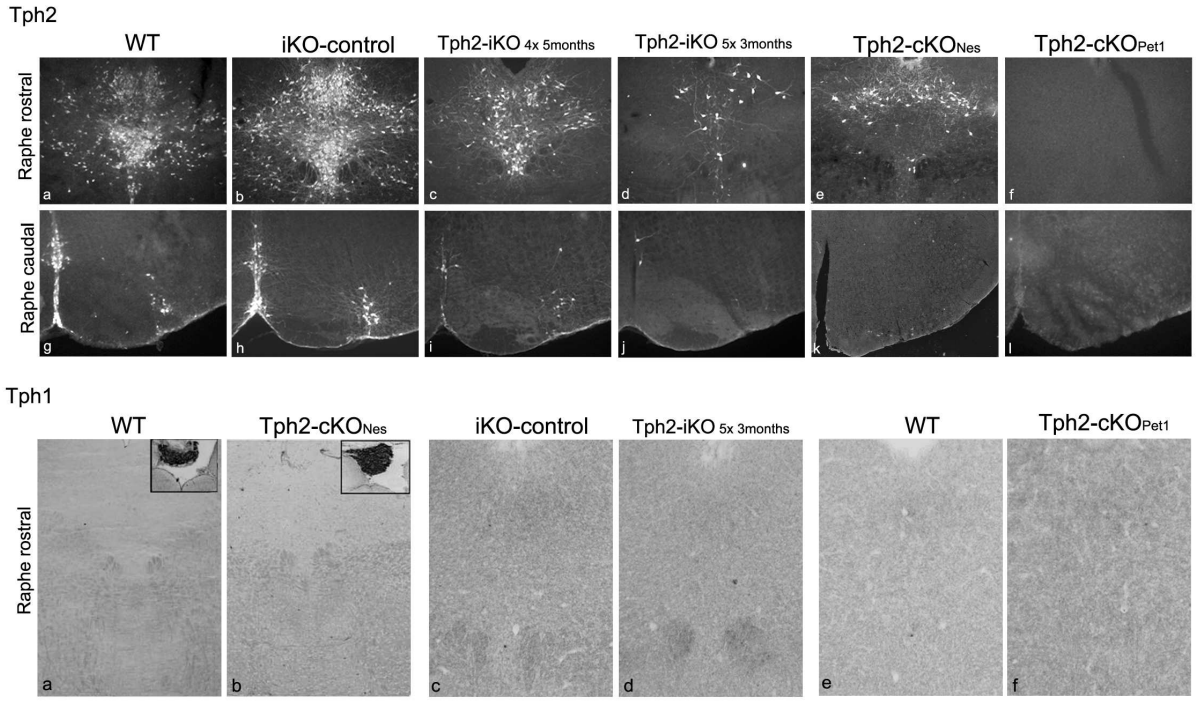


Fig. 3-11 Tph2 and Tph1 immunohistochemistry in different Tph2 knockout models vs. specific controls.
The upper panel *illustrates IHC with specific Tph2 antibodies in the rostral (a-f) and caudal (g-l) raphe region. Highest amounts of Tph2-positive cells were detected in wildtype (WT) and iKO control (a, g and b, h). The Tph2 iKO of five months age which was treated four times with tamoxifen also possessed a relative high amount of Tph2-positive cells, mainly in the dorsal part of the rostral raphe region (c) and likewise in the caudal raphe region (i), whereas a three months old Tph2 iKO which was injected five times showed less Tph2-positive cells in both raphe regions (d, j). In comparison to Tph2 cKO$_{Pet1}$ which exhibited no Tph2-positive cells neither in the rostral nor in the caudal raphe nuclei, Tph2 cKO$_{Nes}$ possessed Tph2-positive cells mainly in the dorsal part of rostral raphe region.* ***The lower panel*** *shows IHC with specific Tph1 antibodies in the rostral raphe region using WT (a and e) and injected WT (iKO-control) (c) for comparison with Tph2 cKO$_{Nes}$ (b) and Tph2 cKO$_{Pet1}$ (f) or a three months old Tph2 iKO which was injected five times (d). In none of the investigated groups, Tph1-positive cells were detected, indicating no compensatory upregulation of Tph1 expression in brains of Tph2 cKOs. Sections of pineal gland where used as positive control for Tph1 IHC (small inserts of a and b).*

In search for differences between the three different Tph2 deficient mouse models, qRT-PCR studies were done to detect expressional changes of seven genes specific for the serotonergic system: *5-HT$_{1a}$, 5-HT$_{1b}$, 5-HT$_{2a}$, 5-HT$_{2c}$ receptors, serotonin transporter (Sert), Tph2,* and *Tph1* in eight different brain regions of interest: frontal and prefrontal cortex (marked as cortex frontal), striatum, hippocampus, hypothalamus, amygdala, rostral and caudal raphe region, and the entorhinal cortex. qRT-PCR experiments were carried out on a 384-well plate with one single PCR run including all investigated groups (male/female: cKO-controls, iKO-controls, Tph2 iKO, Tph2 cKO^{Nes} and Tph2 cKO^{Pet1}). The quantity values (Q-values) from the PCR run were normalized with the two most stable of four different housekeeping genes. Since there were no significant differences between males and females, statistical evaluation concerning genotype differences were done on means of females and males to increase the power of the study. For statistical analyses, both the non-parametric Kruskal-Wallis test and the parametric ANOVA test were used (Statview program, SAS institute Inc., version 5.0)(table 3-1). If parametric and non-parametric tests yielded p-values smaller 0.05, groups were further analysed by using the accurate Fisher test.

Table 3-1. Statistical evaluation of serotonergic gene expression in different Tph2 cKO models.
Significant findings are highlighted

Gene of interest	Brain region	Kruskal-Wallis test	ANOVA test
5-HT$_{1a}$	Cortex frontal	p = 0.0713	p = 0.0689
	striatum	p = 0.9711	p = 0.9567
	hippocampus	**p = 0.0303**	**p = 0.0308**
	hypothalamus	p = 0.8731	p = 0.7657
	amygdala	p = 0.0235	p = 0.0891
	Raphe rostral	p = 0.1790	p = 0.2593
	Raphe caudal	p = 0.0539	p = 0.2664
	cortex entorhinal	p = 0.1533	p = 0.6409
5-HT$_{1b}$	Cortex frontal	p = 0.4493	p = 0.8349
	striatum	p = 0.5516	p = 0.5741
	hippocampus	p = 0.2206	p = 0.2124
	hypothalamus	p = 0.9331	p = 0.9399
	amygdala	p = 0.2569	p = 0.3502
	Raphe rostral	p = 0.5553	p = 0.8735
	Raphe caudal	p = 0.6837	p = 0.7068
	cortex entorhinal	p = 0.1369	p = 0.1166
5-HT$_{2a}$	Cortex frontal	p = 0.5083	p = 0.8895
	striatum	p = 0.7381	p = 0.6696
	hippocampus	p = 0.0842	p = 0.0784
	hypothalamus	p = 0.8682	p = 0.8441
	amygdala	p = 0.3668	p = 0.5794
	Raphe rostral	p = 0.7536	p = 0.9206
	Raphe caudal	p = 0.0909	p = 0.0696
	cortex entorhinal	p = 0.2844	p = 0.3131
5-HT$_{2c}$	Cortex frontal	p = 0.5691	p = 0.7867
	striatum	p = 0.1195	p = 0.0847
	hippocampus	p = 0.0716	p = 0.0658
	hypothalamus	p = 0.0679	p = 0.2672
5-HT$_{2c}$	amygdala	p = 0.1721	p = 0.2583
	raphe rostral	p = 0.1786	p = 0.0905
	raphe caudal	p = 0.0988	p = 0.1438
	cortex entorhinal	p = 0.4900	p = 0.1499
Sert	cortex frontal	p = 0.8379	p = 0.9240
	striatum	p = 0.7578	p = 0.7268
	hippocampus	p = 0.0657	p = 0.2717
	hypothalamus	p = 0.7756	p = 0.3120
	amygdala	p = 0.7449	p = 0.8106
	raphe rostral	p = 0.0962	p = 0.5100
	raphe caudal	p = 0.3226	p = 0.3848
	cortex entorhinal	p = 0.2223	p = 0.1571
Tph2	cortex frontal	p = 0.2051	p = 0.9869
	striatum	p = 0.8215	p = 0.5887
	hippocampus	p = 0.1804	p = 0.2305
	hypothalamus	p = 0.4365	p = 0.3944
	amygdala	p = 0.4258	p = 0.4349
	raphe rostral	**p < 0.0001**	**p < 0.0001**
	raphe caudal	**p < 0.0001**	**p < 0.0001**
	cortex entorhinal	p = 0.9511	p = 0.9919
Tph1	cortex frontal	p = 0.9535	p = 0.3982
	striatum	p = 0.4113	p = 0.3337
	hippocampus	p = 0.3068	p = 0.3265
	hypothalamus	p = 0.6301	p = 0.6539
	amygdala	p = 0.8112	p = 0.6302
	raphe rostral	p = 0.1446	p = 0.1447
	raphe caudal	p = 0.5575	p = 0.6209
	cortex entorhinal	p = 0.0713	p = 0.0575

Strong expression differences ($p < 0.001$) of Tph2 mRNA were detected in rostral and caudal raphe regions of the different genotypes confirming previous IHC results (Fig. 3-12, table 3-2). qRT-PCR as well shows residual Tph2 expression in all Tph2 cKOs except for Tph2 cKO^{Pet1} (Fig. 3-12, table 3-2 a). In accordance to IHC, residual Tph2 expression of approximately 20% indicated that Tph2 cKO^{Nes} and Tph2 iKO strains in fact represent a brain-specific knockdown model, whereas Tph2 cKO^{Pet1} can be considered a complete Tph2 knockout featuring very

low and probably non-relevant baseline expression detectable only by the highly sensitive qRT-PCR method (see also Fig. 3-13 F).

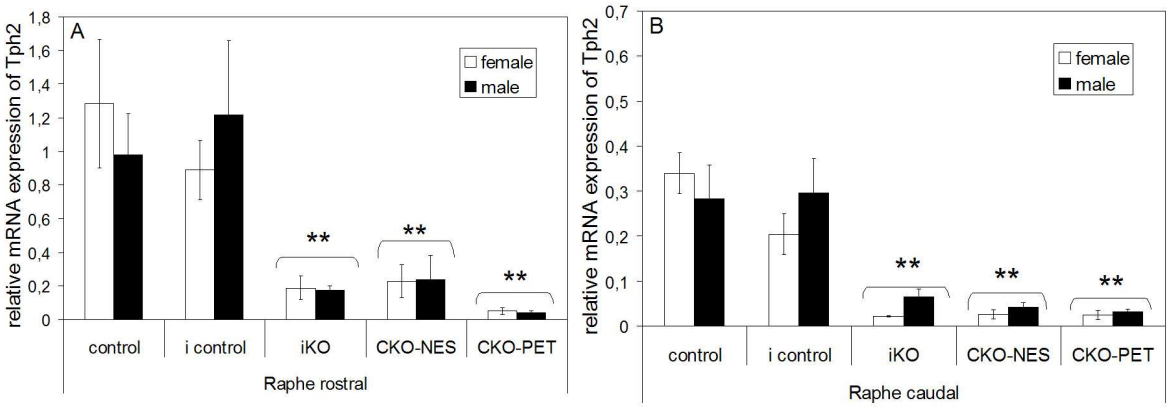


Fig. 3-12 Quantitative real time PCR of Tph2 on different Tph2 conditional knockout (cKO) models.
*Illustration of the relative mRNA expression of Tph2 in the rostral (**A**) and caudal (**B**) raphe region. Calculated quantity values (Q-values) were normalized by GeNorm (Vandesompele et al., 2002) with the two most stable housekeeping genes of four. Data are means ± SEM of 4-5 mice. * shows significant differences (** p≤0,0001) of both females and males of the particular genotype from their respective controls.*

Table 3-2 a) ***Percent of Tph2 mRNA reduction against respective controls:***

	raphe rostral	**raphe caudal**
Tph2 iKO	82,86 %	82,66 %
Tph2 cKO^{Nes}	79,43 %	89,06 %
Tph2 cKO^{Pet1}	96,01 %	90,83 %

Table 3-2 b) **P-values of the *Fisher test* between different genotypes:**
Significant findings are highlighted

Fisher-PLSD Effect: genotype	Tph2 in raphe rostral	Tph2 in raphe caudal	5-HT$_{1a}$ in hippocampus
cKO^{Pet1}, cKO-controls	**p < 0.0001**	**p < 0.0001**	**p = 0.0374**
cKO^{Nes}, cKO-controls	**p = 0.0001**	**p < 0.0001**	p = 0.0730
iKO^{Pet1}, iKO-controls	**p = 0.0023**	**p < 0.0001**	p = 0.0585
cKO^{Pet1}, iKO-controls	**p = 0.0002**	**p < 0.0001**	**p = 0.0070**
cKO^{Nes}, iKO-controls	**p = 0.0013**	**p < 0.0001**	**p = 0.0151**
iKO^{Pet1}, cKO-controls	**p = 0.0003**	**p < 0.0001**	p = 0.2013
cKO^{Pet1}, cKO^{Nes}	p = 0.3984	p = 0.8757	p = 0.7896
cKO^{Pet1}, iKO^{Pet1}	p = 0.5984	p = 0.6166	p = 0.5978
cKO^{Nes}, iKO^{Pet1}	p = 0.8200	p = 0.7179	p = 0.7693
cKO-controls, iKO-controls	p = 0.5645	p = 0.1217	p = 0.4265

Again confirming IHC data, Tph1 expression in the brain was virtually not detectable in comparison to specific Tph1-control tissues like small and large intestine and pineal gland (Fig. 3-13 G/H). In addition, Tph1 expression values in the different brain regions of all genotypes was negligible as compared to Tph2, suggesting no physiologically relevant Tph1 expression in Tph2 cKOs. With respect to the other investigated genes, significant changes of the expression between different genotypes could only be detected for 5-HT_{1a} receptor expression in the hippocampus. However, further refined statistical comparison between Tph2 cKOs and their corresponding controls yielded only a trends or borderline significant p-values (cKO^{Pet1} p = 0,0374; cKO^{Nes} p = 0,0730; iKO^{Pet1} p = 0,0585; table 3-2). Thus, the expression of 5-HT_{1a} receptor in hippocampus as measured by qRT-PCR seems as well not importantly altered in the different Tph2 cKO models.

Although no significant changes between the different genotypes could be detected in different genes of the serotonergic system, general expression differences between the different brain regions could be seen independently from genotype. The highest amount of mRNA was measured for the 5-HT_{1a} receptor in the hippocampus with the lowest amount in the striatum (Fig. 3-13 A), whereas the opposite applies for the 5-HT_{1b} receptor (Fig. 3-13 B). For the 5-HT_{2a} receptor, the most intense expression was detected in the frontal cortex which in turn expressed the lowest amount of 5-HT_{2c} receptor. The lowest level of 5-HT_{2a}-mRNA was detected in the entorhinal cortex (Fig. 3-13 C). 5-HT_{2c} receptor mRNA expression was slightly higher in raphe nuclei containing regions than in the other brain regions (Fig. 3-13 D). Serotonin transporter mRNA could only be detected in raphe nuclei and also showed no significant difference between the distinct genotypes (Fig. 3-13 E).

3.3 The effect of acute stress on the expression of Tph1/2 and oxytocin in wildtype and serotonin transporter (Sert) KO mice

3.3.1 Tph1/2 expression of wildtype and Sert KO mice in relation to acute stress

To investigate whether there is an effect of acute stress on the expression of Tph1 and/or Tph2 in the murine raphe region, adult female and male WT C57/BL6 as well as mice lacking the serotonin transporter (Sert KO mice) were exposed to 1 hour of immobilization stress and immediately euthanized by cervical dislocation afterwards. QRT- PCR was performed on cDNA isolated from raphe nuclei and using a 384-well plate. One PCR run included all four

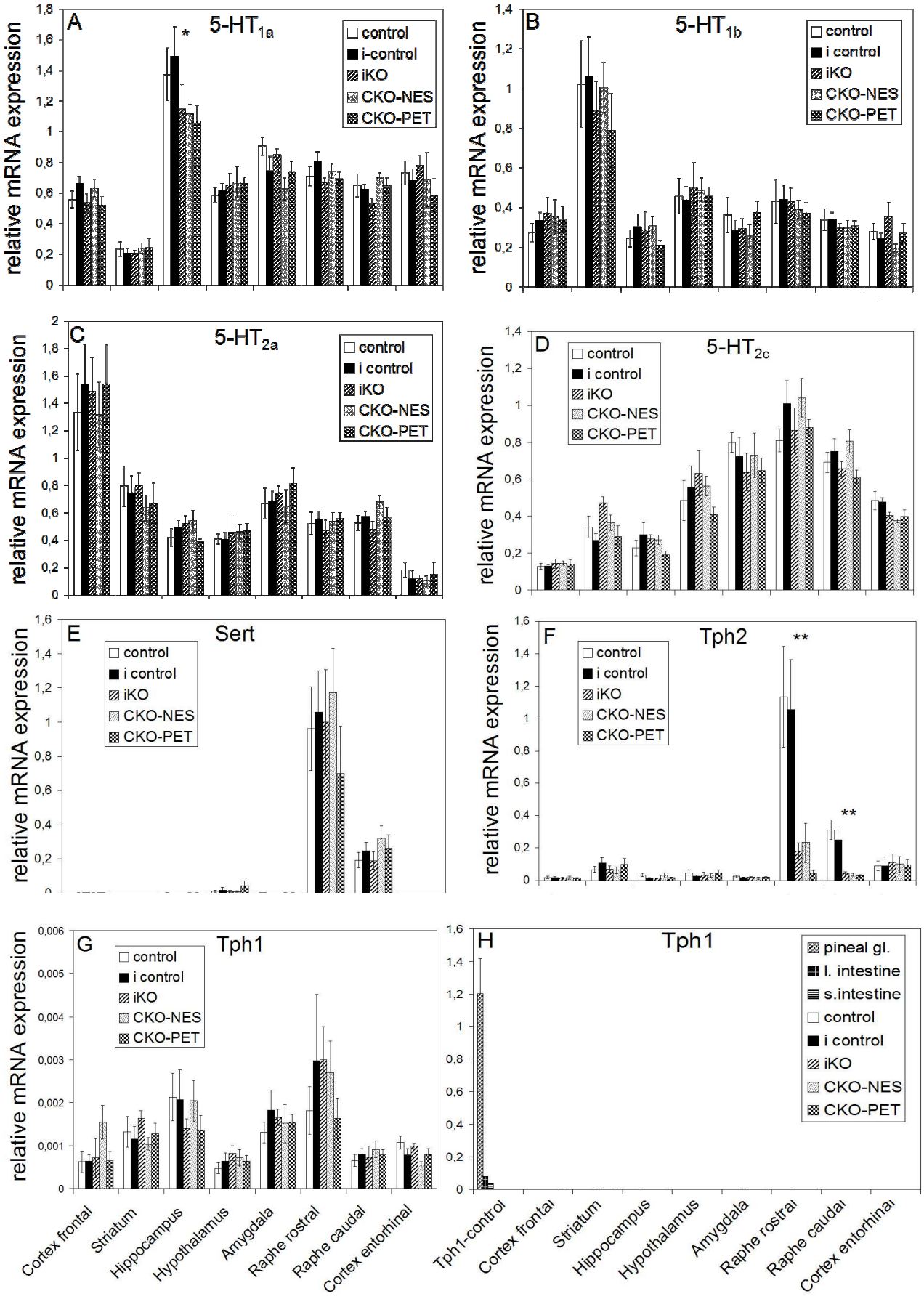


Fig. 3-13 Quantitative real time PCR on distinct brain regions of different Tph2 conditional knockout (cKO) models. *Illustration of the relative mRNA expression of 5-HT$_{1a}$ (**A**), 5-HT$_{1b}$ (**B**), 5-HT$_{2a}$ (**C**), 5-HT$_{2c}$ receptor (**D**), serotonin transporter (Sert) (**E**), Tph2 (**F**), and Tph1 (**G** and **H**) in different murine brain regions. Calculated quantity values were normalized by GeNorm (Vandesompele et al., 2002) with the two most stable housekeeping genes of four. Data are means ± SEM of 6-10 mice. * shows significant differences (** $p \leq 0{,}0001$) of the particular genotype from their respective controls.*

investigated groups (WT stressed/unstressed, Sert KO stressed/unstressed) with up to 10 mice per group for both females and males (see chapter 2.1.2). Q-values calculated from the qRT-PCR values were normalized with the two most stable of four housekeeping genes resulting in relative mRNA values for the respective genes. Relative values were then statistically evaluated using the non-parametric Mann-Whitney test (Statview program, SAS institute Inc., version 5.0). No differences were observed of the relative mRNA expression of neither Tph2 nor Tph1 between genders, genotypes as well as the different treatments i.e. acute stress exposure versus the respective controls in the raphe nuclei region (see Fig. 3-10 A, B). In figure 3-10 C the means of females and males is displayed showing that Tph1 mRNA is not detectable in the brain as compared to Tph2 in all animals (Fig. 3-10 C).

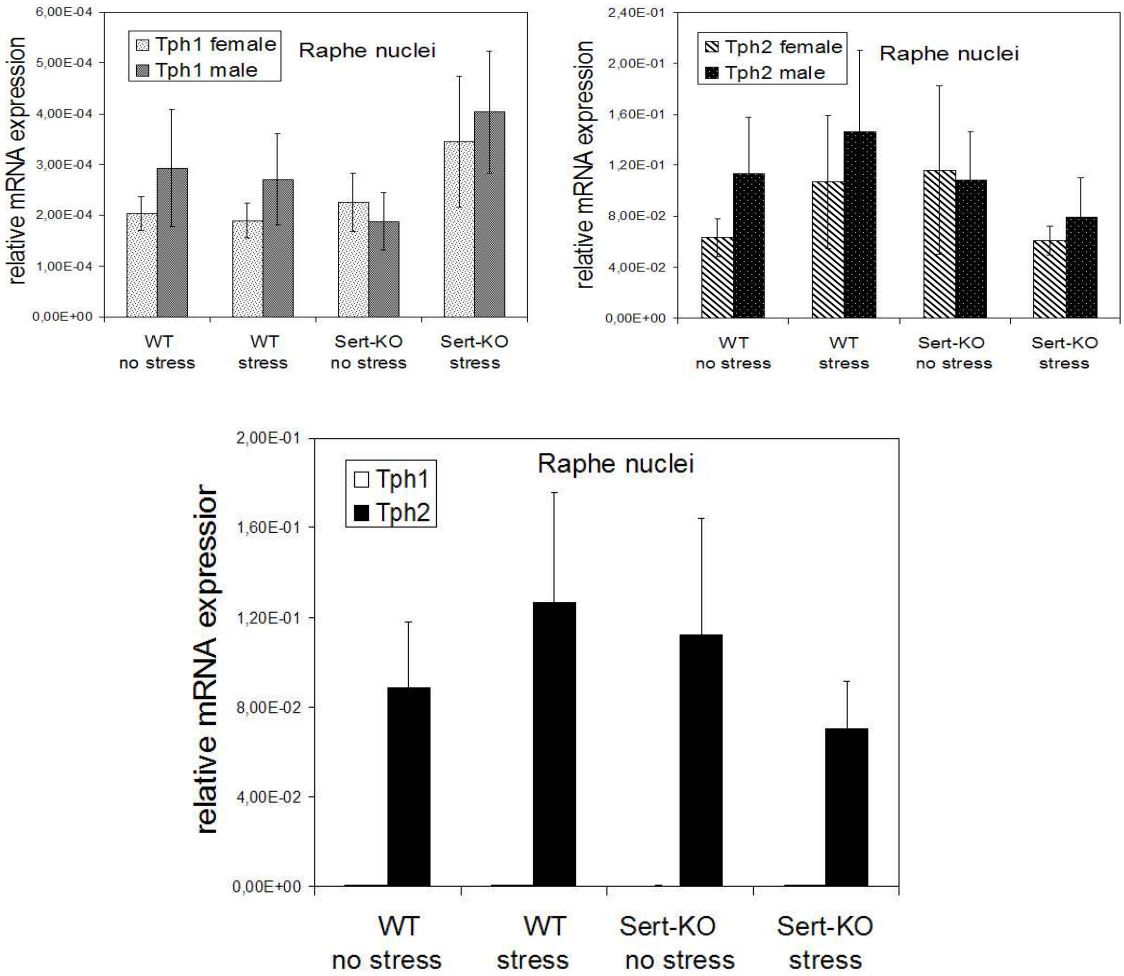


Fig. 3-10 Quantitative real time PCR of adult murine raphe region in relation to gender, stress and the serotonin transporter. *Comparison of relative mRNA expression of Tph1 and Tph2 shown by normalized quantities in the raphe region of wildtype (WT) or serotonin transporter knockout (Sert KO) mice exposed to 1 hour of immobilization stress. Calculated values were normalized by GeNorm (Vandesompele et al., 2002) with the two most stable housekeeping genes of four. No statistical significant differences in the relative expression of Tph1 mRNA (**A**) and Tph2 mRNA (**B**) between gender, genotype or stress exposure were detected. Data are means ± SEM of 8-10 mice. In comparison to Tph2 mRNA levels, Tph1 mRNA levels were virtually not detectable (**C**). Fig. C shows the mean of the relative expression values of females and males (n =14-18). The statistical test used was the Mann-Whitney test (Statview program, SAS institute Inc., version 5.0).*

3.3.2 Oxytocin expression in adult murine brain regions in relation to gender, stress and the Sert

In this study, we investigated the expression levels of oxytocin in different brain regions of interest (cortex, hippocampus, amygdala, hypothalamus, raphe nuclei) from female and male WT and Sert KO mice with or without exposure to 1 h of acute immobilization stress. qRT-PCR studies were performed in an all-in-one-run, which allows to test all groups at the exact using identical PCR conditions. After normalization of the quantity values with the two most stable of four different housekeeping genes and subsequent statistical evaluation (Statview program, SAS institute Inc., version 5.0), significantly higher expression levels of oxytocin in brain regions which are involved in the processing of emotional stimuli (amygdala, hippocampus) were detected in stressed male WT mice, whereas male Sert KOs as well as female WT and Sert KOs lack these stress-induced changes (Fig. 3-14 A/B). Both the non-parametric Mann-Whitney test and the parametric ANOVA test yielded p-values below 0.05 (see table 3-3).

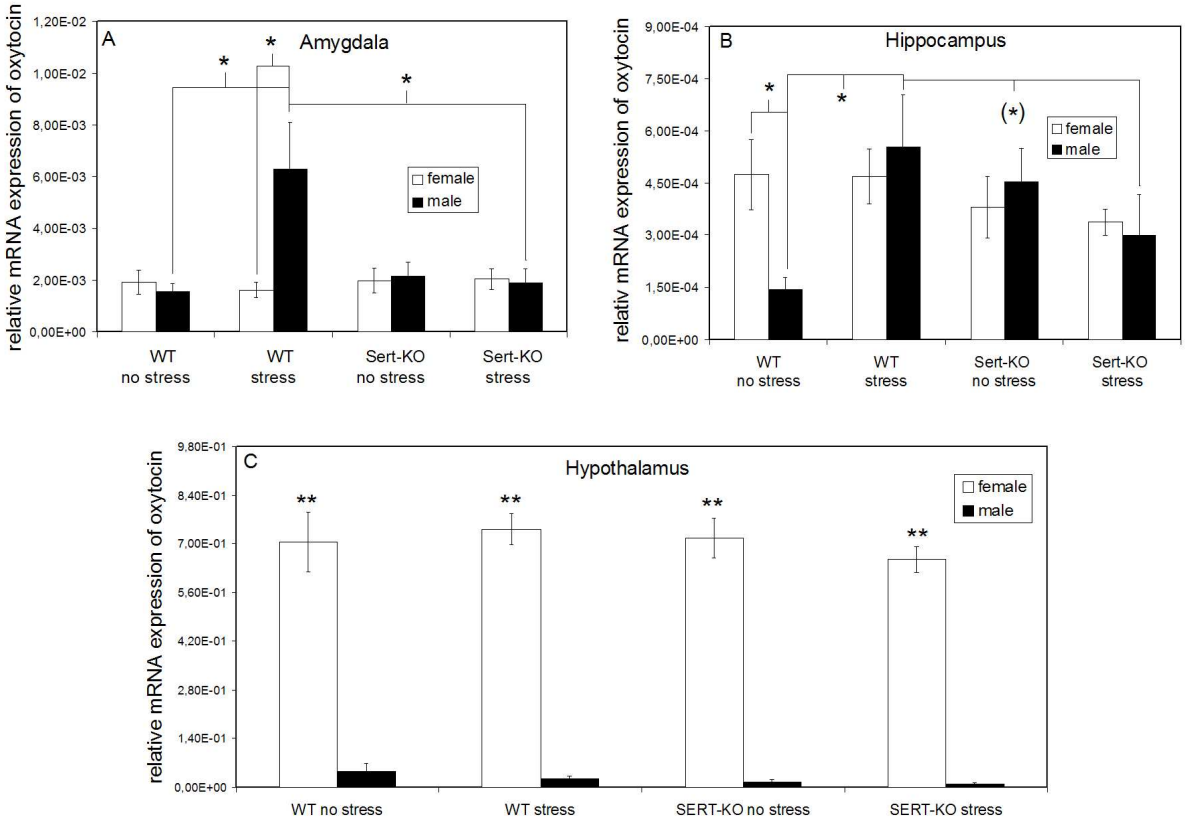


Fig. 3-14 Relative oxytocin expression after acute stress exposure measured by qRT-PCR in different murine brain regions. *Comparison of oxytocin expression in wildtype (WT) and serotonin transporter knockout (Sert KO) mice with and without acute stress exposure shown by relative mRNA expression levels in amygdala (**A**), hippocampus (**B**) and hypothalamus (**C**). Calculated values were normalized by GeNorm (Vandesompele et al., 2002) with the two most stable housekeeping genes of four. Stressed male WT mice showed significant higher expression levels of oxytocin in the amygdala (**A**) and the hippocampus (**B**), whereas male Sert KO as well as female WT and Sert KO mice lack these stress-induced changes. A significant higher oxytocin expression in females compared to males was detected in the hypothalamus (**C**), which is known to contain the majority of oxytocinergic neurons. Data are means ± SEM of 8-10 mice. Statistical tests were Mann-Whitney, ANOVA and Fisher (Statview program, SAS institute Inc., version 5.0).* shows significance (** p<0,00001), (*) shows tendency.*

In contrast to the significant gender- as well as genotype-dependent differences in oxytocin expression in the hippocampus and the amygdala following acute stress exposure (see table 3-3), all other investigated brain regions did not possess any significant alteration of oxytocin expression between the different groups (data not shown). However, there was a significant increased oxytocin expression in females as compared to males independent of genotype in the hypothalamus, the brain region which is known to contain the majority of oxytocinergic neurons (Fig. 3-14 C).

Table 3-3. **Oxytocin mRNA expression as assessed by qRT-PCR and analysed using the Fisher test:**

Significant findings are highlighted

Fisher-PLSD

	amygdala	hippocampus
stressed male Sert KO vs. WT	**p = 0.0121**	p = 0.0826
male WT stressed vs. control	**p = 0.0190**	**p = 0.0285**
stressed WT males vs. females	**p = 0.0074**	p = 0.6327
untreated WT males vs. females	p = 0.2411	**p = 0.0071**

4 DISCUSSION

4.1 Spatio-temporal expression of TPH isoforms

Given the discrepancies in recent literature regarding the expression of tryptophan hydroxylase (TPH) isoforms as well as the relevance of this topic for the field of neuropsychiatric research, a systematic spatio-temporal analysis of TPH1 and TPH2 expression was performed to clarify this issue. For the animal models, care was taken in investigating mice of the same background strain as there is evidence for the presence of a proline-to-arginine mutation of Tph2 in some mouse strains, which is accompanied by a two-fold lower activity of Tph2 enzyme and substantially lower levels of 5-HT in the brain (Zhang et al., 2004). Furthermore, mice exhibiting the arginine-form of Tph2 have less 5-HT in medial prefrontal cortex and dorsal hippocampus dialysate (Calcagno et al., 2007), and changed aggressive behaviour (Kulikov et al., 2005) as well as altered SSRI responsiveness (Cervo et al., 2005; Invernizzi, 2007). Hence, in this study mice were used with either a pure C57/BL6 or a mixed C57/BL6, 129/SvEMS and FVB/N strain background, strains, which all possess the proline variant of the Tph2 enzyme.

Four different methods (quantitative real-time (qRT)-PCR, *in situ* hybridization (ISH), immunohistochemistry (IHC), and Western blot analysis) were carried out during pre- and postnatal development and adulthood of mice as well as in adult human tissues to systematically investigate temporal, tissue-, species- and isoform-specific expression differences both on the pre- and the posttranslational level. To confirm data on mRNA expression, the highly sensitive qRT-PCR method which provides quantitative results was complemented by data from ISH which yields better spatial resolution at the disadvantage of a lower level of sensitivity. These date supplement previous studies from (Clark et al., 2006) which reveal differences in the density of expression of Tph2 in different subnuclei of the dorsal raphe nucleus (DRN) as well as studies from (Malek et al., 2005) showing varying Tph2 mRNA levels within both median and dorsal raphe nuclei.

The high sensitivity of PCR-based methods in quantifying gene expression requires extreme caution in the design of the assay and the normalization procedure. Therefore, the guidelines provided by Vandesompele, who recommended to control the nucleic acid quantity and quality at each step, to use several reference genes, and to apply an adapted algorithm (GeNorm) to select only the most consistently expressed reference genes across samples for the final data analyses (Vandesompele et al., 2002) were strictly followed. Similarly, to confirm

data on the protein level, both IHC and the binding of isoform-specific antibodies generated considering internal characteristics of the respective protein were validated by Western blot analysis providing quantitative results and additionally controlling for reactivity and specifity of antibodies. In general, to ensure specific binding and functioning of primers, probes, and antibodies, both negative controls referring to the detection method itself and positive controls using tissue with known TPH1- or TPH2 expression, respectively, were investigated in every set of experiments. The sensitivity of our procedure to detect TPH1 mRNA in mouse and man is underscored by the high expression level found in the control tissues, i.e. pineal gland and jejunum. Taken together, Western blot and IHC experiments not only ensure that the antibodies developed for this study are able to discriminate among the two isoforms of TPH but also exclude cross-reactivity with other enzymes belonging to the same protein family such as tyrosine and phenylalanine hydroxylases by the lack of any significant staining of neural cells apart from serotonergic neurons. The amino acid sequences selected for the generation of anti-Tph2 antibodies are identical in several species and thus have the potential to react with both mouse and human proteins. The positive control experiment in human jejunum demonstrates that they are able to detect the human protein (Fig. 3-9 f).

Based on findings from several studies, there is little doubt that TPH2 is abundantly expressed in the raphe nuclei. However, its expression in peripheral organs including the pineal gland is far from being well-defined. Additionally, data are equivocal regarding the presence of TPH1 in the brain, as it was reported to be detectable at low levels in mouse brains (Gundlah et al., 2005), rat brains (Patel et al., 2004; Abumaria et al., 2008) and human brain (Zill et al., 2007) by some groups, while others reported exclusive expression in the periphery including the pineal gland (Côté et al., 2003; Walther and Bader, 2003). Interestingly, one group (Nakamura et al., 2006) found Tph1 expression in mouse brains at P21, whereas Tph1 mRNA was not detected at P7 and in adults indicating a transient expression increase of Tph1 mRNA during the late brain development. Although this might provide an explanation for the association of *TPH1* variants with neuropsychiatric conditions including suicidal behaviour found in some studies, our data do however not support this notion as we could not detect *Tph1* expression in the raphe at any time point during development.

4.1.1 Localisation of TPH2 expression

The results of the present work clarify the tissue-specific expression of both TPH isoforms and their putative expression changes during early and late brain development. As expected, TPH2 mRNA and protein could be demonstrated in the brain during all developmental stages abundantly in the raphe containing regions of WT mice and of adult humans showing no

physiologically relevant expression in other brain regions. Although TPH2 mRNA was not detected in the human peripheral tissues heart, liver, lung, duodenum, adrenal gland and kidney by (Zill et al., 2004), we found Tph2 expression in the intestine as evidenced by qRT-PCR and ICH (Fig. 3-2) and, at lower levels, in several other peripheral organs of the mouse. In addition, Tph2 expression was magnitudes higher as Tph1 expression in all peripheral organs except for samples from the gastrointestinal tract (intestine and stomach; shown by qRT-PCR, Fig. 3-2). Despite the fact that Tph2 was termed the neuronal isoform, detected previously in brain and myenteric plexus neurons by ISH (Walther et al., 2003; Côté et al., 2007), our IHC and qRT-PCR experiments revealed an hitherto unknown expression of TPH2 in peripheral murine organs as well as in the epithelium of human jejunum (Fig. 3-9 f). Even though Tph2 mRNA is sparsely expressed in the pineal gland (only detectable with qRT-PCR, yet not with ISH) and no Tph2 protein was detected in the soma of pinealocytes, Tph2-positive fibres were detected in the deep pineal gland, but not in its superficial part (data not shown). The strong innervation of the habenular nuclei and the presence of Tph2-positive fibres projecting along the habenular and posterior commissure suggest that axons originating in the raphe are likely to enter the pineal gland via this pathway. These findings are in accordance with findings from Sugden and associates (Sugden, 2003) who reported Tph2 expression within the pineal gland, showing (in contrast to Tph1) no significant diurnal variation indicating Tph2 expression in the small proportion of "non-pinealocyte" cells. Neuronal projections from the dorsal raphe to the pineal complex were previously described in rats by neuronal tracing (Moller and Hay-Schmidt, 1998). It is also possible that raphe serotonergic neurons receive direct or indirect input from the pineal gland via reciprocal connection of the two structures.

4.1.2 Localisation of TPH1 expression

In contrast to TPH2, expression analysis targeting TPH1 mRNA and protein did not reveal any physiologically relevant expression in the brain neither in adult C57/BL6 mice, housed under regular conditions, nor in human brain. This was true for any stage of embryonal and postnatal development. The extremely low TPH1 mRNA concentration detected in the brain by the highly sensitive qRT-PCR in adult human brains (Fig. 3-8) most likely represents artifactual traces of expression without physiological relevance, since TPH1 values in brain regions are several magnitudes smaller as TPH2 brain values or values of the TPH1 control samples. Conversely, TPH1 mRNA and protein is intensely expressed in tissues from the gastrointestinal tract in both murine and human tissue and highly intense in samples from pineal glands. No difference was found between mouse brains with and without blood components, which allows us to infer that the level of TPH1 mRNA detected in the brain in

other studies using PCR-based methods cannot readily be explained by the presence of non-neural circulating blood cells remaining in the blood vessels and expressing TPH1.

4.1.3 Tph1/2 expression during development

During brain development, Tph2 expression started from E11.25 on, increased until the end of gestation and peaked at birth (P0.5) with the second highest level occurring around P21. Young and older adults seemed to display slightly lower level of Tph*2* expression (qRT-PCR, Fig 3). In accordance with the findings by Cote (Côté et al., 2007) in mice, Tph2-positive neurons in the brain of E9.5 embryos could not be detected, but already a considerable number of neurons at E11.25 expressed Tph2 which indicates their rapid proliferation and suggesting differentiation processes following initial Tph2 expression around E10–10.5. Neither Tph2 mRNA nor Tph2 protein expression could be detected in peripheral organs and in the pineal body of whole-body slices from embryos of E11.25 to E.15.5 (as shown by ISH and IHC). However, there is evidence that the density of Tph2-positive fibre tracks in the pineal stalk varies during postnatal development, since they first appear at birth, increase in number during the subsequent postnatal stages, and regress before P21 where, as in adults, fewer fibres were detected (IHC data, not shown). Although definitely no Tph1 expression was detected in the brain of any of the developmental stages, it could be observed in the periphery of the pineal body from E15.5 on (Fig. 3-4), thence in the pineal gland at every phase of development. Slices from adult murine intestine furthermore expressed Tph1 protein as well as Tph2 protein, which was shown by IHC (Fig. 3-2 B). Hence, these findings have implications for the interpretation of data derived from previously published studies on the role of the two TPH isoforms in the etiopathogenesis of a wide variety of neuropsychiatric disorders and provide a framework for further studies on how differential expression of TPH isoforms is related to their intrinsic physiological roles during brain development and function.

4.1.4 TPH2 protein activity in axons and dendrites

TPH2 mRNA could be shown in high quantities in the soma of serotonergic neurons yet was lacking in fibres of the serotonergic tract (as shown by ISH), whereas positive staining of TPH2 protein representing branching of serotonergic fibres could be observed in brain regions adjacent to the raphe nuclei , suggesting that most of the TPH2 protein is assembled in the raphe nuclei and then transported along the axon to diverse projection areas. 5-HT synthesis is regulated via complex interconnected mechanisms, including the availability of L-

tryptophan, the transcription rate of the *TPH* gene, which occurs in the soma, and the TPH enzymatic activity. The abundance of Tph2 protein in fibres all over the brain indicates that a considerable fraction of brain 5-HT is produced on site in axon terminals where the synthesis rate can be specifically regulated by the enzymatic activity of TPH2. Experiments performed prior to the discovery of the second TPH isoform reported an increase of brain TPH enzymatic activity by stimulation of raphe neurons (Herr et al., 1975; Boadle-Biber et al., 1983) and a decrease by activation of hyperpolarizing autoreceptors such as the 5-HT_{1A} receptor located on somatodendritic membranes (Hamon et al., 1988; Invernizzi et al., 1991). Since the data presented here revealed that TPH2 is the only isoform consistently expressed in the brain, these findings most likely specifically refer to TPH2, but further experiments have to be conducted to validate this assumption experimentally. Kinetic properties and enzymatic activity differ to a large extent between the two isoforms considering the fact that TPH1 seems to be the "*quick-and-dirty*" worker as compared to TPH2, which possesses increased substrate preference for L-tryptophan. This on the other hand goes along with decreased affinity for L-tryptophan and decreased catalytic turnover (McKinney et al., 2005). Indeed, *in vitro* studies with recombinant enzymes (McKinney et al., 2005; Nakamura et al., 2006) showed three- to four-fold higher catalytic efficiency of TPH1 over TPH2 in the synthesis of its direct product, 5-hydroxytryptophan (5-HTP), from tryptophan. As evidenced by our qRT-PCR analysis, the level of TPH1 mRNA in the raphe is about 10^3-fold lower than TPH2 and 10^4-fold lower than in the pineal gland. It thus can be assumed that TPH1 mRNA detected by qRT-PCR represents either artifacts or trace amounts of mRNA having no physiological meaning whatsoever.

4.1.5 Possible causes for spurious TPH1 detection in the brain

To explain conflicting data demonstrating TPH1 expression in the brain (Gundlah et al., 2005; Nakamura et al., 2006; Zill et al., 2007; Abumaria et al., 2008), alignment of TPH1 riboprobes with the TPH2 mRNA sequence of the particular species was done. The two TPH isoforms are substantially homologous in their sequence and almost identical in their catalytic domains. They exhibit an overall sequence identity of 71%, and even 82% identity at the amino acid level of the catalytic domain in humans. ISH probes used in other studies which detected low levels of Tph1 mRNA in the raphe display significant overlap with conserved sequences (i.e. studies from (Patel et al., 2004; Gundlah et al., 2005; Malek et al., 2005). Alignment of the different Tph1 riboprobes used in those studies with the entire Tph2 mRNA sequence (using the EMBL-EBI Water program, http://www.ebi.ac.uk/emboss/align/) demonstrates a nucleotide identity of 62–71%, whereas the probes in our study as well as the probes used by

Côté and co-workers (Côté et al., 2003; Côté et al., 2007) both targeted the specific 3' UTR sequence. These regions are less than 40% identical and by using these more specific probes, both Côté and we failed to detect Tph1 mRNA in the brain. Hence, Tph1 riboprobes with nucleotide identity below 40% provide better discriminative specificity for Tph1 versus Tph2 mRNA. In addition, aberrant methodological handling like lower hybridization temperature (< 55 °C), longer exposure or radioactive labeling might also explain lower specificity and increased probability to detect a non-specific signal. In support of this notion, a more specific oligoprobe outside homologous sequences also failed to detect a Tph1 signal in raphe nuclei (Malek et al., 2005). Aside from this, IHC experiments as performed in the present study using antibodies raised against two epitopes located on both C- and N-terminals did not reveal presence of TPH1 protein in the raphe region which strongly supports the ISH data. Therefore, several lines of evidence clearly converge to the notion that TPH2 is the exclusive enzyme responsible for the synthesis of 5-HT in the brain across the lifespan, thus playing the predominant role in the maintenance of serotonergic neurotransmission. In contrast, TPH1 expression appears unlikely to play a role in development and function of the brain.

There are plenty of reports associating single nucleotide polymorphisms (SNPs) in the human *TPH1* gene with several neuropsychiatric conditions using case–control study designs. This seems counter-intuitive at first, considering our finding that TPH2 is the sole isoform expressed in the CNS. However, interesting results from Côté and associates (Côté et al., 2007) demonstrate that heterozygous (+/–) Tph1 KO embryos and newborns resulting from a null (Tph1-/-) mother and a WT father display dramatic abnormalities in the development of the brain and other tissues, very unlike heterozygous resulting from a null (Tph1-/-) father and a WT mother. This suggests a crucial role for peripheral maternal 5-HT and therefore functional maternal Tph1 rather than embryonal 5-HT from functional embryonic Tph2 for normal development of the brain. In addition, this points to a very early function of 5-HT in CNS development, as the majority of 5-HT available for the embryo (before it is able to produce 5-HT itself) seems to be primarily under the influence of circulating maternal 5-HT during gestation depending on maternal Tph1 expression. These findings provide a rationale how *TPH1* polymorphisms might be linked to neuropsychiatric dysfunction – although the mother's rather than the offspring's genotype has to be considered. Apart from usually small gene effects in complex disorders and a lack of power of most studies, this might also explain the inconsistent results from previous association studies.

Besides detecting low Tph1 expression in the brain of untreated controls some groups additionally reported on increased Tph1 mRNA levels in the dorsal raphe under conditions putatively influencing Tph expression, such as increased stress or hormonal alterations

(such as an increase in estrogen levels) (Abumaria et al., 2008 and Gundlah et al., 2005, respectively). As previous studies provide various examples how ovariectomy and estrogen replacement affects the expression of several serotonergic genes (several examples are given in (Rubinow et al., 1998), an effect of estrogen on Tph expression seems indeed plausible. However, since rather unspecific riboprobes were used by Gundlah et al., their findings have to be treated with caution and the effect of estrogen on the expression of the different Tph isoforms has to be specifically proven by more Tph isoform-specific investigations.

4.2 Tph1/2 expression under certain conditions

4.2.1 Tph1/2 expression is not affected by gender

Several studies have indicated that there is an enhanced responsiveness of the HPA axis to a stressor in the presence of estradiol. This goes along with enhanced cortisol release. Furthermore, women are twice as likely as men to experience a major depressive episode, and they tend to suffer from a more severe form of depression along with greater functional impairment (Angold and Worthman, 1993). Although we did neither perform ovariectomy nor estrogen treatment, global gender differences in Tph1/2 expression were examined along with a potential effect of environmental variables such as stress exposure or serotonin transporter knockout. Performing gender-controlled experiments revealed no significant differences in Tph1/2 expression under any of the conditions examined. Thus, all data were finally analysed by grouping together both genders as this also increases the statistical power of the study.

4.2.2 Influence of stress on Tph1/2 expression in a rodent model of depression

Current knowledge implicates not only genetic factors, but also environmental conditions like acute or chronic stress in the etiopathogenesis of diverse psychiatric disorders and the regulation of the serotonergic system as well. Several lines of evidence document an increased amount of ACTH pulses resulting in enhanced cortisol release in some depressed patients (Deuschle et al., 1997) indicating HPA axis hyperactivity. Clinical studies consistently link a modified stress hormone system, as well as alteration of the HPA axis function, with the

pathophysiology of affective disorders. Serotonergic projections of the raphe nuclei modulate the physical stress response which normally is self-terminated after cessation of stress exposure by negative feedback. However, under some conditions such as chronic stress exposure or in patients with altered stress behaviour found in affective disorders, an inability to terminate the stress response turns into pathological dysfunction. (Lesch and Lerer, 1991) thus suggested a dysfunctional interaction between the 5-HT and HPA systems in affective disorders due to findings on unipolar depressed patients with the selective 5-HT_{1A} receptor agonist ipsapirone.

Sert KO mice were examined with respect to their Tph1 and Tph2 expression following acute stress exposure. Sert KO mice generally possess increased 5-HT levels in the extracellular synaptic cleft and decreased 5-HT levels within serotonergic cells; the amount of serotonergic cells in the raphe nuclei is diminished, resulting in several behavioural changes including increased anxiety-like behaviours (Li et al., 2000), reduced aggression, and exaggerated stress responses (Holmes et al., 2003). In analogy to behavioural changes in Sert KO mice, altered functional expression of SERT in humans (Lesch, 2001) is associated with emotional dysregulation and a higher risk for anxiety, reduced stress adaption and depression (Canli and Lesch, 2007). Therefore, Sert KO mice have a good face and construct validity as a model for affective disorders. An association of Tph2 with stress response could already be shown by Clark and co-workers (Clark et al., 2005; Clark et al., 2008) who revealed decreased raphe Tph2 protein in response to a synthetic corticosterone as well as by Chen and associates (Chen et al., 2006) who identified several polymorphisms affecting Tph2 expression and HPA axis function in rhesus monkeys. Additionally, increased TPH2 mRNA and protein in neurons of the raphe nuclei was detected (Bach-Mizrachi et al., 2006; Bach-Mizrachi et al., 2008) in post-mortem studies on brains of depressed suicides. However, TPH1 also seems to have a role in stress and regulation of the HPA axis. This was not only suggested in rodents (Abumaria et al., 2008), where contrary to Tph2 an upregulation of Tph1 mRNA in serotonergic neurons of the rat raphe was detected after chronic stress treatment, but also in humans (Gizatullin et al., 2008), as it was suggested that TPH1 might be of relevance in a particular group of stress-induced depressed patients.

In contrast to previous evidence for modified TPH1 and/or TPH2 expression in association with stress and depression, we failed to detect changes of either Tph1 or Tph2 expression in a mouse model of depression subjected to acute stress. Up- or downregulation of Tph1/2 could neither be observed in untreated Sert KO versus WT mice nor between any of the groups including Sert KO and WT mice treated with one hour immobilization stress. However, since acute stress lasted for only one hour, no possibility for counter-regulation was provided, as

mice were instantaneously sacrificed after treatment. As "stress" is an extremely heterogeneous and complex phenomenon, our study – investigating acute stress and not allowing counter-regulation – can only examine a certain aspect of stress and thus does not necessarily contradict previous studies. Furthermore, chronic stress might more relate to the human condition. Anyhow, the use of an acute stress paradigm allows us to obtain a glimpse on the very first step of stress-induced changes of gene expression. Moreover, since Tph2 inducible (i)KO mice and their respective controls have to be repeatedly injected with tamoxifen, known to produce stress reactions in mice, this procedure can be regarded as a chronic stress model. Thus, analysis of different brain-specific Tph2 iKO mice and their respective controls can also serve as chronic stress models and provide conclusions about Tph1 and Tph2 expression changes during chronic stress in both WT as well as Tph2 iKO mice. Although statistical evaluation of expression levels from qRT-PCR with the non-parametric Kruskal-Wallis and the parametric ANOVA test, respectively, demonstrated no significant differences in Tph1 expression (see table 3-1; p-values > 0.14 in raphe rostral and > 0.5 in raphe caudal), we additionally applied an explorative post-hoc Fisher test, detecting as well no significant differences between the injected group and untreated controls, neither in rostral nor caudal parts of raphe region in both WT and KO mice. Similarly, Tph2 expression in the WT mice showed no significant variation between the injected group and untreated controls as validated by the Fisher test (see table 3-2, p-values > 0.56 in raphe rostral and > 0.12 in raphe caudal). Even more controversial, but in line with our prior investigations, Tph1 expression could not convincingly be detected in the raphe nuclei as there were only extremely low levels of Tph1 expression in the raphe nuclei which most likely do not have a physiological relevance. This is further supported by the absence of any positive IHC signal of Tph1 protein. Neither brain-specific Tph2 iKO mice nor the respective controls comprising repeatedly injected animals display detectable Tph1 protein in the raphe region (Fig. 3-11). However, the influence of chronic stress on the Sert KO mouse model should be further investigated considering the association between the short variant of SERT length polymorphism and impaired stress coping in humans. The lack of Tph1/2 mRNA expression changes after exposure to acute stress in Sert KO mice does neither rule out an influence of chronic stress to Tph1/2 mRNA levels nor an impact of both acute and chronic stress exposure on Tph1/2 protein levels in Sert KO mice.

4.3 Gene expression in different brain-specific Tph2 cKO models

Summarizing the quintessence of all previous investigations, TPH2 appears to be the relevant isoform maintaining 5-HT synthesis in the brain also under conditions like stress. Thus, the genetic set-point of CNS 5-HT synthesis, presumably resulting in behavioural abnormalities, seems due to variation at the TPH2 rather than the TPH1 locus. Bearing this in mind, different brain-specific Tph2 conditional knockout (cKO) models were generated and the expression of several genes of the serotonergic system was examined. These models include one model specific for *Tph2* deletion in all neuronal cells (Tph2 cKO^{Nes}) and two models specific for *Tph2* deletion only in serotonergic cells (Tph2 cKO^{Pet1} and Tph2 iKO), the latter featuring the advantage of iKO providing a time-specific deletion of *Tph2*. In contrast to non-inducible cKO models (cKO^{Pet1} and cKO^{Nes}) which both produce gene deletion from embryonal stages E10 - E11 on, the iKO model was tamoxifen-injected at the stage of adulthood avoiding putative compensatory effects from other systems during brain development which probably might balance reduced 5-HT availability. Comparison of all three Tph2 cKO models considering the induction of gene deletion at different points of time provides a means to further study brain-specific modulation of 5-HT system.

4.3.1 Effectiveness of cre recombination linked to *Tph2* gene deletion

Effective working of cre recombination was controlled by crossing the different cre-strains with the ROSA26 cre reporter strain using X-Gal as substrate or specific ß-gal antibodies resulting in the detection of ß-galactosidase enzyme, whose expression is then dependent on cre-mediated recombination (compare chapter 2.2.4 and 3.2). Double labelling of ß-gal and Tph2 antibodies in the brain-specific Tph2 iKO mice controls the efficiency of both cre recombination as well as *Tph2* gene deletion, which should be linked to each other. Results of fluorescence IHC double labelling were in accordance with this assumption. Although some residual Tph2-positive cells remained in the iKO raphe nuclei, detection of cells double labelled for ß-gal and Tph2 could barely be found in raphe region of iKO mice, thus displaying effective functioning of cre recombination linked to *Tph2* gene deletion (shown for Tph2 iKO, Fig. 3-10).

Efficiency of Tph2 knockout was additionally tested in all Tph2 cKO models with IHC and validated quantitatively by qRT-PCR. As expected, all knockout models possess a significantly reduced amount of cells and fibres staining positive for Tph2 in the raphe region, including a complete loss of Tph2 enzyme in the brain of Tph2 cKO^{Pet1}. Interestingly, both Tph2 cKO^{Nes} and

Tph2 iKO represent more a brain-specific knockdown model. For as yet unknown reasons Tph2 cKO^{Nes} exhibits some residual Tph2-positive cells mainly in the dorsal part of the rostral raphe, which could also be observed in Tph2 iKO mice. In the latter, this results presumably from less effective, however time-specific knockout. Additional experiments demonstrate as well that a variation of the first time point of tamoxifen injection and the number of repetitions cause alterations in the amount of Tph2-positive cells, in that earlier and repetitive injection resulted in a higher efficacy of the gene knockout (see Fig. 3-11 upper panel c/i and d/j). Accordingly, we used mice at the stage of young adulthood (3-4 months) for qRT-PCR and injected them five times, providing a higher reduction of Tph2-positive cells. The Tph2 mRNA levels obtained by qRT-PCR are in complete accordance with IHC and show a deletion of Tph2 in both the rostral and the caudal part of the raphe region (see table 3-2). Based on the values of the respective controls, an 80% knockdown of Tph2 mRNA was obtained in the rostral and caudal raphe regions of Tph2 iKO mice as well as in the rostral part of Tph2 cKO^{Nes}. In the caudal raphe of Tph2 cKO^{Nes} this was even more pronounced featuring approximately 90% of knockdown. The assumption of an absolute and brain-specific knockout in Tph2 cKO^{Pet1} was verified by a reduction of Tph2 mRNA over 90% in the caudal and more than 95% in the rostral raphe. To our best knowledge, this is the first report on a brain-specific inducible gene deletion with a user-controlled first onset in the stage of young adulthood.

4.3.2 No compensatory increase of Tph1 expression in Tph2 cKO models

Expecting a compensatory boost of Tph1 expression in the brain at least in Tph2 cKO^{Pet1} mice, completely lacking Tph2 protein from gestation on, prompted us to investigate Tph1 expression on both the transcriptional as well as the translational level. No significant differences of Tph1 expression however was detected by using qRT-PCR in any of the investigated brain regions, neither in the iKO nor in the cKO models. Moreover, loss of Tph1 enzyme in the raphe nuclei was detected by specific Tph1 antibodies across all different mouse genotypes and their respective controls, whereas pineal glands presented robust labelling. Therefore, compensatory up-regulation of Tph1 in the brain of animals lacking neuronal Tph2 protein does not occur. The absence of immunodetection of either Tph isoform in complete Tph2 (-/-) KO mice, resulting from a spontaneous germline transmission of Tph2 cKO^{Nes}, further supports this conclusion. Even under this condition, Tph1 remained undetectable in the raphe nuclei rendering any spontaneous brain Tph1 expression unlikely (Gutknecht et al., 2008; Gutknecht et al., 2009).

4.3.3 Animal models for human affective disorders?

Since long, 5-HT has been implicated in the etiopathogenesis of affective disorders. In addition, most of the currently used antidepressants interfere with the serotonergic system. Further data supportive of this notion are around since the early seventies, where it was observed that depressive symptoms could be ameliorated by administration of 5-HT precursors like 5-HTP and tryptophan (Mendels and Frazer, 1975). On the other hand, aggravation of symptoms could be evoked in depressed patients by administering a tryptophan depleting diet (Delgado et al., 1990; Van der Does, 2001). Likewise, also in healthy subjects this causes gloomy mood (Young et al., 1985). In contrast to monoamines like 5-HT, amino acids like tryptophan are able to cross the blood brain barrier. Thus, modulation of availability of precursors for neuronal 5-HT synthesis might have an important influence in developing depression-like behavioural changes during adulthood. Our inducible knockdown of Tph2 might be a model for both reduced availability of 5-HT in the brain (i.e., mirroring tryptophan depletion) and modulation of the 5-HT system during adulthood.

4.3.4 Expression of serotonergic genes in different brain-specific Tph2 cKO models

By studying the expression of several genes of the serotonergic system using qRT-PCR we further tested the proper formation of the serotonergic system in Tph2 cKO mice. As we performed our studies using a 384-well system, providing an all-in-one run including all genotypes within the respective brain region, possible measurement errors were avoided. Different 5-HT receptor subtypes and the serotonin transporter were investigated in eight different brain regions of interest including prefrontal and frontal cortex, striatum, hippocampus, hypothalamus, amygdala, raphe region (rostral and caudal separately) and entorhinal cortex. Interestingly, there was no significant change of any of the genes in any brain region, apart from the hippocampus featuring a significant alteration of $5\text{-}HT_{1a}$ receptor expression detected by the non-parametric Kruskal-Wallis as well as the parametric ANOVA test. Further statistical analyses using *the accurate Fisher test showed* only borderline significant differences between Tph2 cKOs and their respective controls (cKO^{Pet1} $p = 0{,}0374$; cKO^{Nes} $p = 0{,}0730$; iKO^{Pet1} $p = 0{,}0585$; compare table 3-2). Hence, variation in hippocampal $5\text{-}HT_{1a}$ receptor expression most likely is not physiologically relevant and rather negligible. These results demonstrate normal development of the serotonergic system on the transcriptional level not only (as expected) in the time-specific iKO model but also in the tissue-specific Tph2 knockdown/-out models where gene deletion occurred from the early embryonic

development preceding 5-HT-mediated neuronal formation on. Tph2 cKO^{Pet1} mice show no immunodetectable 5-HT in the brain, whereas the Tph2 iKO feature at least a residual 5-HT in the brain as evidenced by IHC (Dr. Ding, ION, Shanghai, China, personal communication).

Taken together, normal development of genes of the 5-HT system was not expected in a brain-specific Tph2 knockout model. One possible explanation might be that *Tph2* deletion does not start from the beginning of embryonic development on but at the time of expression of the serotonergic transcription factor Pet1, which operates rather at the end of a transcription factor cascade necessary for proper formation of serotonergic phenotypes and brain development (Ding et al., 2003). Thus, if a cell once is programmed to express the serotonergic phenotype (a process which is starting already before Pet1 is switched on), it might not stop the programme until its end. Thus, serotonergic proteins are produced in a normal fashion and localized to their specific sites, although they are presumably not needed. Alternatively, a growing body of literature supports the idea of promiscuity among neurotransmitter-associated proteins, e.g. the monoamine transporters which provide uptake of multiple amines in addition to their "native" transmitter. The dopamine and noradrenaline transporter (DAT, NET) e.g. transport 5-HT under conditions in which SERT activity is eliminated by pharmacological inhibition or gene knockout, and also SERT can accomplish low dopamine and noradrenaline transport, respectively (for a review see (Daws, 2009)). Thus the absence of the neurotransmitter 5-HT in the brain of Tph2 cKO models might not lead to disruption of 5-HT-associated proteins like the Sert and 5-HT receptors in the brain, as they still might be required for functions not related to 5-HT.

Furthermore, general expressional differences could be detected between the investigated genes within different brain regions, independently from genotype. Thereby, previous findings on the localisation of 5-HT receptors could be confirmed and further specified. Every receptor subtype seems to have a particular region where its expression has the highest level: the hippocampus in the case of the 5-HT$_{1a}$, striatum for the 5-HT$_{1b}$, frontal and prefrontal cortex for 5-HT$_{2a}$ and finally the raphe region for the 5-HT$_{2c}$ receptor. For Sert, as expected, expression could only be detected in raphe nuclei as well as in peripheral intestine tissue.

4.4 Oxytocin

Oxytocin is known as a gestational hormone regulating labour and milk-ejection which in concert give rise to mother-child bonding and maternal care. In recent years, oxytocin was implicated as well not only in establishing trust between sexual partners thereby facilitating pair bonding, but also more generally in establishing trust in fellow men. Neuroeconomic

studies (Kosfeld et al., 2005) identified enhanced trust in business partners during financial affairs induced by oxytocin which was also accompanied by reduced activation of the amygdala (Baumgartner et al., 2008). As evidenced by rodents exposed to an anxiety paradigm, oxytocin seems to have positive effects in the regulation of anxiety and fear. Furthermore, intraventricular injections of CRH into male or female rats lead to increased oxytocin secretion (Bruhn et al., 1986) which can be blocked by lesions of the PVN of the hypothalamus (Jezova et al., 1995). As CRH is known to be released from the hypothalamus as response to stress, it is likely that oxytocin-producing cells are stimulated by CRH neurons and that oxytocin is released to modulate the expression of fear during stress.

Oxytocin is also uniquely associated with social recognition and affiliative (or pro-social) behaviours which are missing in autism (Insel et al., 2001; Choleris et al., 2004). Oxytocin KO mice show pronounced deficits in olfactory-based social recognition which might result from the loss of oxytocin in the central and medial nucleus of the amygdala (Ferguson et al., 2001). 5-HT agonism during a critical time in development in a negative feedback loop leads to loss of 5-HT terminals, an effect which could also be seen in subjects with autism, and results in "autistic-like" behaviours in rodents. Rats treated prenatally with an 5-HT agonist have less oxytocin-containing cells in the hypothalamus, as well as less of oxytocin projections towards brain regions like the amygdala and show behaviours such as decreased social bonding, sensory hyper-responsiveness, seizures and motor changes, i.e. behaviours which might relate to autism (Whitaker-Azmitia, 2005). In line with these animal studies, there are reports of decreased oxytocin in the blood of autistic patients (Green et al., 2001) and infusion of oxytocin into autistic children reverses some behaviours (Hollander et al., 2003). Connections between the amygdala, the hypothalamus and the serotonergic system could represent a part of a neurocircuit involved in the emotional response towards social stressors which is impaired in autism (Whitaker-Azmitia, 2005).

The influence of the oxytocinergic system on social encounters and anxiety might well include the modulation of serotonergic neurotransmission. Furthermore, an impact of oxytocin in the etiology of neuropsychiatric disorders like depression, anxiety and panic disorders was postulated. Projections from serotonergic cells of the DRN, innervating both the hypothalamus and the amygdale, may present a link between the serotonergic and oxytocinergic systems to modulate neuronal networks implicated in social, emotional and sexual behaviours.

4.4.1 Stress-induced changes of oxytocin expression

Following this hypothesis, oxytocin expression in an animal model for depression (Sert KO mouse) exposed to acute immobilization stress was studied. qRT-PCR was applied to investigate expressional differences in five brain regions of interest, including the frontal cortex, hippocampus, hypothalamus, amygdala and raphe nuclei. The experiments were controlled for intervening variables, i.e. females and males of the same age were used, and stress exposure for every group was carried out at the same time of the day (8:00-12:00) to ensure a comparable level of peripheral corticosterone in each animal. Interestingly, qRT-PCR detected significantly higher expression levels of oxytocin in brain regions which are involved in the regulation of emotional stimuli (amygdala, hippocampus) in stressed male WT mice, whereas male Sert KO as well as female WT and Sert KO mice lack these stress-induced changes. Thus, on the one hand Sert KO mice of both genders do not respond to acute stress with increased oxytocin levels while on the other hand, during acute stress a gender-specific increase in oxytocin expression takes place in WT mice in brain regions known to be involved in emotional regulation.

4.4.2 Gender differences in oxytocin expression

As known previously, qRT-PCR demonstrated a marked expression of oxytocin in the hypothalamus (which is known to contain the majority of oxytocinergic neurons) of female mice as compared to males independent from genotype or treatment. Gender differences of oxytocin expression in the hypothalamus are in accordance with previous studies suggesting that oxytocin is more important in females, whereas vasopressin appears to play a more important role in males. However, both oxytocin as well as vasopressin are associated with the establishment of trust and bonding. While vasopressin acts excitative on a population of neurons in the amygdala thereby enhancing aggressiveness, stress levels and the consolidation of fear memory, oxytocin decreases anxiety and stress and facilitates bonding and trust probably by acting inhibitory on the very same neurons (Huber et al., 2005). Furthermore, there are also gender-differences in behaviour of mice lacking the gene for oxytocin. Female oxytocin KO mice are unable to use olfactory signals to choose male partners and react to their behaviour (Kavaliers et al., 2006), whereas male oxytocin KO mice display decreased social recognition which seems to be associated with loss of oxytocin in the amygdala (Ferguson et al., 2001). Males and females might react differently to stress, which has probably been shaped during evolution. One might speculate that a more "male" behaviour might be to behave aggressively in a flight-or-fight decision, whereas the "female"

behaviour might encompass to enforce trust in social interactions and to build strong social networks in order to achieve assistance and support. In the condition of immobilization stress, where attacks or aggressiveness might be less sensible than saving energy by giving up to struggle, an increase of oxytocin might prevent an excessive fight reaction in males. Female WT mice exposed to immobilization stress might be protected from a potentially disadvantageous fight reaction due to their higher overall oxytocin levels, presumably generated in the hypothalamus, and thus do not have to compensate by an increase of hippocampal and amygdala oxytocin levels. This suggests that oxytocin dampens fear presumably in stress situations in males where fighting behaviour would be disadvantageous.

Stress-induced increase of oxytocin expression could only be detected in the hippocampus and the amygdala; other brain regions - including the hypothalamus, containing the majority of oxytocinergic neurons - lack those stress-induced changes independent of gender and genotype. These findings were in accordance to Jezova et al. (Jezova et al., 1995) detecting no differences of oxytocin mRNA after two hours of immobilization stress neither in the PVN nor in the SON of the hypothalamus; however, at a later time point, they could detect a significant increase of oxytocin mRNA in the PVN. Regarding gender differences, they could detect increased plasma oxytocin levels in adult male rats 30 minutes after immobilization stress (Jezova et al., 1995), yet no increase of vasopressin levels, while in female rats a rise in plasma-vasopressin levels was observed even after a short period of immobilization (Jezova et al., 1996). Comparing the present study with the findings of Jezova et al., both oxytocin and vasopressin seem to be early genes reacting to stressors differentially dependent on the gender of the animal.

4.4.3 Oxytocin expression in Sert KO mice exposed to immobilization stress

Our findings are in accordance with the hypothesis of oxytocin being necessary for protection against stress, depressive mood and anxiety but suggest gender-dependent differences. Gender-differences in stress coping could also be shown to be affected by the short allele of 5-HTTLPR, known to predispose to depression under adverse psychosocial circumstances. Brummett and colleagues could show higher depression scores for females carrying the short allele of 5-HTTLPR whereas for males, the long allele was associated with depression in combination with a stressor only (Brummett et al., 2008). Comparing those to our results, further investigations are needed not only regarding the kind of stressor but also regarding the impact of neurohormones like oxytocin and vasopressin in interaction with 5-HTTLPR genotype.

A further result of this study is that in an animal model for depression (Sert KO mouse), which might correspond to presence of the short allele of 5-HTTLPR, both genders lack stress-induced oxytocin expression changes. Thus, Sert KO mice seem to be either incapable of responding properly to stressors and thus are less able to permit an oxytocin response which is probably protective against prolonged stress response (then inducing anxiety and depression) or vice versa, Sert KO mice might be more protected from stress-induced fear expression and do not necessarily require increased oxytocin levels apparently decreasing enhanced fear. However, the latter assumption lacks experimental support as increased anxiety-like behaviours (Li et al., 2000) and exaggerated stress responses (Holmes et al., 2003) were detected in Sert KO mice. The lack of altered oxytocin expression in Sert KO mice anyway indicates a modulation of the oxytocin response by the serotonergic system and provides a novel research avenue with respect to an altered response of Sert KO mice to stress and anxiety inducing stimuli.

4.5 Outlook

As outlined above, dysregulated tryptophan hydroxylase (TPH)-dependent 5-HT synthesis is implicated in altered emotional behaviour and stress, which points to the relevance of the two isoforms TPH1 and TPH2 for the etiology and pathogenesis of various neuropsychiatric disorders. Yet the impact of genetic variation of either TPH1 or TPH2 on human disorders as well as their tissue-specific expression pattern in different species remains controversial. The main goal of this study was therefore to clarify the tissue- and time-specific expression pattern of TPH1 and TPH2 in mice and humans. These experiments were not only carried out to resolve inconsistencies in the pertinent literature, but also to shed some light on equivocal results from genetic association studies regarding TPH1 and/or TPH2 polymorphisms in diverse psychiatric disorders. In addition to this, the influence of stress on the expression of both isoforms was studied in an animal model for depression (Sert KO mouse) to gain insight about the developmental regulation of TPH1/2 expression in stress and depression.

Due to the importance of 5-HT in early brain development, different tissue-specific and inducible knockout mice lacking the *Tph2* gene were generated to further establish the role of the 5-HT system for neuronal development and, as a consequence, the regulation of emotions later in life. Expression studies of 5-HT-associated genes were performed to approach the first steps in a long-term journey studying neuronal 5-HT deficiency during different developmental stages. The interesting findings of unchanged expression of 5-HT associated genes give rise to a range of follow-up studies in mice lacking 5-HT but expressing

a normal 5-HT system. This includes behavioural, epigenetic and neuro-imaging studies as well as investigation of double-knockout models like a Tph2-Sert-double KO.

The second project concerning oxytocin expression in stressed Sert KO mice aimed to identify a link between the serotonergic and the oxytocinergic systems in stress behaviour which might provide a novel target for psychopharmacological treatment in affective disorders. As stress-induced changes of oxytocin on the mRNA level could only be detected in male WT (but not in female as well as Sert KO mice), this data might provide an attractive approach to discuss subtypes of depression or the impact of gender on the development of affective disorders. Further studies however are needed to validate stress-induced changes of oxytocin expression on the protein level.

5 APPENDIX

5.1 References

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5.2 List of figures and tables

FIGURES

1 INTRODUCTION

2 MATERIAL AND METHODS

3 RESULTS

TABLES

1 INTRODUCTION

2 MATERIAL AND METHODS

3 RESULTS

5.3 List of abbreviations

°C	degree Celsius
µ	micro (10^{-6})
5-HIAA	5-hydroxyindol acetic acid
5-HT	5-hydroxytryptamine (serotonin)
5-HTP	5-hydroxy tryptophan
8-OH-DPAT	8-hydroxy diprolamino tetralin

A

AA	arachidonic acid
aa	amino acids
AAAD	aromatic L-amino acid decarboxylase
AA-NAT	arylalkylamine N-acetyl transferase
AC	adenylyl cyclase
ACTB	actin beta
ACTH	adrenocorticotropic hormone
ADHD	attention-deficit/hyperactivity disorder
aP	alkaline phosphatase
A-P	anterior-posterior
Aq	aqueduct
Arbp	acidic ribosomal phosphoprotein P0
Arg	arginine

B

BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BH_4	6R-L-erythro-5, 6, 7, 8-tetrahydrobiopterin
BLA	basolateral nucleus of the amygdala

C

C	cortex
c	centi (10^{-2})
cDNA	complementary desoxyribonucleic acid
CeA	central nucleus of the amygdala
cKO	conditional knockout
CLi	caudal linear raphe nucleus
CNS	central nervous system
CP	caudate-putamen
cre	cyclization recombinase
CRH	corticotropin releasing hormone
cRNA	complementary ribonucleic acid
C_T	threshold cycle

D

DAB	3,3'-diaminobenzidin
DAG	diacylglycerol
DIG	digoxigenin
DNA	desoxyribonucleic acid
DR	dorsal raphe
DRD	dorsal raphe nucleus dorsal
DRI	dorsal raphe nucleus inferior
DRN	dorsal raphe nucleus

DRV	dorsal raphe nucleus ventral
DRVL	dorsal raphe nucleus ventrolateral

E

E	embryonic day
E. coli	Escherichia coli
EDTA	ethylendiamintetraacetat
e.g.	exempli gratia (for example)
ER	human estrogen receptor
etc.	et cetera

F

F	frontal part of the brain
or F	female
FAD	flavin adenine dinucleotid
Fig	figure
floxed	loxP-flanked
fMRI	functional magnetic resonance imaging

G

GAPDH	glyceraldehyde-3-phosphate dehydrogenase

H

h	hour
HPA	hypothalamic-pituitary-adrenal
HPLC	high performance liquid chromatography
HYP	hypothalamus

I

i.e.	id est (that is)
IHC	immunohistochemistry
iKO	inducible knockout
IP_3	Inositoltrisphosphate
ISH	*in situ* hybridization
ITT	*in vitro* transcription and translation

K

k	kilo (10^3)
KLH	limpet haemocyanin
KO	knockout

L

l	litre
LA	lateral nucleus of the amygdala
LD	light-dark cycle
LNAA	large neutral amino acid
loxP	locus of X-over of bacteriophage P1
LS	lateral septum
LSD	lysergic acid diethyl amide

M

M	male
m	milli (10^{-3})
or m	meter
MAO-A or MAO-B	monoamine oxidase A or B in humans
Mao-a or Mao-b	monoamine oxidase a or b in rodents
MdThal	mediodorsal thalamus
MeA	medial nucleus of the amygdala
min	minute
MRN	median raphe nucleus
mRNA	messenger ribonucleic acid

N

n	nano (10^{-9})
NAcc	nucleus accumbens
NAD	nicotin amide adenine dinucleotid
NBT	4-nitro-blue-tetrazolium-chloride
Nes	nestin
NGS	normal goat serum

O

OB	olfactory bulb
OCD	obsessive compulsive disorder

P

P	postnatal day
p	pico (10^{-12})
PAH	phenylanlanine hydroxylase
PBS	phosphate buffered saline
PFA	paraformaldehyd
PFC	prefrontal cortex
PG	pineal gland
Phe	phenylalanine
Pi	preimmune serum
PIP2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLA2	pospholipase A2
PLC	phospholipase C
Pn	pontine nuclei
PPIA	peptidylprolyl isomerase A
Pro	proline
PVN	paraventricular nucleus
qRT PCR	quantitative real-time polymerase chain reaction

Q

Q-values	quantity values

R

R	raphe nuclei region
RC	raphe cap
Rn18s	18S RNA
RNA	ribonucleic acid
RPL13A	ribosomal protein L13a
RT	room temperature

S

SCN	suprachiasmatic nuclei
SDS	sodium dodecylsulfate
sec	second
SEM	standard error mean
SERT	serotonin transporter in humans
Sert	serotonin transporter in rodents
Shh	sonic hedgehog
SNP	single nucleotide polymorphism
SOV	supraoptic nucleus
SSC	saline sodium citrate
SSRI	selective serotonin reuptake inhibitor

T

TBS	tris buffered saline
TH	tyrosine hydroxylase
TPH1 or TPH2	trypthophan hydroxylase 1 or 2 in general and humans
Tph1 or Tph2	trypthophan hydroxylase 1 or 2 in rodents
Tris	Tris-(hydroxymethyl)-aminomethan
Trp	tryptophan
Tyr	tyrosine

U

U	unknown
UTR	3'-untranslated region

V

VMAT	vesicular monoamine transporter
VP	ventral pallidum
VTA	ventral tegmental area

W

WT	wildtype

X

X-Gal	5-brom-4-chlor-3-indoxyl-β-D-galactopyranosid

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Psychologen (siehe oben) und Psychiater
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5.5 Declaration

I hereby declare that this dissertation is my own work, that it contains no other sources and tools than those given, that all quotations from literature are indicated and that, to the best of my knowledge and belief, it contains no material previously published or written by another person.

The present dissertation contains no material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning.

Erklärung

Hiermit versichere ich, dass ich die vorliegende Dissertation selbständig angefertigt habe, keine anderen als die angegebenen Quellen und Hilfsmittel verwendet und alle aus der Literatur entnommenen Stellen als solche kenntlich gemacht habe. Ich versichere, dass die vorliegende Arbeit mit bestem Wissen und Gewissen nicht in gleicher oder ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat und keine Ergebnisse enthält, die zuvor durch andere Personen ohne meine Beteiligung veröffentlich oder beschrieben wurden.

Die vorliegende Dissertation wurde weder in gleicher noch in ähnlicher Form einer anderen Fakultät zur Erwerbung eines akademischen Grades vorgelegt.

Würzburg, den 27.08. 2009

Claudia Kriegebaum