TARGETING THE TRYPTOPHAN HYDROXYLASE 2 GENE FOR FUNCTIONAL ANALYSIS IN MICE AND SEROTONERGIC DIFFERENTIATION OF EMBRYONIC STEM CELLS

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I INTRODUCTION

1. DISCOVERY AND DUALISM OF SEROTONERGIC SYSTEM

1.1 Discovery of serotonin

In 1930s, Dr. Vittorio Erspamer from The Institute of Comparative Anatomy and Physiology, University of Pavia, in Italy, was interested in the smooth muscle constriction properties of various amine substances isolated from different animal species. One amine, that was causing strong contractions of the smooth muscles of the rat uterus, was isolated from the enterochromaffine cells of the gut and, thus, named enteramin (Vialli and Erspamer 1937, reviewed in Whitaker-Azmitia 1999).

In 1948, Dr. Maurice Rapport and Dr. Arda Alden Green, in the laboratory of Dr. Irvin Page at the Cleveland Clinics, USA, were following evidence present from the middle of the nineteenth century - an endogenous constricting substance in the blood that is possibly causing hypertension, and isolated an unknown amine from beef serum. According to its presence in serum and its tonic activity, the substance was named serotonin (Rapport et al. 1948, Rapport 1949).

Until 1952, the final deduction of the chemical structure revealed that enteramine and serotonin are, actually, the same substance - 5-hydroxytryptamine (5-HT). The name serotonin prevailed in the scientific community, as the synthetic 5-HT under the name serotonin was already on the market before the enteramine structure was published (Rapport 1949, Erspamer and Asero 1952).

Betty Mack Twarog, based on the availability of synthetic serotonin, identified it as the relaxing substance in the catch phenomenon of molluscs. Joining later the lab of Dr. Page, she proved its presence in the mammalian brain, setting a milestone in acknowledging serotonin as neurotransmitter (Twarog and Page 1953, Twarog 1954).

In 1964, the presence of serotonin was verified in neurons of the brain stem (Dahlströhm & Fuxe, 1964, Fuxe 1965, Weber & Horita, 1965).

Being a potent vasoconstrictor and a neurotransmitter, serotonin is involved in many complex physiological and psychological activities. This dualism will be highlighted in the chapters that follow.

Present all over the animal and the plant kingdom, and in a variety of the vertebrate tissues, serotonin has an essential role in various physiological processes. I would be glad if the research I performed during my graduate studies helps elucidating its diverse, yet integrative role.

1.2 Evolution of serotonin

Serotonin is an ancient chemical synthesized from an indole - containing precursor, tryptophan. Tryptophan has the ability to convert solar energy into biological energy by being able to easily give and receive the ions from metals and organic molecules, oscillating between different redox states. As oxygen began to be a major component of the atmosphere on Earth, enzymes that served a central function in conversion of CO_2 into glucose, now evolved to hydroxylate many substrates. Hydroxylation of tryptophan produces 5-hydroxytryptophan (5-HTP), prone to subsequent deamination to 5-hydroxytryptamine (5-HT) or serotonin. Tryptophan can hydroxylate to many other indole alkaloids used for medicinal purposes today (reviewed in Azmitia 2007).

Unicellular organisms use the synthesis of serotonin to, converting the energy from the sun, capture the energy from O_2, and protect the cell from this highly reactive molecule. Serotonin is, thus, one of the first antioxidants on earth. The consequences of this process made tryptophan and its associated molecules involved in all aspects of the organism's life: mitosis, movement, and maturation (Azmitia 2001).

Plants are able to synthesize tryptophan in chloroplasts, and thus are the primary source of this essential amino acid. They convert Trp in 5-HT in the cytoplasm, converting later to melatonin and auxin. All three products of Trp are then used for growing and maturation of

the roots, as well as for the rotation of the leaves towards the sun. The evolutionarily oldest serotonin receptor, 5-HTR_{A1}, is found on plant cells. This was the first "transmitter" system involving serotonin.

Animals don't have chloroplasts, and for them, Trp is an essential amino acid that must be taken up by the food and then converted to 5-HT. This system is present even in the most primitive metazoans like sponges, which are not only synthesizing 5-HT in specialized epithelial cells, but also express 5-HT receptors (5-HTRA1). The signaling pathway that involves protein kinase C (PKC) did not change for almost a billion years - ketanserin and clozapine can be used to inhibit this signaling from sponges to humans. After the stimulation of the receptor the sponges undergo a pronounced metamorphosis during which they develop a variety of specialized cells involved in feeding and movement.

The evolution of a neuronal center for the integration of responses to the complex environment, kept serotonin as the main "transmitting" molecule. Only few cell types specialized to synthesize serotonin, due to inavailability of large quantities of Trp, but they evolved to promote many key biological functions.

In vertebrates, serotonin has a homeostatic and holistic function, enabling the complex organism with specialized organs and tissues to function as a whole. Acting as a simple chemical antioxidant in unicellular organism, whose conformational changes where used to shape the morphology of a single cell organism, serotonin evolved to a very potent messenger involved in many physiological processes.

The homeostatic concept of serotonin function is supported by the results produced in this study.

1.3 Distribution and physiological role of serotonin in mammals

Enterochromaffine cells of the intestine are the source of serotonin circulating in the blood - they produce around 90% of total serotonin in a mammalian organism (Walther et al. 2003). The serotonin from the enterochromaffine cells can enter the circulation or the intestinal lumen, and exerts its action on almost all the organs. Historically discovered and

characterized as a vasoconstrictor involved in hypertension, in the last decades role in almost all physiological processes was attributed to serotonin - ontogenesis, heart development, hemostasis, liver regeneration, activation of immune response and reproduction (Gaspar et al. 2003, Azmitia 1991, Azmitia 2001, Baluda et al. 1969, Lesurtel et al. 2006, Mössner and Lesch 1998, Sheng et al. 2004, Shuey et al. 1993, Aragon et al. 2005). Table 1.1 shows tissue distribution of serotonin in the mammalian organism.

Besides its physiological role, it was very early recognized that serotonin may influence psychological processes, by the similarity of its structure to the synthetical derivate with known psychotropic effects - LSD (reviewed in Whitaker-Azmitia 1999) and its presence in the brain of vertebrates (Twarog 1954). It required several decades, however, before the role of serotonin in panic attacks (Fekkes et al. 1997), anxiety (Griebel et al. 1996), obsessive-compulsive disorders (Dolberg et al. 1996), administration of drugs of abuse (Rocha et al. 1998), sexual behavior (Giammanco et al. 1997), appetite regulation (Mauri et a. 1996), sleep (Cases et al. 1995), and regulation of the body temperature (Hodges et al. 2008) was acknowledged. The way serotonin exerts its action in those processes is still largely unknown, and is the matter of extensive investigations.

Cell type	*Biomolecule*	*Source*
Raphe neurons	5-HT and TPH	Weber and Horrita 1965
Pineal gland	5-HT and TPH	Weber and Horita 1965
Retinal cells	5-HT and TPH	Green and Besharse 1994
Enterochromaffine cells	5-HT and TPH	Weber and Horrita 1965
Enteric neurons	5-HT and TPH	Gershon et al. 1965
Adrenochromaffine cells	5-HT and TPH	Dealrue et al. 1992
Proximal renal tubuli	5-HT and TPH	Sole et al. 1986
Thrombocytes	5-HT and TPH	Champier et al. 1997
Lymphocytes	5-HT and TPH (mRNA)	Finocchiaro et al. 1988
Macrophage and	5-HT and TPH	Finocchiaro et al. 1988
Mast cells	(mRNA)	
Spleen	5-HT and TPH	Young et al. 1993
Beta cells in the islands of Langerhans	5-HT and TPH	Barbosa et al. 1998
Neuroendocrine cells	5-HT and TPH	Newman et al. 1993
Leydig cells	5-HT and TPH	Frungieri et al. 1999
Taste buds	5-HT	Fujimoto et al. 1987
Epithelial cells	5-HT and TPH	Matsuda et al. 2004
Zygote	5-HT and TPH	Walther and Bader 1999
Blastocyst	5-HT and TPH	Walther and Bader 1999

Table 1.1 Distribution of serotonin in the mammalian organism.

1.4 Biochemistry of the serotonergic system

1.4.1 Biosynthesis of serotonin

Serotonin (5-hydroxytryptamine (5-HT)) is synthesized in two steps from the essential amino acid tryptophan (Trp), a derivative of indole (Figure 1.1). The first step is the rate limiting hydroxylation of tryptophan to 5-hydroxytryptophan by tryptophan hydroxylase (TPH) that requires Fe^{2+} as a cofactor and O_2 and tetrahydrobiopterine (BH_4) as co-substrate (Lovenberg et al. 1967). The second step is the immediate decarboxylation of 5-hydroxytryptophan to 5-hydroxytryptamine by a non-specific aromatic amino acid decarboxylase (AAAD) (Jung 1986, Uchida et al. 1992).

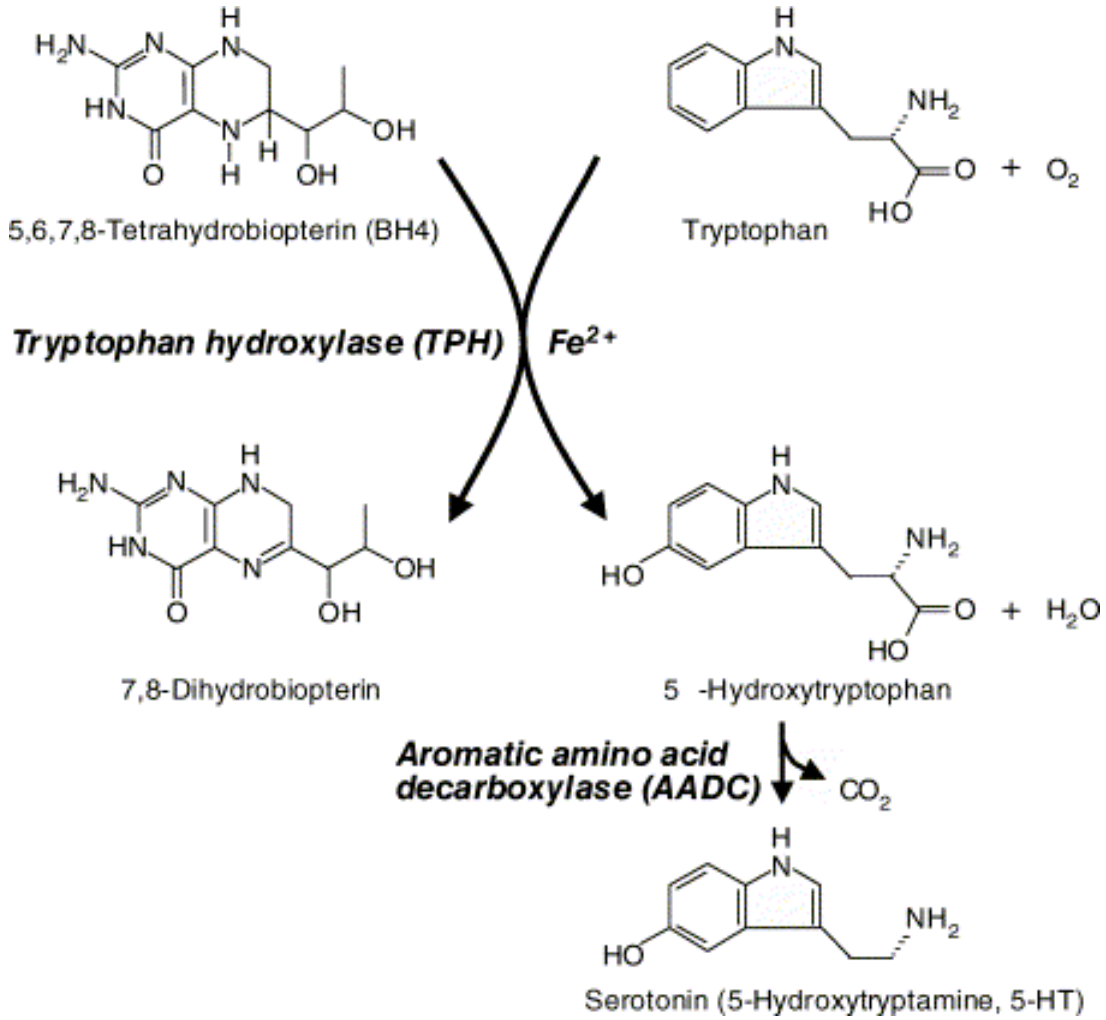


Figure 1.1 Biosynthesis of serotonin

1.4.2 Catabolism of serotonin

The rate of serotonin synthesis is influenced by the enzymatic activity of TPH, and the availability of its substrate Trp, O_2 and BH_4 (Boadle-Biber 1993).

Tryptophan is imported in the cell from the blood by large neutral amino acid transporter (LAT) (Leathwood and Fernstrom 1990) where it can be converted to tryptamin, N-formyl-kinurenin or 5- hydroxytryptophan, the intermediate in the serotonin synthesis. The synthesis of serotonin, thus, should be observed as a dynamic process, the rate of which is influenced by all three metabolic pathways of tryptophan. Not all of that pathways, however, exert the same activity in the different tissues: melatonin biosynthesis occurs mostly in the pineal gland (Coon et al. 1996), while the intermediates of the indol/skatol pathway exerts its physiological role in smooth muscle tissue (Bosin et al. 1976, Hixson et al. 1977).

The kynurenin pathway converts Trp to NAD+/NADP+ redox metabolites, involved in many important redox processes in the cell. The rate limiting step is catalyzed by Trp-2,3-dioxygenase (indoleamine-2,3-deoxygenase) or TDO/IDO, that can metabolize many other indoleamines, including melatonin and serotonin, but only the catabolism of Trp leads to production of NAD+/NADP+. The intermediate products of the kynurenin pathway, N-formyl-kynurenin, kynurenin, kynurenic acid and cholinergic acids are potent neurotoxins, and able to cause epileptic seizures (Gal and Sherman 1980, Perkins and Stone 1982).

The degradation of Trp to indol and skatol, via tryptamine, is physiological most important in the periphery, as tryptamine can bind to 5-HT receptors and act as a potent contractor of smooth muscles.

Melatonin biosynthesis is a two step process that includes 5-HT-N-acetyl-transferase and N-acetyl-5-HT-O-methyl-transferase. Melatonin synthesis takes place in the epiphysis, retina, pituitary and some parts of the brain (Coon et al. 1996), and is important for circadian rhythm and sleep awake state (Cassone 1990).

When not metabolized to melatonin, serotonin is directly degraded to 5-hydroxy-indole-acetic-acid (5-HIAA) by monoamineoxidase A (MAO-A) and monoamineoxidase B (MAO-B) (Lenders et al. 1996, Grimsby et al. 1997). The organs not involved in the synthesis of melatonin, like the brain, excrete serotonin in cerebrospinal fluid, where it is taken out by the blood through active transport, and finally excreted by the kidney.

The activity of these three different pathways (Figure 1.2) is determined by the regulation of rate limiting enzymes of each pathway through the inhibition of the products of another pathway - serotonin allosterically inhibits TDO/IDO, melatonin competitively inhibits TDO/IDO, while 5-hydroxytryptophan inhibits TPH activity in epiphysis and mast cells, but not in the brain.

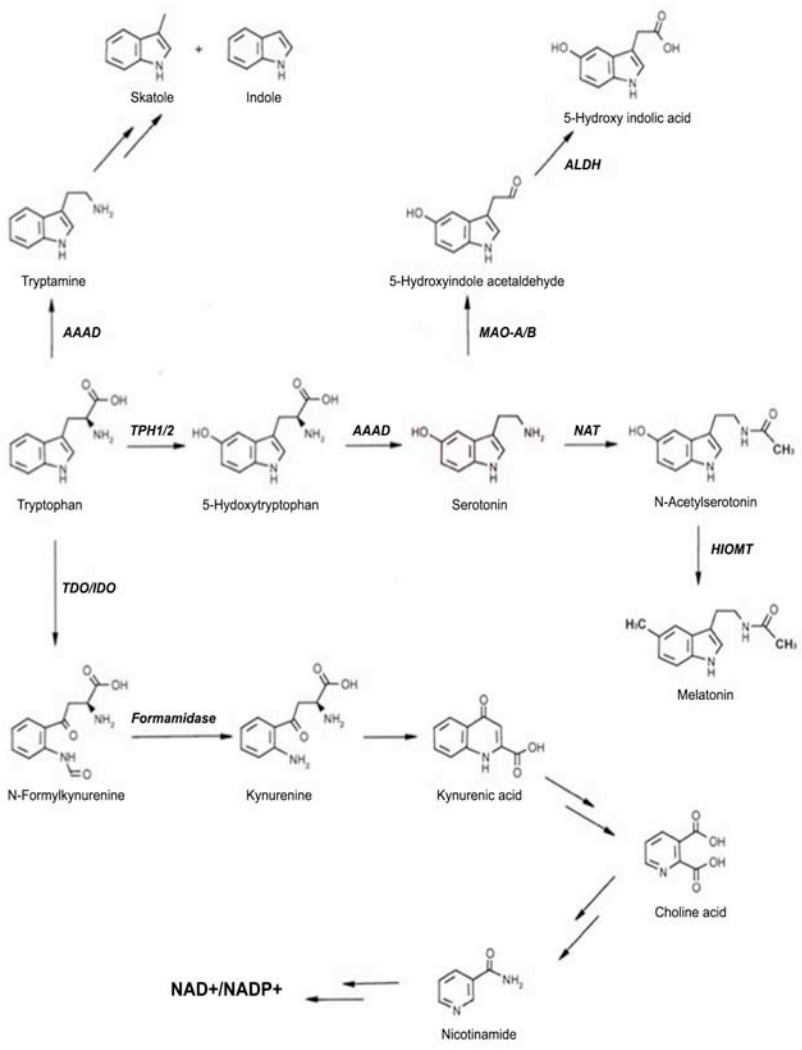


Figure 1.2 Metabolic pathways of tryptophan. For details see text above.

1.5 Monoaminergic system

Biochemically, serotonin is a monoamine, containing an amino group ($-NH_2$) connected to an aromatic ring by two-carbon chain ($-CH_2-CH_2-$). Some other neurotransmitters and neuromodulators share the same structure. They all derive from aromatic amino acids like phenylalanine, tyrosine, tryptophan, or thyroid hormones, by the action of aromatic amino acid decarboxylase (Henry and Bowsher 1986, Brodie et al. 1962, Juorio and Boulton 1982, Jaeger et al 1984, Dyck et al. 1982). All biogenic monoamines act synergistically to maintain the homeostasis of the organism. They have a common origin, similar biochemical pathways, use the same or highly homologous enzymes and transporters, and are involved in integrating the same biological processes, such as the regulation of the

body temperature, heart rate, breathing, circadian rhythm, depression, cognition, learning or emotions (Hellon 1974, Lydic 1996, Penev et al. 1994, Heninger et al 1996, Rogers et al. 1999 (Robert et al. 1997, Kim et al. 1997) .

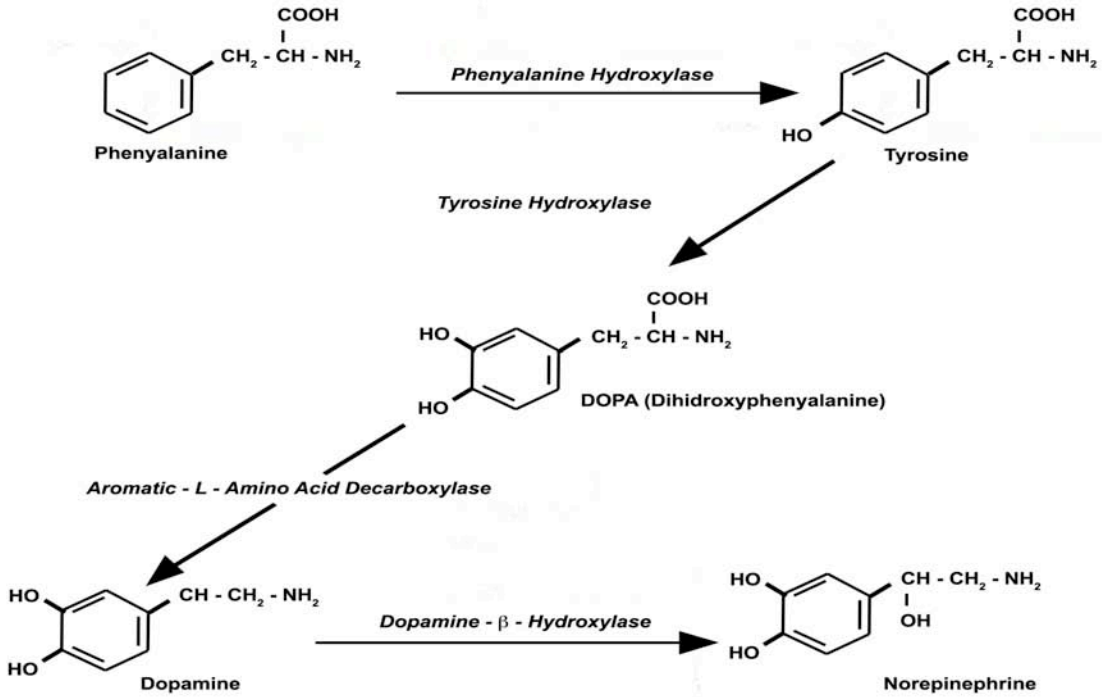


Figure 1.3. Biosynthesis of catecholamines. Phenylalanine hydroxylase (PAH), tyrosine hydroxylase (TH) and dopamine β hydroxylase (DBH) belong to the same family as tryptophan hydroxylase (TPH). Epinephrine is synthesized in an additional methylation step from norepinephrine, involving phenylethanolamine N-methyltransferase (not shown).

Serotonin and the catecholamines (Figure 1.3): noradrenaline (norepinephrine), adrenaline (epinephrine) and dopamine, represent the monoaminergic system of the brain.

The dual role of serotonin is also observed for other catecholamines: besides acting as neurotransmitters between neurons with highly defined and specific projections, catecholamines are synthesized by the adrenal medulla, present in the circulation, and involved in numerous physiological processes (Purves et al. 2008).

Having an integral monoaminergic system whose specific neurotransmitters are used to fine tune similar biological processes may be an evolutionary advantage - once one of

them fails, the enzymes or the final products of the other might be able to complement for the loss of function. This possibility will be discussed further in the course of this work.

1.5.1 Enzymes of the monoaminergic system

There are 3 types of enzymes involved in anabolism and catabolism of monoamines: aromatic amino acid hydroxylases (AAAH), aromatic amino acid decarboxylases (AAAD) and monoamineoxidases (MAO). Additional transporters and receptors are involved in transport of specific neurotransmitters and their precursors through the membranes of the cells, and for specific signaling, respectively.

1.5.1.1. Transporters of the monoaminergic system

To synthesize the monoamines the neurons have to get the essential amino acids from the blood (Figure 1.4). The blood brain barrier, a specific tight endothelial layer covering the capillary network in the brain, does not permit diffusion. Tight junctions of the vascular endothelial cells and the glial cells of the nerve tissue are forming an unsurpassable protective layer, letting only small lipophilic substances and gas molecules to circulate freely between the blood and the brain (Abbott et al. 2006).

Large amino acids like phenylalanine, tyrosine and tryptophan have to be transported actively. The large neutral amino acid transporter (LNAAT) on the membranes of endothelial cells of the blood vessels (Figure 1.4) is using Na^+ current to transport tyrosine, phenylalanine and tryptophan through the blood brain barrier (Leathwood and Fernstrom 1989).

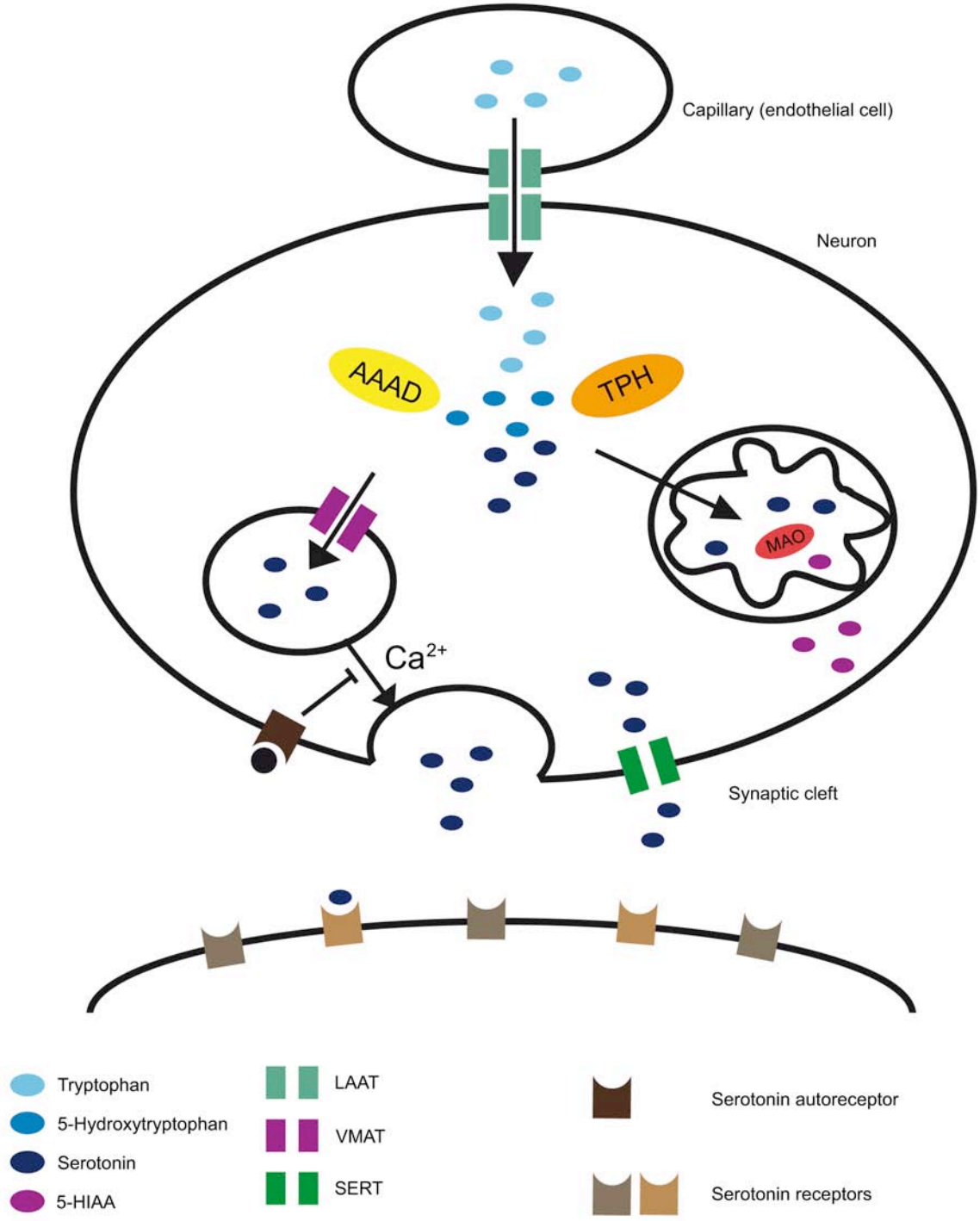


Figure 1. 4. Serotonergic system in the cell.

Serotonin and other monoamines are synthesized in the cytoplasm of the cell body. They are transported to the axonal terminals packed in vesicles (Furuya et al. 1985), waiting for an action potential and Ca^{2+} influx to be released in the synaptic cleft (Figure 1.4). Packing 5-HT and catecholamines in the vesicles requires energy from ATP and a proton

gradient maintained through the membrane of the vesicle, as well as a specific vesicular monoamine transporter (VMAT), present in two isoforms, VMAT1 and VMAT2 (Weihe and Eiden 2000, review). The VMATs are the sites of action of metamphetamines: by blocking the package of monoamines in the vesicles, they stimulate the constant release of the transmitters from the cell, independent of the usual phasic activity of the presynaptic neuron (Rang 2003).

Once in the synaptic cleft, each of the monoamines can be recycled and reused. The specific transporters: serotonergic (SERT), dopaminergic (DAT) and norepinephrine (NET) are importing the redundant neurotransmitters back in the presynaptic neuron to be again stored in vesicles. These transporters are blocked by antidepressants, selective serotonin reuptake inhibitors (SSRIs), as Fluoxetine (Prozac), or are the sites of actions of drugs of abuse, like cocain and amphetamines (George et al. 1998).

1.5.1.2 AAAHs

Aromatic amino acid hydroxylases (AAAHs): phenylalanine hydroxylase (PAH), tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH), form a superfamily of highly conserved enzymes that synthesize the rate limiting step in the anabolism of monoamines: the hydroxylation of the aromatic amino acid. They consist of a variable N-terminal regulatory domain, and a conserved C-terminal catabolic domain (Grenett et al. 1987).

AAAHs are derived from one single PAH gene by gene duplication events: 750 million years ago emerged TH, and around 600 million years ago TPH (Zhao et al. 1994). The PAH gene from bacteria does not have the regulatory domain, as prokaryotic organisms do not use the same regulatory mechanisms as eukaryotes (phosphorylation) (Zhao et al. 1994, Onishi et al. 1991), but the catalytic domain is highly homologous to eukaryotic PAH or PAH, TH and TPH of higher organisms. The three different forms can be found already in *C. elegans,* suggesting its early specialization in evolution (Boularand et al. 1998). Some higher organisms, however, like *D. melanogaster*, has kept enzymes with the ability to hydroxylate both, tryptophan and phenyalalanine (Coleman and Neckameyer 2005), suggesting that only the vertebrates enzymes may have exclusive functions (Neckameyer and White 1992). This question, however, remains open, as it is proven that, at least *in vitro*, the vertebrate PAH can hydroxylate tryptophan (Renson 1962).

AAAHs are metalloproteins that require a non-haem Fe^{2+} ion for their catalytic activity (in some lower organisms Cu^{2+} ion are found (Onishi et al. 1991)). At the C terminal end is a leucine zipper tetramerizing domain. The quaternary structure of AAAHs is deduced from the crystallographic analysis of the rat TH protein (Goodwill et al. 1997). Four monomers form a putative basket structure with Fe^{2+} ion set via His331, His336 and Glu376 and 2 molecules of water in the center of quadratic pyramid. Point mutations that influence the tetramerization of AAAHs might be connected with Parkinson´s disease (TH gene) (Ludecke et al. 1996), phenylketonuria (PAH gene) (Goodwill et al. 1997) or psychiatric diseases (TPH gene) (Bellivier et al. 1998). Until now, TH and TPH were exclusively detected as homotetrameres (Markey et al. 1980, Nakata and Fujisawa 1982, Tong and Kaufman, 1975), while PAH can function as monomer, dimer or tetramer (Kaufman and Fisher 1970, Iwaki et al. 1986). Most probably, the aminoterminal regulatory domain lies as a hinge lid over the catalytic basket (Kumer et al. 1997, Abate et al. 1988), preventing the activity of the enzyme before the activating signal binds and changes its conformation.

Aminoterminal regulatory domain shows remarkable phylogenetic variance. For example, the human TH gene can be found in 4 splice variants in different brain regions (Kaneda et al. 1987), and the isoforms are sex specific, while the closest primates, gorillas, have only 2 isoforms of the same gene (Ichinose et al. 1993). Lower vertebrates, like fishes, have only one (Boularand et al. 1998). The regulatory domain contains the binding sites for the different activators and the sites of phosphorylation that are determining enzyme activity (Mockus and Vrana 1998, Mockus et al. 1997a, Mockus et al. 1997b, Isobe et al. 1991). It is, therefore, easy to imagine that different splice variants of the regulatory domain determine the fine tuning of the activity of the enzyme.

Until recently, functionally active splice variants of TPH in mice were unknown and the sequences of all investigated mice strains were showing 100% similarity to the published *Tph* sequence (Stoll et al. 1990). The variance in the human untranslated 5' region (Boularand et al. 1990, 1995) is very similar to ones found by TH and PAH genes, and may play regulatory role (Dumas et al. 1989, Hart et al. 1991, Kim et al. 1991). Recently, however, it was discovered that TPH in vertebrates is transcribed from 2 different genes: *Tph1* and *Tph2* (Walther et al. 2003). Analysis of the newly discovered *Tph2* gene showed several splicing variants *in vitro* and *in vivo* (Walther 2003) of still unknown function and significance.

1.5.1.2.1 TPH1 and TPH2

The existence of more then one TPH form was speculated in the scientific literature for several decades (Mockus and Vrana 1998, Cash 1998). Different physico-chemical properties of TPH protein isolated from different tissues (Nakata and Fujisawa 1982a, Nakata and Fujisawa 1982b, Yang and Kaufman 1994, Kuhn et al. 1980), antibodies that were detecting TPH protein only in gut but not in the brain (Hasegawa et al. 1987, Chung et al. 2001, Haycock et al. 2002), and different isoelectric points of the protein from the brain tissue that was most probably including pineal gland (Cash et al. 1985), were pointing to at least 2 different forms. More then 100 fold different mRNA/protein ratio detected in the pineal gland and the brain stem (Dumas et al. 1989, Wang et al. 2002, Hart et al. 1991, Austin and O´Donnell 1999) was speculated to be due to different translational efficiencies or post-translational modifications (Cash 1998, Hufton et al. 1995), but it also revealed the possibility of a second TPH isoform, which, at this time, lacked evidence, except in different levels of *lacZ* expression under the *Tph* gene regulation, that showed organ specificity (Huh et al. 1994, Son et al. 1996).

The generation of a mouse deficient in Tph synthesis revealed that all the peripheral organs gut, spleen, thymus and pineal gland were ablated of Tph and serotonin synthesis, but the brain serotonin levels remained normal (Walther et al. 2003). Screening of the Human Genome Database revealed homology of TPH with an unknown gene. The putative *Tph2* cDNA was isolated from mouse, rat, human and zebrafish and the expression of *Tph2* cDNA *in vitro* enabled the expressing cells to synthesize 5-HT, confirming the identity of the newly discovered *Tph* gene.

The expression of Tph isoforms is mutually exclusive: the old *Tph*, now known as *Tph1*, is expressed in the gut, pineal gland, thymus and spleen, while the newly discovered *Tph2* could be detected exclusively in the serotonergic neurons in the brain. Table 1.2. shows the homology of two isoforms between different species.

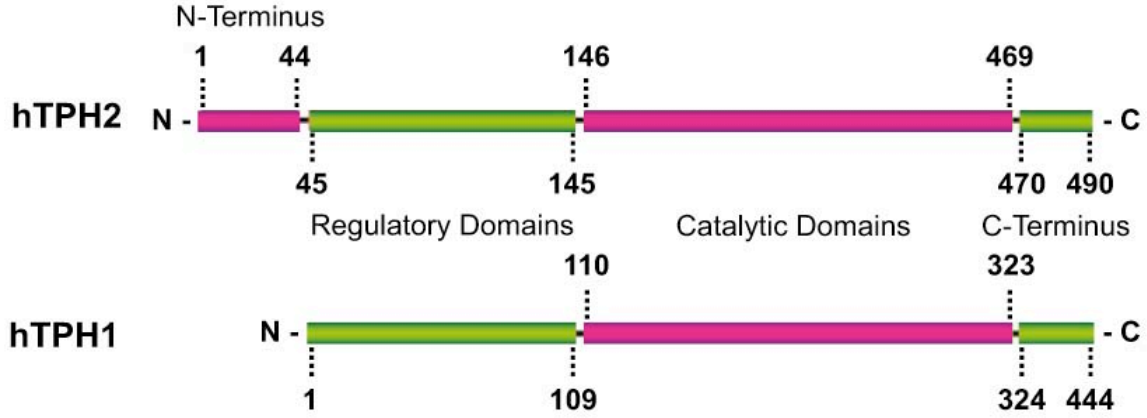
Tph1 and Tph2 share about 66% homology and seem to have highly conserved catalytic domain structure, with conserved binding sites for BH4, Fe^{2+} and Trp, as well as a leucin zipper motif and a hydrophobic domain responsible for multimerization, suggesting the

formation of a tetrameric complex in both TPH variants (Mockus et al. 1997, McKinney et al. 2005, Nakata and Fujisawa 1982). The aminoterminal regulatory domain is most variable and of different length, and most probably responsible for different biochemical properties (Figure 1.6. a, b). However, phosphorylation sites of Ca^{2+} calmodulin kinase II (CaMKII) and protein kinase (PKA) are present in both isoforms (Figure 1.6. b).

	hTPH1	***hTPH2***	***mTPH1***	***mTPH2***	***rTPH1***	***rTPH2***
hTPH1		70%	89%	70%	91%	70%
hTPH2			66%	92%	68%	94%
mTPH1				66%	95%	67%
mTPH2					68%	95%
rTPH1						68%
rTPH2						

Table 1. 2. Sequence homology between different Tph isoforms in different species. Tenner 2008.

a)



b)

```
hTPH1:   1                  M-------------------------IE----------------   3
                            M                          E
hTPH2:   1                  MQPAMMMFSSKYWARRGFSLDSAVPEEHQLLGSSTLNKPNSGKN  44

                                                                  14-3-
           BH4                 hydrophobic interaction           CamKII/PKA
hTPH1:   4  DNKENKDHS-----LERGRASLIFSLKNEVGGLIKALKIFQEKHVNLLHIESRKSKRRNS  58
            D+K NK  S       E G+ +++FSLKNEVGGL+KAL++FQEK VN++HIESRKS+RR+S
hTPH2:  45  DDKGNKGSSKREAATESGKTAVVFSLKNEVGGLVKALRLFQEKRVNMVHIESRKSRRRSS 104

           3                                                 border
hTPH1:   59 EFEIFVDCDINREQLNDIFHLLKSHTNVLSVNLPDNFTLKEDGMETVPWFPKKISDLDHC 118
            E EIFVDC+  + + N++  LLK  T ++++N P+N   +E+ +E VPWFP+KIS+LD C
hTPH2:  105 EVEIFVDCECGKTEFNELIQLLKFQTTIVTLNPPENIWTEEEELEDVPWFPRKISELDKC 164

              BH4
hTPH1:  119 ANRVLMYGSELDADHPGFKDNVYRKRRKYFADLAMNYKHGDPIPKVEFTEEEIKTWGTVF 178
            ++RVLMYGSELDADHPGFKDNVYR+RRKYF D+AM YK+G PIP+VE+TEEE KTWG VF
hTPH2:  165 SHRVLMYGSELDADHPGFKDNVYRQRRKYFVDVAMGYKYGQPIPRVEYTEEETKTWGVVF 224

                                                                BH4
                                                                Trp
hTPH1:  179 QELNKLYPTHACREYLKNLPLLSKYCGYREDNIPQLEDVSNFLKERTGFSIRPVAGYLSP 238
            +EL+KLYPTHACREYLKN PLL+KYCGYREDN+PQLEDVS FLKER+GF++RPVAGYLSP
hTPH2:  225 RELSKLYPTHACREYLKNFPLLTKYCGYREDNVPQLEDVSMFLKERSGFTVRPVAGYLSP 284

                                          BH4
                          Trp             Trp
            BH4       Fe    CamKII        Fe   Fe
hTPH1:  239 RDFLSGLAFRVFHCTQYVRHSSDPFYTPEPDTCHELLGHVPLLAEPSFAQFSQEIGLASL 298
            RDFL+GLA+RVFHCTQY+RH SDP YTPEPDTCHELLGHVPLLA+P FAQFSQEIGLASL
hTPH2:  285 RDFLAGLAYRVFHCTQYIRHGSDPLYTPEPDTCHELLGHVPLLADPKFAQFSQEIGLASL 344

                    BH4   Fe
                BH4 Trp   Trp              Trp
hTPH1:  299 GASEEAVQKLATCYFFTVEFGLCKQDGQLRVFGAGLLSSISELKHALSGHAKVKPFDPKI 358
            GAS+E VQKLATCYFFT+EFGLCKQ+GQLR +GAGLLSSI ELKHALS  A VK FDPK
hTPH2:  345 GASDEDVQKLATCYFFTIEFGLCKQEGQLRAYGAGLLSSIGELKHALSDKACVKAFDPKT 404

hTPH1:  359 TCKQECLITTFQDVYFVSESFEDAKEKMREFTKTIKRPFGVKYNPYTRSIQILKDTKSIT 418
            TC QECLITTFQ+ YFVSESFE+AKEKMR+F K+I RPF V +NPYT+SI+ILKDT+SI
hTPH2:  405 TCLQECLITTFQEAYFVSESFEEAKEKMRDFAKSITRPFSVYFNPYTQSIEILKDTRSIE 464

              Leucine zipper
hTPH1:  419 SAMNELQHDLDVVSDALAKVSRKPSI 444
            + + +L+ DL+ V DAL K+++   I
hTPH2:  465 NVVQDLRSDLNTVCDALNKMNQYLGI 490
```

Figure 1. 6. a) Schematic structure of human *Tph1* and *Tph2* gene b) Sequence comparision of human *Tph1* and *Tph2*. The central line indicates identical and similar (+) amino acid residues. Functionally important residues of TPH1 are marked. Fe: iron (Fe^{2+}) binding site, Trp, tryptophan binding site, BH4, co-substrate binding site, 14-3-3, binding site for 14-3-3 proteins, PKA: protein kinase A phosphorylation site; CaMKII: Ca^{2+}/calmodulin-dependent protein kinase II phosphorylation site, also, the hydrophobic

interaction domain and the leucine zipper involved in multimerization and the border between the regulatory and the catalytic domains are shown (Walther and Bader 2003).

Serotonin is not able to cross the blood brain barrier, which, together with exclusivity of the TPH isoforms in peripheral and central tissues, marks a new level of the dualism of the serotonergic system. Seventy years after its discovery in the blood, and 40 years after its detection in the raphe neurons, the physical and physiological separation of the serotonin synthesis was confirmed on the genetic level (Walther et al. 2003).

The function of serotonin, its synthesis and role in ontogenesis and homeostasis, thus, must be regarded in the scope of this dualism. Now, it is confirmed that *Tph1* and *Tph2* do not complement each others function, as the activity of *Tph1* in the brain and *vice versa,* is low, and not of physiological relevance (Walther et al 2003 and this thesis). The role of serotonin in ontogenesis, however, stays to be elucidated: serotonin is synthesized from the zygote to blastocysts stage (Walther and Bader 1999), before the blood brain barrier is formed, enabling the possibility that serotonin synthesized by both isoforms may contribute to the development of the embryo. There are evidences that serotonin is also actively transported through the placenta, where high concentrations of SERT were detected (Padbury et al. 1997), and, thus, the status of the peripheral serotonin synthesis of the mother may influence the development of the embryo.

By the generation of a Tph2 deficient mouse we expected to provide some answers to those questions in this thesis.

1.5.1.3 Aromatic amino acid decarboxylase (AAAD)

AAAD catalyzes the decarboxylation of aromatic amino acids and their α-methyl derivatives in the anabolic pathway of catecholamines and 5-HT. It is involved in decarboxylation of L-DOPA to dopamine (Henry and Bowsher 1986), 5-HTP to 5-HT (Brodie et al. 1962), but also in synthesis of tryptamine from tyrosine (Juorio and Boulton 1982), 2-phenylalanine from phenylalanine (Jaeger et al. 1984) and tryptamine from tryptophan (Dyck et al. 1983). Its presence in other neuronal types and organs, such as adrenal gland, kidney and liver (Sourkes et al. 1979, Voltattorni et al. 1987), suggests its role in the

metabolic pathways outside the monoaminergic neurons (Kitahama K et al. 1988, Jaeger et al. 1984, Eaton et al. 1993, Skageberg et al. 1988) or the CNS.

1.5.1.4 Monoamineoxidase (MAO)

Monoamineoxidases catalyze the oxidative deamination of primary, secondary and tertiary amines. They are responsible for the degradation of 5-HT to 5-HIAA, after its reuptake in the cell. They exist in 2 isoforms MAO-A and MAO-B, that differ in the specificity of substrate and the inhibitors: MAO-A metabolizes norepinephrine and 5-HT, while MAO-B metabolizes benzamidine (Johnston et al. 1968). Both MAO metabolize tyramine and dopamine (Johnston et al. 1968). Its distribution in the brain reflects its substrate specificity (Collins et al. 1970).

1.5.1.5 Receptors of the serotonergic system

Serotonin exert its effects via the specific receptors. 16 different serotonergic receptors are isolated up to date, that are divided in 7 families (Table 1. 2) by homology, signal transduction pathways and sensitivity to pharmacological modulations (Lucas and Hen 1995, Barnes and Sharp 1999).

Signalling pathway	***Receptor***	***Distribution***	***Physiological role***	***Agonist***	***Antagonists***
cAMP ↓	**5-HT$_{1A}$**	CNS (raphe nuclei, hippocampus, amygdala, septum, enthorinal cortex, hypothalamus) gut (myenteric plexus) spleen	autoreceptor (blocks neurotransmission)	Spiperone Methiothepin 8-OH-DPAT decrease core temperature and impairs shivering: hypotensive, antidepressive, anxiolytic, tachycardic	WAY 100635 Reverse temperature effects – increases T and shivering
	5-HT$_{1B}$	CNS blood vessels	presynaptic autoinhibitory receptor blocks proliferation	GR 55562 SB 224289 Methiothepin Sumatriptan	

cAMP ↓	**5-HT$_{1B}$**	CNS blood vessels	presynaptic autoinhibitory receptor blocks proliferation	against migraine	
	5-HT$_{1D}$	CNS (substantia nigra, basal ganglia, superior colliculus) heart	autoreceptor	Sumatriptan against migraine Anpirtoline analgesic and antidepressant	
	5-HT$_{1E}$	CNS (frontal cortex)	unknown		
	5-HT$_{1F}$	CNS (cortex, hippocampus, striatum, gyrus dentatus, nucleus tractus solitarius, bulbus olfactorius, spinal cord) uterus	autoreceptor	LY 334370 against migraine	
IP_3 ↑	**5-HT$_{2A}$**	CNS (cortex, claustrum, nucleus accumbens) smooth muscles trhombocytes	vasoconstrictor, contractile response of urinary tract, gut, uterus, increase capillary permeability, impairs throbocytes aggregation, stimulates hormone secretion, responsible for some behavioural effects		Ketanserine Ritanserine MDL 100907 against hypertension schizophrenia antipsychotic drugs
	5-HT$_{2B}$	CNS (cerebellum, cortex, amigdala, caudate, substantia nigra, hypothalamus, thalamus, retina) gut heart kidney lung blood vessels endothelial cells of the	smooth muscles contraction vasorelaxation through NO release	anxyolytic effects hyperphagia reduction in grooming efficiency	anti migraine?

		pulmonary arteries			
	5-HT$_{2C}$	CNS (plexus choroidei, globus pallidus, cortex, hypothalamus, septum, substantia nigra, spinal cord)	hypoactivity hypophagia increase penile grooming/erection oral dyskinesia neuroendocrine secretion inhibits dopaminergic and noradrenergic neurotransmission	anxiolytic	increases food intake
Ion channel	**5-HT$_{3A}$** **5-HT $_{3B}$** **5-HT$_{3C}$**	CNS (hippocampus, entorhinalcortex, nucleus accumbens, nucleus motorius dorsalis, amygdala, area postrema, nucleus tractus solitarius, spinal cord) peripheral autonomal and sensory neurons	quick depolarization through Na+ K+ influx, motility of the gut, intestinal secretion increases dopaminergic transmission		Ondansetron Granisetron anti-vomitting antipsychotic anxiolytics
cAMP ↑	**5-HT$_4$**	CNS (hippocampus, striatum, substantia nigra, pre-Boetzingercomplex) gut bladder adrenal gland heart	increases memory through higher neurotransmitter release, contracts oesophagus and gut, triggers acetylcholinic release	Cisapride Tegaserod	Tropisetron
cAMP ?	**5-HT$_{5A}$** **5-HT$_{5B}$**	CNS(cortex, cerebellum, septum, corpus callosum, hypothalamus, hippocampus, fimbria, cerebral ventricules, olfactory bulb, nucleus raphe dorsalis, glial cells)	adaptive behaviour to stress		

cAMP ↑	**5-HT$_6$**	CNS (striatum, amygdala, nucleus accumbens, hippocampus, cortex)	adaptive behaviour and learning	stimulate cetylcholinic transmission	Clozapine antipsychotic Clomipramine antidepressant
cAMP ↑	**5-HT$_7$**	CNS (cortex, septum, hypothalamus, thalamus, amygdala, superior colliculus) smooth muscles	vasodilatation respond to acute stress and antidepressants treatment		Clozapine Risperidon antipsychotic antidepressants

Table 1.2. Tissue distribution, physiological function and pharmacological modulations of serotonergic receptors (Barnes and Sharp 1999, Pauwels 2003).

The complexity of the serotonergic receptor system mirrors the variety of the physiological roles it exerts to maintain the homeostasis of the organism (Table 1.3). The divergence of the receptors spreads further and involves: posttranslational modifications (phosphorylation and glycosylation), tissue specific RNA editing (5-HT2C) and splicing (5-HT4 and 5-HT7), homo- and heterodimerization (5-HT1B/1D) (Hoyer et al. 2002).

G-protein coupled serotonergic receptors posess the usual 7-transmembrane domain structure, and can inactivate (Gi/o 5-HT1, 5-HT5) or stimulate (Gs: 5-HT4, 5-HT6) adenylate cyclase that produces cAMP and stimulates protein kinase A (PKA). 5-HT2 receptors are coupled with Gq that activates phospholipace C, increases the concentration of IP3 and DAG, which increases the concentration of Ca2+ and activates protein kinase C (PKC), respectively (Raymond et al. 2001).

Targeted molecule	***Adult phenotype***	***Developmental defects***	***References***
5-HT1A	Increased anxiety, decreased exploration, reduced effects of antidepressants, altered sleep patterns	Adult neurogenesis, Dendritic maturation (hippocampus) in first 3 weeks of postnatal life	Gross et al. 2002, Cases et al. 1996, Gross et al. 2000, Sibille et al. 2000, Boutrel et al. 2002
5-HT1B	Increased aggression, increased exploration, increased response to	Axon connection defects (retinotectal)	Upton et al. 2002, Saudou et al. 1994, Brunner et al. 1999

	cocaine, altered sleep patterns		
5-HT2A	Decreased response to hallucinogens, enteric nervous system raectivity	Dendritic maturation (phrenic motor neurons)	Bou-Flores et al. 2000, Fiorica-Howells et al. 2002
5-HT2B	Dilated cardiomyopathy	Cardiovascular and enteric neuron embryogenesis (cell survival)	Nebigil et al. 2000, Nebigil et al. 2001
5-HT2C	Feeding behavior, late-onset obesity, audiogenic seizures, altered LTP in hippocampus	Synaptic plasticity	Edagawa et al. 2001, Heisler et al. 1998, Heisler et al. 2003
5-HT3A	Reduced pain behavior	Not known	Zeitz et al. 2002
5-HT4	Altered response to stress, hypersensitivity to seizures	Not known	Compan et al. 2003
5-HT5A	Increased exploration	Not known	Greihe et al. 1999
5-HT7	Abnormal thermoregulation	Not known	Hedlund et al. 2003
Sert	Altered 5-HT homeostasis, modified response to drugs of abuse	Axon connection defects (retinal, thalamic, barrel field), decreased apoptosis	Salichon et al. 2001, Persico et al. 2003, Bengel et al. 1998, Persico et al. 2001
MAOA	Increased aggression, Increased fear conditioning, reduced exploration, altered beam walking	Axon connection defects (first postnatal week), dendritic exuberance (medulla), neuropeptide expression (hypothalamus)	Cases et al. 1995, Upton et al. 1999, Cases et al. 1996, Rebsam et al. 2002, Bou-Flores et al. 2000, Vacher et al. 2003
MAOB	Increased reactivity to stress	Not known	Grimsby et al. 1997
VMAT2	Lethal, altered feeding/growth	Increased apoptosis in telencephalon, altered migration	Fon et al. 1997, Persico et al. 2001, Wang et al. 1997, Takahashi et al. 1997, Alvarez et al. 2002

Table 1.3. Mouse mutant phenotypes for the enzymes of monoaminergic system (Gaspar et al. 2003).

2. SEROTONIN AS NEUROTRANSMITTER

2. 1 Serotonergic system in the brain

Of about 10^{10} neurons building the rodent brain, only 20 000 are immunoreactive for serotonin (Jacobs and Azmitia, 1992). The ratio is even bigger in humans – 300 000 serotonergic neurons are detected among approximately 100 billion brain neurons (Dahlström and Fuxe 1964, Glencoe Health 2nd Edition 1989). The small bunch, however, projects to virtually all brain regions and spinal cord (Azmitia and Whitaker-Azmitia, 1991), and is involved in almost everything the brain does: from integrating the signals of the environment and regulating the basic physiological functions such as consistent body temperature or autonomic function (sympathetic/parasympathetic response), to reaction control through some of the highly tuned features of the brain, as anxiety, learning, depression or stress adaptation (reviewed in Azmitia 2007). Located in a narrow line along the brain stem (Figure 1.8), spreading from medulla oblongata to midbrain, the cell bodies of serotonergic neurons are grouped in so called raphé nuclei (B1-B9) and considered to be the medial portion of the reticular formation (Dahlström and Fuxe 1964). A small number of immunoreactive cells can be found scattered outside the nuclei, in the brain stem and reticular formation (Jacobs and Azmitia 1992). Raphé nuclei can be divided into a caudal group in the medulla (including the raphé pallidus, raphé magnus and raphé obscurus in the midline and the parapyramidal region on the VLMS), and a rostral group in the pons and midbrain (including the pontine raphé, dorsal raphé and median raphé). These two groups have different embryological origin and projections (Lidov and Molliver 1982, Gaspar et al. 2003, Cordes 2005, Jensen et al. 2008). Those in the medulla project throughout the medulla and spinal cord, where they influence breathing, cardiovascular control, autonomic output, motor control and pain processing. Those in the midbrain raphé project throughout the forebrain, and are associated with arousal, anxiety and aggression, and control of cerebral blood flow. Raphé nuclei do not consist of serotonergic neurons only, but have intermingled catecholamine, glutamate and substance P producing neurons, that may be found even in the same vesicles that transport serotonin (Pelletier et al. 1981).

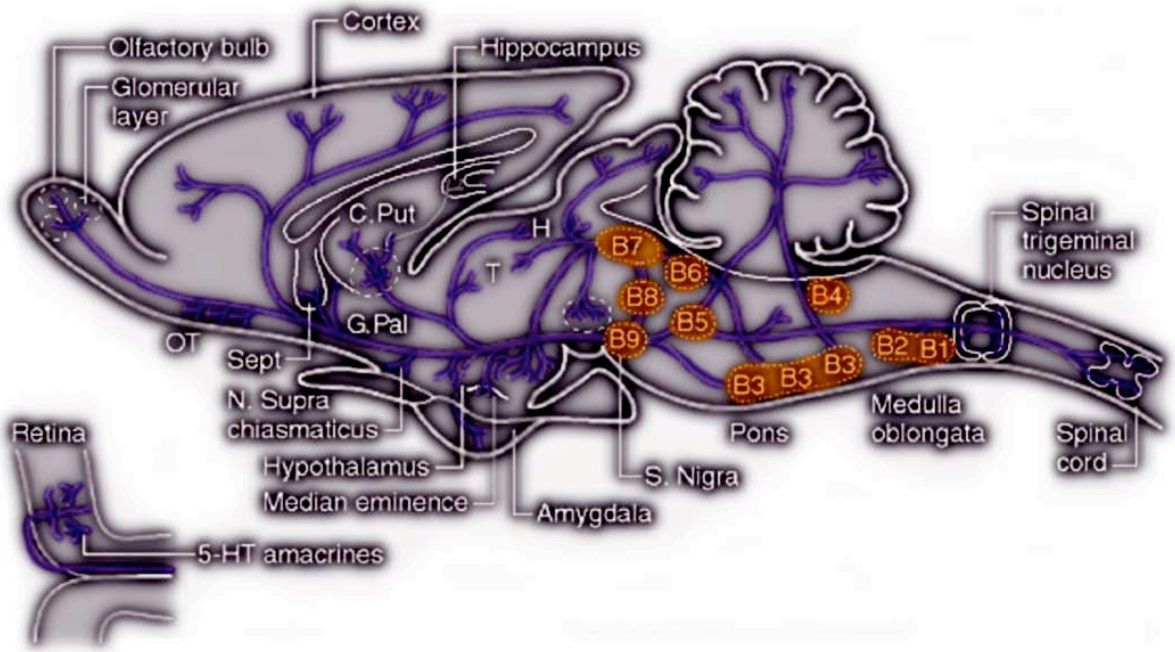


Figure 1.8. Serotonergic projections of a rat brain (sagittal section). Raphé nuclei are marked B1-B9, OT - olfactory bulb, Sept - septum, C. Put - Nucleus caudatus putamen, G. pal - globus pallidus, T - thalamus, H - habenulae, S. nigra - substantia nigra (Modified from Consolazione and Cuello 1982).

Creation of Tph2 deficient mouse during the course of this work made available a live model system for elucidating the role of serotonin in the control of the autonomous nervous system function, its influence on physiological functions, like ion homeostasis or temperature regulation, and involvement in many behavioral responses, such as maternal care, aggression and sexual behavior, to mention just a few.

3. EMBRYONIC STEM CELL TECHNOLOGY:
KNOCK-OUT MOUSE MODEL AND IN VITRO DIFFERENTIATION

3.1 Embryonic stem cells

Embryonic stem cells (ESC) are cells derived from the outgrowth of the inner cell mass (ICM) of the pre-implantation blastocyst stage of the embryo (Evans and Kaufman 1981, Martin 1981). ESC can be cultured *in vitro*, either on feeder layer or by addition of cytokines. If handled properly, they maintain proliferative potential and stable karyotype for a long time. ESC are pluripotent and have the capacity of self-renewal (Silva and Smith 2008, Ying et al. 2008, Conti et al. 2005).

Self-renewal is an asymmetrical cell division, where one cell keeps the self-renewal capacity, while another one is getting the signals to undergo differentiation. It is a life maintaining process involved in development, regeneration and plasticity of the organism, and the signaling involved in the equilibrium between the self-renewal state and the differentiation is one of the most intriguing questions of science today (Ying et al. 2008).

Pluripotency is the possibility of differentiation to all three germ layers: ectoderm, endoderm and mesoderm. It is an intrinsic property of ICM, and, thus, excludes only the possibility to develop into trophectoderm and extraembryonic endoderm, that are surrounding ICM before and after implantation. Pluripotency can be recapitulated *in vitro*, but its timing and the signaling pattern is still to be elucidated (Chambers et al. 2007, Silva et al 2006, Smith 2005).

3.1.1 Investigating function through ablation: knock-out mouse

The ability to propagate ESC in culture maintaining a stable karyotype, enabled *in vitro* transgenesis and mutagenesis. Genetically modified ESC could then be injected into a new blastocyst (the same stage from which they were isolated - 3.5 day post fertilization), and transplanted to a pseudopregnant female mouse, that will give birth to chimeras: a mosaic offspring that has cells with the genotype of the ESC and the blastocysts used for injection. If

the ESC in culture were from a white mouse, and the host blastocysts originated from a black mouse, chimeras born after the injection will be black and white, as the ESC will contribute to all tissues including epidermis and hair colour. The mosaicism of chimera colour reflects the mosaic genotype of the animal (Figure 1.9), and may be extrapolated to all tissues - all white haired cells are originating from ESC, while all black cells are originating from the donor blastocysts.

The extent of ESC contribution to chimera is a random process - if they contribute to the germ line differentiation in the gonads, the genetic manipulation will be transmitted to the offspring. If integrated stably in the genome, the manipulation can be inherited indefinitely, and different mouse strains with a particular mutation can be generated after backcrossing for several generations.

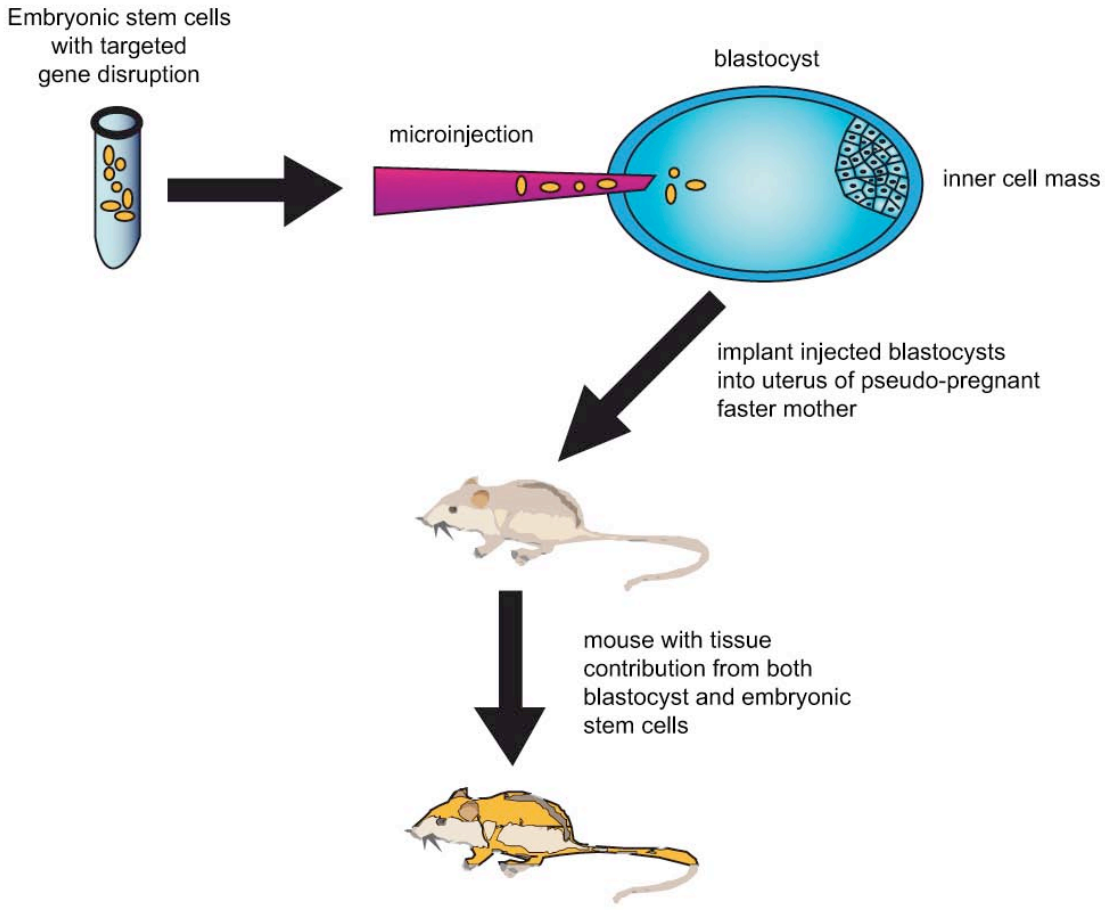


Figure 1.9. Chimera formation. For details see text.

The technology of homologous recombination in ESC in vitro was developed by Oliver Smithies and Mario Capecchi, several years after Evans and Kaufmann succeeded in isolating, maintaining, manipulating and injecting ES cells to obtain a chimeric mouse. They also augmented its efficiency by introducing a negative (thymidine kinase selection (TK)) and positive selection method (neomycine resistance (neo^R)) (Thomas et al. 1986, Wong and Capecchi. 1986, Smithies et al. 1985). This enabled the creation of the first knock-out mouse (KO) model by exchanging the exon 8 of the *Hprt* gene with neo^R (Thomas and Capecchi 1987). Homologous recombination can be used not only for the ablation of a gene function, but for any genetic manipulation that requires the integration in the particular place of the genome, like the first point mutation exchange in the Hprt gene in the O. Smithies lab (Doetschman et al. 1987). Today, many different approaches are used to target a particular locus in a mouse genome, like gene trapping or creating conditional KO strains, where the expression of the gene is driven in particular cell type or the genes of interest are deleted in chosen tissues only, respectively (Clarke 2000).

Since the late eighties many KO mouse models were generated, and became a common tool of biomedical research. Both, EU and the United States are creating the facilities for keeping KO mouse stocks and making it available to public research. International Knockout Mouse Consortium (Gondo 2008), founded in 2007, has as an aim to establish mouse KO strains for all the mouse genes and to overcome the gap between genetics that provided us with the complete sequence of the mouse genome in 2002, and functional genomics that culminated with the development of bioinformatics. Studying the function of the gene in its physiological surrounding is the first step in determining the penetration and the complexity of the phenotype. It is, also, a valuable tool for evaluating its therapeutic potential. The recent drug development is tightly connected with creating KO mouse models with defined pathologies (Zambrowicz and Sands 2003).

O. Smithies, M. Evans and M. Capecchi shared the Nobel Prize for Medicine in 2007, for developing the technology for isolation, maintainance in culture and re-injection of ESC into a new blastocyst, that were able to attribute to chimerism of the offspring (Evans), and for integrating the foreign DNA by homologous recombination into ESC (Capecchi and Smithies) and creating the first KO mouse.

3.1.2 In vitro differentiation from embryonic stem cells (ESC)

The phenomenon of in vitro differentiation was first noticed and described by Martin and Evans, 1975, while they were still working with teratocarcinomas. They noticed that embryonal carcinoma (EC) cells devoid of feeder layer tempt to form aggregates referred to as embryoid bodies (EBs) (Martin and Evans 1975) that are similar to teratocarcinomas (containing the cells of all three germ layers). If kept as floating cell clumps, this embryoid bodies were developing further in a cystic formation, ressembling the morphology of a developing embryo, and after attachment to a tissue culture surface they were able to give rise to cells of all three germ layers, but not trophectoderm. ESC, after isolation, showed a similar behavior in culture, and the ability to form almost all cell types when stimulated with specific sets of growth factors and under specific conditions.

Since then, several methods for ESC differentiation were developed. The EB protocol was refined and stayed the first choice for differentiating ESC in culture, as it gives rise to all cell lineages (Doetschman et al. 1985). The flaws of the protocol, such as random fraction of cells of each germ layer and the robustness of the outcome lead to a development of protocols that differentiate ESC in a monolayer, either on extracellular matrix proteins (Nishikawa 1998) or on an additional layer of feeder cells (Nakano et al. 1994). All three protocols (Figure 1.10) have advantages and disadvantages: EBs promote cell-cell interactions in the floating cell clumps, but the inducing factors are too complicated for interpretation, as too many cell types are involved; specific stromal cell lines induce specific differentiation, but provide ESC with unidentified factors and make the separation of the two cell types difficult; monolayer on simple matrix proteins is the 'cleanest' protocol, but it does not give all the cell lineages efficiently enough (Keller 2005).

The isolation of human ESC (hESC) in 1998 (Thomson 1998) opened the possibility of stem cell therapy. The replacement of impaired cells in the adult, like, for example, dopaminergic neurons in Parkinson disease, with differentiated DOPA neurons in culture from the stem cells of a matching donor, would lead to a new era in medicine. However, the controversy following this research, as well as technological and functional uncertainty and batch-to-batch variability of the differentiation outcome, have to be overcome before the cell replacement therapy becomes reality (Lindvall et al. 2004, Strauer and Kornowski 2003).

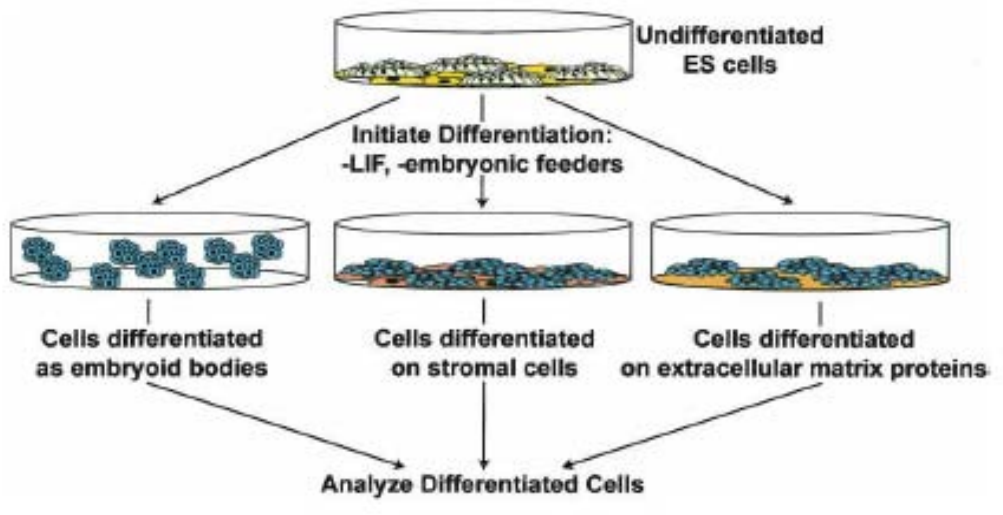


Figure 1.10. Three methods in use for ES cell differentiation in culture. For details see text.

3.1.2.1 In vitro differentiation of embryonic stem cells towards serotonergic neurons

The technology of ESC differentiation has as a main goal to produce a functional specific cell type, in a high percentage and with a high reproducibility, under clear and controlled conditions. This goal is not easily achievable, as ESC require intrinsic and extrinsic signaling for differentiation that always includes more than one cell type, as well as the surface to attach before leaving the mitotic state. For example, neuronal differentiation always include glial cells, as they derive from the same precursor, and all three cell types co-exist in neuronal cultures: neurons, glia, and the mitotic precursors. Those cultures are not safe for transplantation, as the precursor cells may continue the renewal *in vivo* and cause cancer. Trans-differentiation, a process of one cell type transforming into another under the influence of specific signaling, or de-differentiation back to self-renewal state are some of the processes still uncleared and uncontrolled in an artificial system. How the higher organisms keep their plasticity without developing tumorigenicy, is a matter of the current research efforts throughout the scientific community.

Efficient production of neurons was first achieved during EBs differentiation with super-physiological dose of RA (1μM) (Bain et al. 1995, Li et al. 1998). This protocol is still in use, as it gives rise to high proportions of neurons in culture, but has several flaws. RA is perturbing neuronal patterning, as it does *in vivo*, and suppresses production of some neuronal

identities, like forebrain neurons (Soprano and Soprano 1995, Sucov and Evans 1995), and the real inductional force is difficult to be elucidated as the EBs contain mesodermal and endodermal lineages.

Serotonergic differentiation from ESC was until now, performed in detectable percentage in two different conditions: EBs and stromal differentiation on a layer of PA6 cells (Lee et al. 2000, Kawasaki et al. 2000).

The five stages EBs protocol developed in Ronald McKay´s lab (Lee et al. 2000) gave rise to certain percentages of dopaminergic and serotonergic neurons that were shown to be functional, at least *in vitro*. The first stage promotes the expansion of ESC, second stage promotes the formation of EBs, third stage applies a specific medium and the attachment of EBs on a tissue culture surface that leads to expression of nestin, a marker for neuronal precursors, fourth stage expands nestin positive cells after the application of a mitogen, bFGF (FGF2), on the laminin surface and the fifth stage induces the differentiation into dopaminergic and serotonergic neurons, that are developed from the same precursor and can be detected by staining with tyrosine hydroxylase (TH) and serotonin (5-HT), respectively. The percentage is not high: 7% of TH positive and 11% of 5-HT positive cells can be obtained, but the cells are functional and are able to secrete the neurotransmitters after depolarization.

PA6 stromal cells are derived from skull bone marrow (Kodama et al. 1986), and it was discovered through the screening for neuron producing feeder cell lines. Up to 92% of the colonies were positive for neuronal markers, such as NCAM (N-cadherin) by the twelfth day of induced differentiation (Kawasaki et al. 2000). Immunocytochemical studies showed that the colonies were containing 30% of dopaminergic, 9% of GABAergic, 5% of cholinergic and 2% of serotonergic neurons.

The factors that run the differentiation towards those particular cell types are still unknown, but the development of protocols that give a mixture of specific cell types opened the possibility to amplify the ratio of one of the neuronal cell types by adding a defined cocktails of factors in culture, and, thus, simulating the signaling in vivo. Components of such a cocktail would be sonic hedgehog, fibroblast growth factor 8 and fibroblast growth factor 4

(SHH/FGF8/FGF4), morphogens known to promote dopaminergic and serotonergic neurons *in vivo*.

3.2 Serotonergic differentiation during embryogenesis

Serotonergic neurons in the brain are restricted to a brain stem region and, in small part, to the reticular formation. They are generated around embryonic day (E) 10 to 12 in mouse, but the full maturation of the axonal terminal network requires more time and is achieved only after birth in rodents (Lidov and Molliver 1982).

Organogenesis during embryonal development starts after gastrulation and the formation of three germ layers: ectoderm, mesoderm and endoderm. The primitive axis of the embryo is then defined by the notochord of mesodermal origin, that secretes morphogens like SHH to establish the dorso-ventral patterning of the embryo. Dorso-ventral patterning includes neuronal identities in the neural tube, arising from neural ectoderm via the neural plate. The neural tube, in its rostral region, will form the brain, divided in forebrain (prosencephalon), midbrain (mesencephalon) and hindbrain (rhombencephalon), and, in its caudal region, the spinal cord. The serotonergic neurons arise in the hindbrain (Figure 1.11).

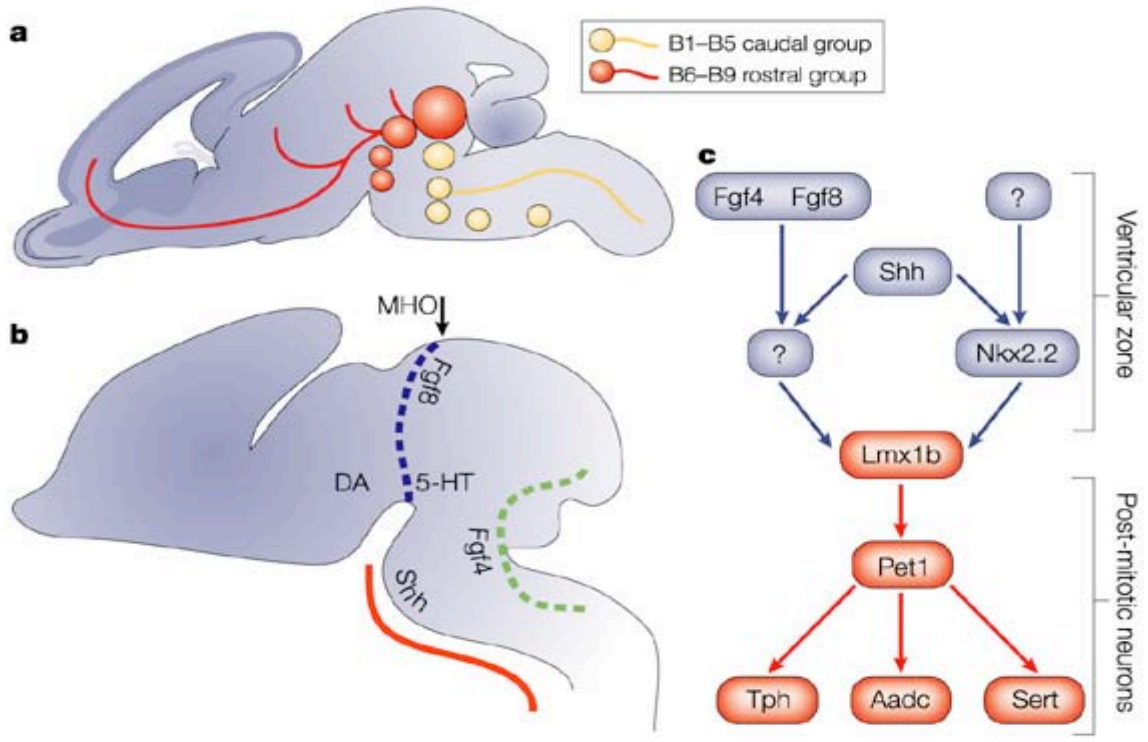


Figure 1.11. a) raphe nuclei in the brain stem (B1-B9) b) specification of serotonergic neurons during embryogenesis is determined by the combination of morphogens. Fgf8 - fibroblast growth factor 8, Fgf4 – fibroblast growth factor 4, Shh – sonic hedgehog c) transcriptional network controlling the serotonergic phenotype in raphe nuclei. Blue labeled factors are specific for neuroblasts in the ventricular zone. Red labeled factors induce terminal differentiation into functional serotonergic neurons (Gaspar et al. 2003).

FGF8, FGF4 and SHH, produced by notochord, the primitive streak and the midbrain – hindbrain organizer (MHO), respectively, act together to produce 5-HT precursor cells in the area defined by this signaling (Ye et al. 2001) – rhombencephalon (Figure 1.11). MHO, defined by the expression of the transcription factors Otx2 and Gbx2 is a major determinant for the development of serotonergic neurons. They appear caudal of it and dopaminergic neurons are generated rostrally. When the organizer is moved, the area of one transmitter is increased to the expense of another (Brodski et al. 2003). However, there are many other determinants of serotonergic phenotype. The rhombencephalon is divided in rhombomeres, morphological subunits of the hindbrain, with different transcriptional programs (r1 - r7). The development of the specific cell type within each rhombomere is time and space specific, and involves the communication of morphogens through the grade of concentration, with the factors secreted by the rhombomeres and some other organizers, like the floor plate or the roof plate of the neuronal tube or prechordal mesoderm (such as RA, BMPs, OTX, WNTs). Serotonergic neurons are developing, from the precursors that already gave rise to branchial and visceral neurons, in all the rhombomeres, except r1, which never generates motoneurons, and r4, which carries on producing motoneurons and never gets serotonergic (Figure 1.12).

3.3 Transcription factors involved in serotonergic neurons differentiation

3.3.1 Phox2b

Phox2b (paired-like homeodomain protein 2b) is a transcription factor belonging to the Q50 paired-like class (Brunet and Pattyn 2002) which represses serotonergic differentiation in r2-r7. Phox2b deficient mice lack all visceromotor neuron precursors and serotonergic neurons are extensively produced in r2-r7, including r4 (Pattyn et al. 2003a), confirming that Phox2b is a central repressor of serotonergic fate. The formation of serotonin

neurons is enabled in r2-r3 and r5-r7 through inhibition of Phox2b by Nkx2.2, whereas in r4, Hoxb1, Nkx6.1, and Nkx6.2 sustain its expression and thereby block serotonergic differentiation (Pattyn et al. 2003b).

3.3.2 Mash1

Mash1 (mouse achaete-scute homolog 1) is a basic-helix-loop-helix (bHLH) transcription factor, which is already detected in r1-r7 during motor neuron generation but becomes only essential when serotonergic neurons are developed in this zone. Thus, in Mash1-deficient mice no cells expressing the downstream factors Pet1, Lmx1b, Gata2, Gata3, and also no serotonergic neurons appear (Pattyn et al. 2004). However, Nkx2.2, Phox2b, and SHH retain their normal pattern of expression in these mice. Furthermore, Mash1 specifies the serotonergic phenotype in neural crest derivatives like enteric and other peripheral neurons (Blaugrund et al. 1996).

3.3.3 Nkx2.2

Nkx2.2 (NK transcription factor related, locus 2) is expressed transiently starting at E10.5 in all serotonergic precursors. Mice lacking this factor do not express Gata3, Lmx1b, and Pet1 in caudal raphe nuclei and no serotonergic neurons develop in this area in contrast to the dorsal raphe nuclei where all these factors and such neurons persist (Pattyn et al. 2003, Briscoe et al. 1999, Ding et al. 2003). Together with Lmx1b and Pet1 it can induce ectopically the development of serotonergic neurons in the chick neural tube (Cheng et al. 2003).

3.3.4 Lmx1b

Lmx1b (LIM homeobox transcription factor 1β) is required for the formation of the entire serotonin system in the hindbrain, since its deletion in mice leads to the absence of such neurons in the brain (Ding et al. 2003, Cheng et al. 2003, Briscoe et al. 1999). It is expressed in developing serotonergic neurons together with Pet1 starting around E11 in the rostral cluster of serotonergic differentiation and one day after in the caudal one consistent with the delayed appearance of serotonergic cells in the latter region (Cheng et al. 2003).Its ablation does not affect the expression of Nkx2.2, Gata3, and SHH and only partly the one of Pet1,

putting these factors upstream or in parallel to Lmx1b (Ding et al. 2003, Cheng et al. 2003). Together with Nkx2.2 and Pet1 it can induce ectopically the development of serotonergic neurons in the chick neural tube (Cheng et al. 2003). In addition, Lmx1b is important for the development of dopaminergic neurons (Smidt et al. 2000).

3.3.5 Pet1

The ETS domain transcription factor Pet1 (Pheochromocytoma 12 ETS (E26 transformation-specific)) is a specific marker for all serotonergic neurons from E11 until adulthood (Scott et al. 2005a). Recently, this unique specificity was confirmed by the use of the Pet1 promoter to target marker genes exclusively to 5-HT neurons in transgenic mice (Scott et al. 2005b). Pet1 binding sites are found in the promotor regions of several genes expressed in serotonergic neurons such as AAAD and SERT (Hendricks et al. 1999). In mice lacking Pet1, 70% of serotonergic neurons fail to differentiate, whereas in the remaining Pet1-deficient neurons diminished expression of VMAT2, TPH and SERT was observed (Hendricks et al. 2003). These animals survive but show anxiety-like and aggressive behavior.

3.3.6 Gata2 and Gata3

Six Gata (GATA-motif binding) transcription factors exist in vertebrates characterized by C4-type zinc-finger motifs and two of them, Gata2 and Gata3, are expressed in the developing brain (Patient and McGhee 2002). Experiments in chicks show, that Gata2 is necessary and sufficient for the induction of Lmx1b and Pet1 and serotonergic neurons in r1, but not more caudally (Craven et al. 2004). In hindbrain explant cultures of Gata2 deficient mice, no 5-HT neurons are developed indicating that Gata2 maybe also pivotal for serotonergic differentiation in general. In contrast, Gata3 is not required for the differentiation of the rostral 5-HT neurons (van Doorninck et al. 1999) and appears unable to substitute for the loss of Gata2 in r1. However, in Gata3-deficient mice, around 80% of serotonergic neurons in the caudal clusters and 30% in the rostral clusters are missing (Pattyn et al. 2004). Nevertheless, the expression of Pet1 and Lmx1b was unchanged in Gata3 knockout mice showing that these factors act in parallel.

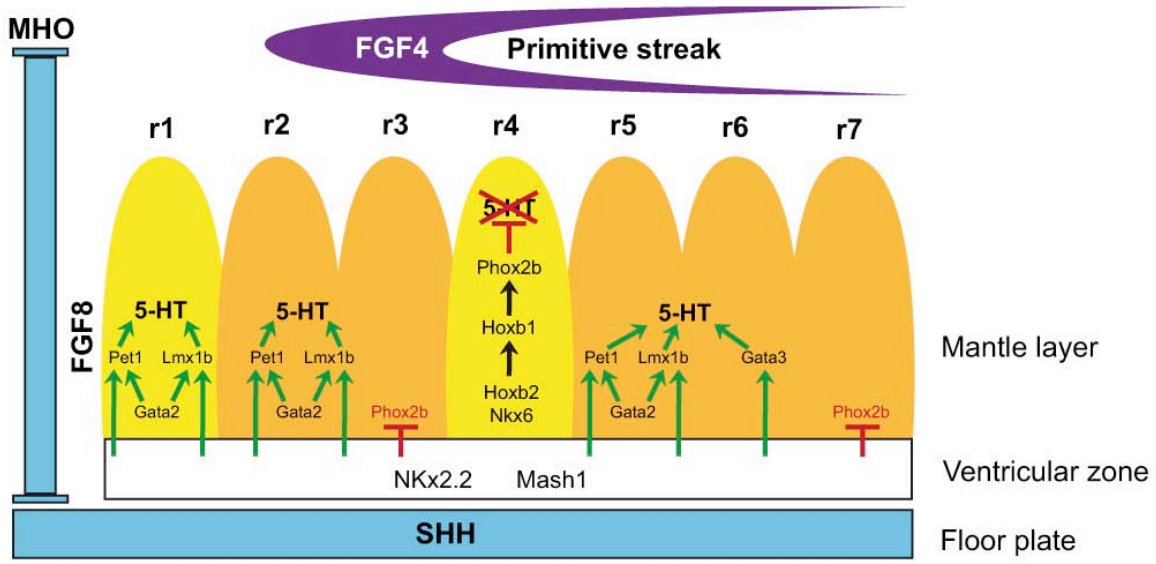


Figure 1.12. Induction and differentiation of serotonergic neurons in vivo (see text for explanation). MHO, midbrain-hindbrain organizer; r1-r7, rhombomeres 1-7; SHH, sonic hedgehog, FGF, fibroblast growth factor (modified from Alenina et al. 2006).

3.4 FunGenES

Functional Genomics in Embryonic Stem Cells (FunGenES) was an Integrated Project funded by the 6th framework program of the European Union, that had the aim to achieve a basic understanding of the processes of self-renewal and differentiation.

This was to be achieved by the development of efficient protocols for differentiation of ESC into lineage commited cells of all three germ layers and further into a multitude of somatic cells. Creating engineered ESC lines, that express marker genes under the control of lineage or cell type specific promoters was supposed to provide a selectable system for each cell type that will be suitable for Affymetrix analysis, and for the discovery of candidate genes for further functional screening. The global data analysis was supposed to show the transcriptional network responsible for self-renewal of ESC, as well as their differentiation towards specific cell types. After the analysis, the data were supposed to be published as a genotypic and phenotypic atlas of each cell type and ESC in self renewal state and given to general and scientific public for further use and investigation.

All transcription factors involved in serotonergic differentiation and their hierarchy were deduced from creating the knock-out mouse models for the genes of interest. Large scale screening during serotonergic differentiation in culture would confirm the data *in vivo*, opening the possibility of investigating the factors with the same expression pattern as the known genes, and, eventually, to the discovery of new inducers or inhibitors of the serotonergic phenotype.

4. AIMS – TARGETING TPH2 GENE

The Tph2 gene can be used as a marker gene for the serotonergic lineage as it is expressed exclusively in serotonergic neurons. Its expression starts around E12 in mice after the expression of the regulatory transcriptional network, that includes *Pet1* as the first, postmitotic, upstream regulator. In CNS, *Pet1* is found in serotonergic neurons exclusively, and can be used as a marker gene for those neurons together with *Tph2* (Hendrick et al, 2003).

The creation of a mouse model with ablation of Tph2 will elucidate the function of this *Tph* isoform in brain serotonin generation and in a variety of psychological and physiological processes.

Several aims were defined at the beginning and during the course of this study:

1) The first aim was to create a construct that targets the *Tph2* gene by the method of homologous recombination (knock-in construct). The construct should recombine at the 5' region of the gene and use the endogenous *Tph2* promoter to express neomycine resistance (neo^R) and dsRed genes (dsRed), enabling the positive selection (by applying neomycine) and fluorescence sorting (FACS) by detecting the red fluorescence. At the same time, the construct will prevent the transcription of the endogenous *Tph2* gene, leading to the ablation of one allele of the gene in the ESC, and creating a knock-out mouse model after obtaining chimeras, heterozygous and homozygous offspring.

2) The selection of serotonergic neurons will allow the isolation of RNA expressed in this cell type, only. This RNA can be used for microarray analysis, that will define the genes essential for development and survival of serotonergic neurons.

3) To this purpose, an efficient protocol of serotonergic differentiation out of ESC was supposed to be established. Starting from the embryoid bodies protocol, obtained as a part of FunGenES consortium, modifications should be established that would lead to effective development of serotonergic neurons in culture.

4) The *Tph2*$^{-/-}$ knock-out mouse should be established and it was expected that the animal will be completely devoid of serotonin synthesis in the brain. Thus, the functionality of this system could be investigated *in vivo*.

II MATERIALS AND METHODS

5. MATERIALS

5.1. Chemicals

Chemical	*Company*
Acetic acid	Roth, Karlsruhe, Germany
Acrylamid-/Bisacrylamid 19:1	Roth, Karlsruhe, Germany
Acrylamid-/Bisacrilamid 37,5:1	Roth, Karslruhe, Germany
Ampicillin	Serva, Heidelberg, Germany
APS	Sigma, Steinheim, Germany
Ascorbic acid	Sigma, Steinheim, Germany
B27 50x	Gibco, Paisley, Scotland, UK
Bactoagar	Difco Microbiology, USA
Bactotrypton	Difco Microbiology, USA
BDNF	Sigma, Steinheim, Germany
Blasticidin	Gibco, Scotland, UK
Bradford reagent	Sigma, Steinheim, Germany
Bromphenolblue	Sigma, Steinheim, Germany
BSA	Sigma, Steinheim, Germany
bFGF	R & D systems, GmbH, Germany
Complete Protease Inhibitor Cocktail Tablets	Roche, Mannheim, Germany
DAPI	Sigma, Steinheim, Germany
DEPC	Serva, Heidelberg, Germany
DMEM:F12	Gibco, Scotland, UK
DMSO	Sigma, Steinheim, Germany
dNTPs	Amersham Biosciences, NJ, USA
D-PBS (Ca^{+}, Mg^{+})	Gibco, Paisley, Scotland, UK
DTT	Sigma, Steinheim, Germany
EDTA	Sigma, Steinheim, Germany
Ethanol	Roth, Karlsruhe, Germany
Ethidiumbromid	Sigma, Steinheim, Germany

FGF8b	R & D systems, Germany
FGF4	R & D systems, Germany
Fluorescent mounting medium	Dako, Denmark
G418 50mg/ml	Gibco, Scotland, UK
Gelatin 2%	Gibco, Paisley, Scotland, UK
Glucose	Sigma, Steinheim, Germany
Glutamine	Gibco, Paisley, Scotland, UK
Glycerol	Roth, Karlsruhe, Germany
GMEM	Sigma, Steinheim, Germany
HCl	Roth, Karlsruhe, Germany
Hygromycin B	Gibco, Scotland, UK
IPTG	Fermentas, Burlington, CDN
Knockout serum replacement	Gibco, Scotland, UK
Laminin	Sigma, Steinheim, Germany
Matrigel	BD Biosciences, Bedford, MA, USA
Methanol	Roth, Karlsruhe, Germany
Milk powder	Roth, Karlsruhe, Germany
Mitomycin C	Sigma, Steinheim, Germany
N2 100x	Gibco, Paisley, Scotland, UK
N2B27	Stem Cell Sciences, Edinburgh, UK
NaCl	Roth, Karslruhe, Germany
NaOH	Roth, Karlsruhe, Germany
Natriumacetat	Sigma, Steinheim, Germany
Non-essential amino acids	Gibco, Paisley, Scotland, UK
PBS 1x	Gibco, Paisley, Scotland, UK
Penicillin/Streptomycin	Gibco, Paisley, Scotland, UK
PFA	Roth, Karlsruhe, Germany
Poly-D-Lysine	Sigma, Steinheim, Germany
Random Hexamer Primer	Boehringer, Mannheim, Germany
Retinoic acid	Sigma, Steinheim, Germany
RNasin	Promega, Madison, WI, USA
RHB-A	Stem Cell Sciences, Edinburgh, UK
Serum (FCS)	Invitrogen, Scotland, UK
Sodium Pyruvate	Gibco, Paisley, Scotland, UK

SDS	Serva, Heidelberg, Germany
SHH	R & D systems, Germany
TEMED	Sigma, Steinheim, Germany
Tris	Roth, Karlsruhe, Germany
Triton X-100	Sigma, Steinheim, Germany
TRIZOL	Invitrogen, Carlsbad, CA, USA
Trypan blue 0.4%	Gibco, Paisley, Scotland, UK
Tween-20	Sigma, Steinheim, Germany
X-Gal	Fermentas, Burlington, CDN
β-Mercaptoethanol	Sigma, Steinheim, Germany

5.2. Enzymes, kits and markers

Nucleic acids manipulation and purification

Product	***Company***
Expand™ Long Template PCR system	Bühlmannn Laboratories, Salzburg, AT
Jet Star Plasmid Purification MAXI Kit 2.0	Genomed, GmbH, Löhne, Germany
QIAquick Gel Extraction Kit	Qiagen, Hilden, Germany
QuickSpin Columns for radiolabeled DNA purification	Roche, Mannheim, Germanyy
Wizard SV Gel and PCR Clean-up System	Promega, Madison, WI, USA
DnaseI	Roche, Mannheim, Germany
M-MLV Reverse Transcriptase	Promega, Madison, WI, USA
Proteinase K	Merck, Darmstadt, Germany
Restriction Enzymes	New England Biolabs, Ipswich, MA, USA
RNase A	Boehringer Mannheim, Germany
T4-DNA-Ligase	Promega, Madison, WI, USA
*Taq*DNA Polymerase	Invitrogen, Carlsbad, CA, USA
Prime-It RmT Random Primer Labeling Kit	Stratagene, LaJolla, CA, USA
RNeasy Mini Kit	Qiagen, Hilden, Germany
RNase-Free DNase Set	Qiagen, Hilden, Germany

QantiTect SYBR Green PCR Kit	Qiagen, Hilden, Germany

Immunoassays

Product	***Company***
R.T.U. VECTASTATIN Universal$_{Elite}$ ABC Kit	Vector Laboratories, Burlingame, CA, USA
SuperSignal West Dura Extended Duration Substrate	Thermo Fisher Scientific, Bonn, Germany
IGFI EIA Kit	Diagnostic System Laboratories, Webster, TX, USA
FSH ELISA Kit	Alpco Immunoassays, Salem, NH
Serotonin ELISA Kit	Raybiotech, Inc., Norcross, GA

Markers

Product	***Company***
Precision Blue Protein^{TM} Standard All Blue	BioRad Laboratories, Richmond, USA
λ DNA/*EcoRI* + *HindIII* Marker, 3	Fermentas, Burlington, CDN
ΦX174 DNA/*BsuRI (HaeIII)* Marker, 9	Fermentas, Burlington, CDN

5.3. Antibodies

PRIMARY ANTIBODIES				
Antibody	***Application***	***Working dilution***	***Dilution buffer***	***Company***
Rabbit anti-serotonin (anti-5-HT)	ICC IHC	1:1000 1:500	1% BSA in PBS 10% donkey serum in TBS	Sigma Aldrich, Steinheim
Mouse anti-TUJ1	ICC	1:500	1% BSA in PBS	COVANCE, Berkeley,

(neuronal class III β- -tubulin)				California
Rabbit anti-nestin (neuronal stem cell marker)	ICC	1:500	1% BSA in PBS	Abcam, Cambridge, UK
Rabbit anti-BLBP (neuronal marker)	ICC	1:500	1% BSA in PBS	Abcam, Cambridge, UK
Rabbit-anti-B-FABP (glial precursor marker)	ICC	1:200	1% BSA in PBS	Santa Cruz Biotechnology, Inc., CA, USA
Sheep anti-GFP	WB	1:1000	5 % milk powder in TBST	Novus Biologicals,CO,USA
SECONDARY ANTIBODIES				
Donkey anti-rabbit Cy3 IgG (H+L)	ICC	1:500	1% BSA in PBS	Jackson Immuno Research,Suffolk, UK
Donkey anti-rabbit Cy2 IgG (H+L)	ICC	1:500	1% BSA in PBS	Jackson Immuno Research,Suffolk, UK
Donkey anti-mouse Cy3 IgG (H+L)	ICC	1:500	1% BSA in PBS	Jackson Immuno Research,Suffolk, UK
Donkey anti-mouse Cy2 IgG (H+L)	ICC	1:500	1% BSA in PBS	Jackson Immuno Research,Suffolk, UK
Rabbit anti-sheep HRP IgG (H+L)	WB	1: 10000	5% milk powder in TBST	Sigma, Steinheim

5.4. Cloning vectors

pGEM Teasy (Promega, Wisconsin, USA)

pIRES-EGFP (Clontech, Mountain View, CA) (Appendix 1)

dsRedN1 (Clontech, Mountain View, CA) (Appendix 2)

SV40-EM7-Bsd-pA (kindly provided by F. Stewart, Technical University, Dresden)

RP23-226H2 Tph2 containing BAC

40kb ePet -β globin-EYFP-pBACe3.6 (kindly provided by Evan S. Deneris, Case Western Reserve University, Cleveland, OH)

PGK-neo (Clontech, Mountain View, CA)

5.5. Cell lines

E14Tg2a (Bay Genomics, San Francisco, CA)

E14Tg2a is ES cell line available from Bay Genomics and shared among the members of FunGenES consortium for standardizing conditions and comparing the results of ES cell differentiation performed in different labs. We used this line to develop an efficient protocol of serotonergic differentiation and for introducing the constructs for obtaining selectable serotonergic neurons in culture.

CGR8: CAG-GFP (GFP+)

The line was kindly provided by Pierre Savatier, Institut National de la Sante et de la Recherche Medical (INSERM), Paris, France, and used as a positive control for detection of GFP expression. It is a transgenic cell line driving ubiquitous GFP expression under CAG (CMV early enhancer/chicken β actin) promoter.

E14Tg2a: CMV-dsRed (C3)

C3 is transgenic cell line with dsRed expression driven by CMV promoter (Clontech, dsRedN1) used as a positive control for detection of dsRed expression.

NB4: Tph2 "knock-in" E14Tg2A line

Tph2-neo-IVS-IRES-dsRed-pA /SV40-EM7-Bsd-pA

The line was created during the course of this study to drive the neoR under the Tph2 endogenous promoter and, thus, enable the selection of serotonergic neurons in culture. An additional fluorescent color (dsRed) was connected through synthetic intron (IVS) and internal ribosomal entry site (IRES) to neoR for simultaneous transcription from Tph2 promoter.

ePet 3kb: transgenic E14Tg2A line

3kb ePet-pEGFP-IRES-gb3-neo-pA cassette/SV40-EM7-Bsd-pA

The line was created as an additional approach to mark serotonergic neurons in culture, after dsRed selection was undetectable (see Results and Discussion section of this work).

ePet 40kb: transgenic E14Tg2A line

40kb ePet-β globin-EYFP/Cm (pBACe3.6)

An additional transgenic cell line created to the same purpose as above, including longer enhancer region (40kb) as in Scott et al. 2005.

5.6. Cell culture media recipes

β-mercaptoethanol stock solution

70µl of β-mercaptoethanol (Sigma)

20ml of ddH_2O (Gibco-BRL)

filter sterilized

stored at 4^oC for 2 weeks

ES cell medium for feeder free ES cells (FFES)

1 x GMEM 500ml

2 mM glutamine 6ml

1mM sodium pyruvate 6ml

1 x nonessential amino acids 6ml

Penicillin/streptomycin (P/S) 6ml

13% (v/v) fetal bovine serum 80ml

β–mercaptoethanol stock solution 600μl

500 – 1000 units/ml LIF

Knock-out serum replacement medium (KSR) (LIF-)

1 x GMEM 500ml

2mM glutamine 6ml

1mM sodium pyruvate 6ml

1 x nonessentialaminoacids 6ml

P/S 6ml

10% (v/v) Knock-out serum replacement 50ml

β–mercaptoethanol stock solution 600μl

N2B27

The media was either purchased from Stem Cell Sciences (SCS, UK) or prepared by the following recipe:

N2

DMEM:F12 500ml

N2 5ml

BSA (7.5%) 333µl

P/S 5ml

β-mercaptoethanol stock solution 500µl

B27

DMEM:F12 500ml

B27 10ml

P/S 5ml

RHB-A

Improved complete neuronal differentiation media modified from N2B27 and purchased from Stem Cell Sciencec (SCS, UK).

PA6 medium

1 x GMEM 500ml

2mM glutamine 6ml

1mM sodium pyruvate 6ml

1 x nonessential amino acids 6ml

P/S 6ml

10% (v/v) fetal bovine serum 50ml

β-mercaptoethanol stock solution 600µl

5.7. Equipment and expendable material

Equipment and material	*Company*
8 – channel – pipette M300	Biohit, Rosbach v.d Höhe
96-well Photometer anthos htII	Anthos Labtech Instruments, Salzburg, AT
Automatic pipette Witoped XP	Witeg Labortechnik, Göttingen, Germany
Agarose gel electrophoresis apparatus	Biometra, Niedersachsen, Germany
Analytic balance	Sartorius Analytic, Göttingen, Germany
Bio-imaging analyzer BAS 2000	FUJIX, Tokyo, Japan
Phospho-imager plates BAS-III	FUJIX, Tokyo, Japan
Bacterial shaker Certomat^{R} H	B. Braun, Melsungen,
Bacterial incubator B6120	Heraeus, Hanau, Germany
Centrifuge 5415C	Eppendorf, Hamburg, Germany
Centrifuge Sorvall RC 5C	Heraeus, Hanau, Germany
Liquid Scintillation system Beckman LS6000SC	Minnesota, USA
Membrane filter (0.25µm, 0.45µm)	Millipore, Morlsheim, Germany
Electroporator 2510	Eppendorf, Hamburg, Germany
Peltier Thermal Cycler PTC-200	Biozym, San Diego, USA
Power supply for the gel chamber	Appligene, Illkirch, France
Falcon tubes	TPP^{R}, Trasadingen, Switzerland
Fluorescent microscope Axioplan 2 imaging	Carl Zeiss, Jena GmbH, Jena
Binocular MZFLIII	Leica Microsystem GmbH, Wetzlar
Microwave 8020	Privileg, Fürth
Quartz-cuvettes	Hellma Optik GmbH, Jena, Germany
Quickspin^{TM} columns, Sephadex G-50	Roche, Mühlheim, Germany
Saran film	Roth, Karlsruhe, Germany
PCR tubes	Biozym Scientific GmbH, Oldendorf, Germany
Superfrost Plus slides	Menzel Gläser, Braunschweig, Germany
Thermomixer 5437	Eppendorf, Hamburg, Germany
UV/visible spectrophotometer	Appligene, Illkirch, France
UV Stratalinker 1800	Stratagene, La Jolla, USA
Vortex: VibroFix	Janke & Kunkel, IKA, Heitersheim,

	Germany
Whatman 3MM paper	Whatman, Madison, USA
Pipettes	Gilson,
Disposable pipettes Cellstar® 1, 2, 5, 10, 25ml	Greiner bio-one, Frickenhausen
Cell culture incubator	Heraeus Instruments GmbH, Düsseldorf
GenePulse® Cuvette	BioRad Laboratories, Richmond, USA
Cryotubes CryoS	Greiner bio-one, France
Cryo 1°C Freezing Container	Nalgene®Nunc, Rochester, NY, USA
Pasteur pipettes	Roth, Karlsruhe
pH Meter pH Level 1	WTW, Weilheim
Photometer GeneQuant pro	Amersham Biosciences, Little Chalfon, UK
Potter-Homogenizator	Roth, Karlsruhe
PVDF membrane	Amersham Biosciences, Little Chalfon, UK
Roller mixer SRT1	Snijders, Tilburg, NL
Röntgenfilm X-ray Retina	Fotochemische Werke GmbH, Berlin
Rotatable platform Polymax 1040	Heidolph Instruments, Scwabach
SDS-PAGE gel electrophorase chamber	BioRad Laboratories Richmond, USA
Voltage supply PowerPac™ HC	BioRad Laboratories, Richmond, USA
SpeedVac SVC100	Savant Instruments, farmingdale, NY, USA
Table centrifuge Biofuge pico	Hareaus Instruments GmbH, Düsseldorf
Transilluminator MultiImage™ Light cabinet	Alpha Innotech Corporation, CA, USA
Ultrasound Sonoplus	Bandelin electronic, Berlin
Ultra-Turrax T25 basic	IKA® Labortechnik, Staufen
Vacuum pump Vacusafe comfort	IBS Integra Bioscience, Chur, Switzerland
Water bath	GFL, Burgwedel
Cell culture plates and flasks	TPP®, Trasadingen, Switzerland

6. METHODS

6.1. Nucleic Acids

6.1.1 Isolation of plasmid DNA from bacterial cultures

Alkalyine lysis (Birnboim and Doly, 1979) is a common method for the isolation of plasmid DNA from bacteria. An over night culture of bacteria, inoculated previously with the plasmid of interest, is pelleted, resuspended in GTE buffer and digested with the same volume of the lysis buffer. Adding 1.7M Potassium acetate precipitates cell debris and genomic DNA that are then pelleted by centrifugation. Plasmid DNA from supernatant is, then, precipitated with isopropanol, washed with cold 75% Ethanol and resuspended in TE-buffer or ddH_20.

The isolation of plasmid DNA from the big over night cultures (300ml of culture medium with bacteria) is done using JetStar Plasmid Purification MAXI Kit 2.0 following the manufacturer instructions.

GTE-buffer

Glucose 50mM

EDTA pH 8.0 10mM

Tris-HCl ph 8.0 25mM

RnaseA 0.4mg/ml

Lysisbuffer

NaOH 100mM

SDS 0.5%

TE-buffer

Tris-HCl pH 8.0 10mM

EDTA 1mM

6.1.2 Isolation of DNA from organs and cells

For the genotyping of the experimental animals DNA was isolated from tail samples. The tails were incubated over night in 100 μl of the ear buffer, at 55°C. Incubation for 10 min at 95°C inactivates Proteinase K from the buffer, following the RNA digestion with 700 μl TE – buffer containing 20μg/ml RnaseA. After 15 min of the room temperature (RT) incubation, 2 μl of genomic DNA was used as a template for PCR reaction.

The genomic DNA from cells was isolated using the same protocol, after pelleting the cells 5 min at RT, at 5000g. The incubation time in the ear buffer was much shorter 1-4h at 55°C.

Ear buffer

Tris-HCl pH 8.0 100mM

Proteinase K 1mg/ml

EDTA 5mM

NaCl 200mM

SDS 0.2%

6.1.3 Isolation of RNA from organs and cells

For the RNA isolation from organs TRIZOL reagent is used following the manufacturers instructions. 100mg of the organs were diluted in 100ml of TRIZOL and homogenized with Ultra Turrax. The RNA was isolated using phenol chloroform solution (1:1), precipitated with isopropanol and diluted in DEPC treated water.

Before performing RT-PCR or Real Time PCR reaction the RNA samples were treated for 30 min with 0.1 u/μl Dnase I, that was, then, inactivated by heating.

DEPC water

DEPC 0.1% at 37°C over night stirred, then autoclaved

For isolation of RNA from the small number of cells QIAGEN Rneasy Kit was used, according to manufacturers instructions, and Dnase I digestion was performed on-column, using Rnase-Free Dnase Set from QIAGEN.

6.1.4 Isolation of DNA from agarose gels

DNA can be isolated from the agarose gel and used for further manipulation like ligation, restriction digestion or sequencing. DNA is, first, visualized by long wave UV light (300nm) and the band of interest cut out with the scalpel and cleaned using Promega Gel Purification Kit or QIAquick Gel Extraction Kit following manufacturers instructions.

6.1.5 Determination of nucleic acid concentration

The DNA and RNA concentration was determined by OD_{260} compared to OD_{280} in a spektrophotometer. When $OD_{260}/OD_{280} > 1.7$, the protein concentration can be neglected and the concentration of nucleic acids determined by the following ratio:

DNA $OD_{260} = 1 = 50$ μg/ml for double stranded DNA

RNA $OD_{260} = 1 = 40$ μg/ml

When $OD_{260}/OD_{280} < 1.7$ the protein concentration of the solution is too high and requires the new phenol extraction of nucleic acids, after which the concentration was re-measured.

6.1.6 Storage of nucleic acids

For long-term storage DNA was diluted in TE buffer and kept at – 20°C, RNA was diluted in DEPC or Rnase-Free water from QIAGEN, and kept at – 80°C. Primer stocks were diluted in TE buffer, and kept at – 20°C. Before performing PCR the stock were diluted to working concentration in ddH_2O and kept at –20°C.

6.1.7 Separation of nucleic acids on the agarose gel

DNA molecules can be separated by length using 1-3% (w/v) agarose gels. DNA is mixed with 0.1V of 10 x Loading buffer, applied on agarose gel chambers and electrophorised at 1 – 8 V/cm. The gels are prepared with 0.5 μg/ml ethidium bromide for

visualizing DNA under 300nm UV light. The size and the approximate concentration of the DNA bands can be determined by comparison with standardized molecular weight markers λ DNA/*EcoRI + HindIII* and ϕX174 DNA/*BsuRI (HaeIII)*.

TAE buffer

Tris-Acetate pH 7.4 40mM

EDTA 1mM

10 x DNA Loading buffer

Sucrose 40%

Bromphenolblue 0.02%

in TE buffer

RNA molecules can be separated and visualized using formaldehyde agarose gels following the solution preparation and the protocol provided in QIAGEN RNeasy Mini Kit Handbook.

6.1.8 Restriction digestion of DNA

Restriction enzymes are supplied with appropriate restriction buffers and were used as suggested by the manufacturer. 200ng - 1μg of DNA was digested in 10 μl volume, with 1 μl of restriction buffer and 1 μl of enzyme (ca. 10U), incubated 1 - 2h at by the supplier stated temperature and the digestion was proved by agarose gel electrophoresis. For higher concentration of digested DNA needed, the reaction volume was manipulated accordingly, using up to 100 μg of DNA in up to 200μl volume with up to 100 U of enzyme. For double digestion the common buffer was used as suggested by the producer.

6.1.9 Ligation of DNA

For the ligation of sticky DNA ends with plasmid DNA about 25ng of the vector was used with 2-5 molar excess of DNA fragment, 1 μl of 10 x ligation buffer and 1 ml of T4-DNA-Ligase (1u/μl) adding ddH_2O to 10 μl. The reaction was incubated over night at 16^oC and 2 μl were used for the transformation of bacteria.

6.1.10 Red/ET recombineering

For the manipulation of bacterial artificial chromosomes (BACs) whose size did not permit easy ligation, Red/ET recombination kit (Genebridges, Dresden, Germany) was used according to the suggested protocol.

Primers used for amplifying and recombining homology regions

ePet up3

5′CAG TTA TTG GTG CCC TTA AAC GCC TGG TTG CTA CGC CTG AAT AAG TGA TAA TTC TAC CGG GTA GGG GAG G 3′

ePet lo3

5′TTC TCA TGT TTG ACA GCT TAT CAT CGA ATT TCT GCC ATT CAT CCG CTT ATC GCC GCA CAC AAA AAC CAA C 3′

Primers for recombining Bsd cassette

R95 Bsd 5

5′ GCT GGG GAT GCG GTG GGC TCT ATG GCT TCT GAG GCG GAA AGA ACC AGC TGG GGT GTC AGT TAG GGT GTG GAA AGT C 3′

R95 Bsd 3

5′ CGC AGT CCA CGA AGA TTT CGA CTT CAG AAC TTC TTC GCC GGG ACC GCC TGG ATT CGA GCT AGA GGT CGA TAT ACA G 3′

Tph2 homology arms

Tph2 up

5′ CAC TGC TCT TCA GCA CCA GGG TTC TGG ACA GCG CCC CGA GCA GGC AGC TGC CAC TAG TGG CCT CGA ACA CCG AGC GAC CCT GCA GCC AAT 3′

Tph2 lo

5′ CGC AGT CCA CGA AGA TTT CGA CTT CAG AAC TTC TTC GTC GGG ACC TCC TGG ATT CGA AGC TAG AGG TCG ACG GTA TAC AG 3′

Homology arms used for shaving RP23 – 226H2 Tph2 containing BAC

(Gene Bridges protocol)

5′ TCC GAC ACT GAT GAA AAT GTT GGC AGC CCT TGT CCT GTA TCT GCG TTT CCA 3′

5′ TGG ATT CAT CCA CGG ACT GGC AAA GTA AGA GAC GTC TAC TGG CTG CAA CC 3

6.1.11 Sequencing of DNA

Sequencing of DNA fragments and PCR products was done by the company Invitek (Berlin Buch). The company BioTez, Berlin-Buch, produced all the primers synthesized for the purpose of this work.

Sequencing primers

EYFP

5′ - ATG ACC ATG ATT ACG CCA CG – 3′

GFP 52

5′ CCT GAA GTT CAT CTG CAC CA 3

GFP32

5′ AAG TCG TGC TTC ATG TG 3′

dsRed 33

5′ CCT TGG TCA CCT TCA GCT TC 3′

dsRed 32

5′ TGG TCT TCT TCT GCA TCA CG 3′

Tph 32

5´CAA TGT TAA CAT ATA CAG TCT TTG C 3´

Neo 3

5´GTG TTC CGG CTG TCA GCG CAG 3´

6.1.12 Reverse transcription (RT)

The synthesis of cDNA using DnaseI treated total RNA template was done using 6.5µl of Random Hexamer primers for 1-2µg of total RNA in 15.25 µl volume. RNA is denaturated for 3 min at 80^{o}C and quickly chilled to 0^{o}C. RNA was then treated with the master mix consisting of:

1st strand buffer (5x) 6µl

dNTPs (5mM) 3µl

Rnasin (40u/ml) 0.75µl

DTT (100mM) 3µl

M-MLV (200u/µl) 2µl

The reaction was incubated for 1h at 42^{o}C. M-MLV was inactivated at 80^{o}C for 10min and kept on ice before 2 µl were used for PCR reaction.

6.1.13 Polymerase Chain Reaction (PCR)

6.1.13.1 PCR for the animal genotyping

For genotyping of Tph2 deficient mice the following PCR reaction was set using *Taq Polymerase* from Invitrogene:

Genomic DNA 2µl

PCR buffer (10x) 5µl

$MgCl_2$ (50mM) 2.5µl

dNTPs (10mM) 1µl

primer neo3 (10mM) 1µl

primer TPH34 (10mM) 1µl

primer TPH54 (10mM) 1µl

Taq polymerase (5u/ml) 0.5µl

ddH_20 33µl

the reaction was kept on ice before transferred to preheated PCR machine.

The program used for genotyping:

Denaturation 5min 95^oC

Denaturation 45sec 94^oC

Annealing 30sec 55^oC

Elongation 30sec 72^oC

Repeat 34 x

Final elongation 10min 72^oC

Forever 4^oC

The PCR products were visualized after the electrophoresis on 3% agarose gel. The following fragments were separated

WT band: primers TPH54 - TPH34: 568bp

KO band: primers TPH54 –neo3: 401bp

The presence of both bands indicated heterozygous genotype, while the presence of longer or shorter band indicated homozygous genotype, for WT or KO allele, respectively.

The primer sequences used for genotyping of Tph2 deficient animals

Neo3 5' CTG CGC TGA CAG CCG GAA CAC 3'

TPH 34 5' AGC TGA GGC AGA CAG AAA GG 3'

TPH 54 5' CCA AAG AGC TAC TCG ACC TAC G 3'

6.1.13.2 RT-PCR for gene expression analysis

For detecting the expression of the genes during ESC differentiation a semi quantitative RT-PCR was performed, using the specific primers. All the PCR reactions were done with *Taq*Polymerase from Invitrogen, and the standard PCR reaction was performed as suggested by the manufacturer. The program for all the fragments included a step of 55^{o}C or 60^{o}C annealing and 20-30sec elongation, with the lowest number of 25 cycles. The band visible on the agarose gel after 25 cycles were considered highly expressed, while the band visualized after 35 cycles were considered as low expressed genes in the course of differentiation. RT reaction was set as described above and 2µl were used for following PCR reaction.

Primer sequences

β actin

fw TAC AAT GAG CTG CGT GTG

rev CAC AGC CTG GAT GGC TAC

Nestin

fw GGA GTG TCG CTT AGA GGT GC

rev TCC AGA AAG CCA AGA GAA GC

Tuj1

fw AAC TAT GTA GGG GAC TCA GAC CTG

rev TCT CAC ACT CTT TCC GCA CGA C

Fgf8

fw CAT GTG AGG GAC CAG AGC C

rev GTA GTT GTT CTC CAG CAG GAT C

Fgf4

fw CGA GGG ACA GTC TTC TGG AG

rev ACC TTC ATG GTA GGC GAC AC

SHH

fw GGA ACT CAC CCC CAA TTA CA

rev GAA GGT GAG GAA GTC GCT GT

TH

fw TGT CAG AGG AGC CCG AGG TC

rev CCA AGA GCA GCC CAT CAA AG

Wnt1

fw ACC TGT TGA CGG ATT CCA AG

rev TCA TGA GGA AGC GTA GGT CC

DAT

fw GTG GGC TTC ACT GTC ATCC CTC A

rev CCC AGG TCA TCA ATG CCA CGA

Ptx3

fw GAG GAA TCG CTA CCC TGA CA

rev GCG GTG AGA ATA CAG GCT GTG AAG

Tgf alpha

fw CTG AAG GGA AGG ACT GCT TG

rev CAA CCC TTT GAG GTT CGT GT

Nkx2.1

fw TAC TGC AAC GGC AAC CTG

rev GCC ATG TTC TTG CTC ACG TC

Nkx2.2

fw CTC TTC TCC AAA GCG CAG AC

rev AAC AAC CGT GGT AAG GAT CG

Nkx6.1

fw TCA GGT CAA GGT CTG GTT CC

rev CGA TTT GTG CTT TTT CAG CA

Nkx6.2

fw TCC TGG ATA AGG ATG GCA AG

rev CCG GTT GTA TTC GTC ATC GT

Gata2

fw GTG GGC TCT ACC ACA AGA TG

rev TGC TCT TCT TGG ATT TGC TGG

Gata3

fw AGA GTG CCT CAA GTA TCA GG

rev GCA GTT CAC ACA CTC CCT G

Mash

fw TGC TGG ACT TTA CCA ACT GG

rev CCC TGT TTG CTG AGA ACA TTG

Nurr

fw TGA AGA GAG CGG AGA AGG AGA TC

rev TCT GGA GTT AAG AAA TCG GAG CTG

Phox2b

fw ATA CCT CCA GCT TGG CTT CA

rev TCA GTG CTC TTG GCC TCT TT

Gbx2

fw GAG CAT CAC ACA GGG TTC TG

rev GAC CTT TAA ATC GCG CGC TCC TC

En1

fw TCA AGA CTG ACT CAC AGC AAC CCC

rev CTT TGT CCT GAA CCG TGG TGG TAG

6.1.13.3 PCR for amplifying long fragments (Long range PCR)

Long range PCR was used to amplify the 3 kb long ePet promoter region immediately upstream of Exon 1 of the *Pet1* gene (Scott et al. 2005). To this purpose Expand

Long Template PCR System was used, containing DNA polymerase with proof-reading activity.

Primer sequences

ePet NheI

5’ ATG CGC TAG TCC AGA ACC CAG AGC TGA ATC 3’

ePet BclI

5’ GCC ACC GGT CGC CAC CTG ATC ATG ACA TAG 3’

6.1.13.4 Quantitative (real-time) PCR (qPCR)

For detecting the relative expression of the genes of interest during the differentiation quantitative (real-time) polymerase chain reaction (qPCR) was performed.

Total RNA was isolated from the cells at different stages of differentiation using QIAGEN Rneasy Mini Kit (Qiagen) and reverse transcribed using Moloney murine leukemia virus reverse transcriptase (M-MLV, Invitrogen) according to the manufacturer's instructions.

For qPCR reaction 5μl of previously synthesized cDNA was used, 5μl of 10μM primer mixture and 10μl of Real Time Master Mix (2x) from Quanti Tect SYBR Green PCR Kit. qPCR was performed on the iCycler IQ^{TM}5 multicolor real-time detection system (Bio-Rad), using absolute SYBR green fluorescein (ABgene). PCR program was carried out following the standard protocol:

1. 95 ^{o}C 5min

2. (95 ^{o}C 15min 55 ^{o}C 30sec 72 ^{o}C) x 40

3. 55 ^{o}C 15sec

4. 95 ^{o}C 15sec

Oligonucleotides were custom made by Invitrogen. Data were analyzed using $2^{-\Delta\Delta}Ct$ method as described in Livak et al. 2001.

Primer sequences

β actin

fw TAC AAT GAG CTG CGT GTG

rev CAC AGC CTG GAT GGC TAC

Tph2

Tph2 Ex10.5 CCA TCG GAG AAT TGA AGC A

Tph2 Ex12.3 GTC CTG CAC CAC ATT CTC A

Pet1

fw ACG CCT ACC GCT TTG ACT TC

rev AAG CTG CCA TCA AGTTGA GTT

ASB4

fw GAG AGG CAA ATA GAT GTG GAC AC

rev TGA CCA AGC AAA ACT GAC AGA T

CACNA2D

fw GTC ACA CTG GAT TTT CTC GAT GC

rev GGG TTT CTG AAT ATC TGG CCT GA

FoxA1

fw ACA TTC AAG CGC AGC TAC CC

rev TGC TGG TTC TGG CGG TAA TAG

GCH1

fw ACT TCA CCA AGG GAT ACC AGG

rev CTT GCT TGT TAG GAA GAT AGC CA

Pldx2

fw CAA GCC TCT CCA GAG TCC AA

rev CAT TTG GTC TAT GTT AAC CCA CAG

3100002J23RIK

fw CTG AAC CTC ATT CAC CAT GC

rev CCA AGC TCC ACA GTT TAG CC

Erg2

few CTC TAC CCG GTG GAA GAC CT

rev GGA GAT CCA GGG GTC TCT TC

Nkx2.2

few TCT ACG ACA GCA GCG ACA AC

rev TTG TCA TTG TCC GGT GAC TC

Nkx 6.1

fw TCA GGT CAA GGT CTG GTT CC

rev CGA TTT GTG CTT TTT CAG CA

6.1.14 Affymetrix analysis

RNAs from brain of 4 Tph2$^{-/-}$ and 4 Tph2$^{+/+}$ 2 months old females were extracted with TRIZOL-reagent (Invitrogen) following the manufacturer´s instruction, DNase I (Ambion) treated, and cleaned with RNeasy Mini Kit (Qiagen). GeneChip® One-Cycle Target Labeling and Control Reagents Kit (Affymetrix, # 900493) was used for labeling of 20µg mRNA with biotinylated UTP and CTP (Bio-11CTP, #42818, Bio-16-UTP, #42814, Enzo) and fragmentation. The labeled mRNA was hybridized against GeneChip® Mouse

Genome 430 2.0 Arrays (Affymetrix) and the results were analyzed as reported previously (Xu et al. 2007). Student's *t*-test (unpaired, two-tailed assumed unequal variance) was used to check the differences between two experimental groups. $P < 0.05$ and a fold change > 1.7 was used in order to select transcripts the expression of which was considered to be significantly changed in *Tph2* deficinet mice.

6.1.15 Targeting constructs

6.1.15.1 Tph2 "expression - selection" cassette

The Tph2 "expression - selection" cassette was recombined into the *Tph2* locus of the ESC line E14Tg2A (Bay Genomics, San Francisco, CA) leading to the deletion of a 5.2 kb long region, which contains the coding part of exon 1 (Ex1) and Ex2, and thus, disabling the transcription of the entire *Tph2* gene.

First, 1 and 6kb-long arms were amplified by PCR from mouse genomic DNA. The pIRES-EGFP vector backbone (Clontech, Mountain view, CA) was used to introduce 5' Tph2 homology arm together with neoR and dsRed2 (dsRedN1 vector, Clontech) genes, creating Tph2 - neoR - IVS - IRES - dsRed2 - pA expression cassette. An additional SV40 - EM7 -Bsd cassette (kindly provided by F. Stewart, Technical University, Dresden) was inserted by homologous recombination in bacteria using Red/ET recombination kit (Genebridges, Dresden, Germany) downstream of dsRed2 to enable the selection in ES cells. The resulting "expression - selection" cassette was then flanked by the 3' Tph2 homology arm and used for homologous Red/ET recombineering with the Tph2 - containing BAC RP23-226H2 to elongate Tph2 - homology arms. After the successful integration, Tph2 - BAC was shaved according to the Red/ET protocol to a 24.6 kb long plasmid with 14.7 kb 5' and 3.8 kb 3' homology regions. The targeting construct was then linearized with I-Sce I and electroporated in ESC (BioRad Gene Pulser, 800mV, 3μF) and clones were picked after 8 days of blasticidin selection. Correct genomic integration was detected by southern blotting.

6.1.15.2 ePet transgenes

An enhancer region immediately upstream of Pet1 gene (Scott et al. 2005) was shown to be able to drive the expression of a marker gene in serotonergic neurons exclusively. We created two different constructs by using two different lengths of the ePet sequence that

was supposed to drive EGFP expression in the serotonergic neurons obtained by differentiation of ESC in culture.

40kb long ePet sequence isolated from the BAC containing the Pet1 gene (RP23-165D11, Research Genomics, Huntsville, AL) was cloned in front of the β globin promoter (β-globin) of the BGZA plasmid (Yee and Rigby 1993) and then introduced in pBACe3.6 (Frengen et al. 1999) vector backbone in the lab of Evan S. Deneris (Scott et al. 2005). The vector, used already to create transgenic mice expressing EYFP under the control of Pet1 enhancer (ePet), was kindly provided for testing in the ESC *in vitro* differentiation system in our lab.

To be able to select the ESC clones that have an integrated transgene, we had to introduce an additional neoR cassette in the ePet - BAC, by homologous recombination, using Red/ET kit (Genebridges, Dresden). NeoR cassette (PGK - gb2 - neo - pA) was amplified by PCR using PGK - neo (Clontech) plasmid as a template. The primers contained flanking 50bp homology regions at the ends, used for Red/ET recombination. The final BAC, introduced in ES cells (BioRad Gene Pulser, 800mV, 3μF), consisted of ePet - β globin - EYFP sequence, and an additional neoR cassette. The clones were isolated after 7 days of neomycine selection (200μg/ml).

The same ePet template was used to amplify a smaller segment (3kb), and prove its enhancer properties *in vitro.* To this purpose 3kb ePet was amplified by long range PCR and cloned in the backbone of pEGFP plasmid (Clontech), to drive the expression of pEGFP - IRES - gb3 – neo - pA cassette. An additional cassette (SV40 - EM7 - Hyg - pA) was used for selection of the ESC containing the transgene.

Before the electroporation in the ESC, ePet - BAC was linearized with PacI, while the smaller ePet - EGFP plasmid was separated from the vector backbone by double restriction (Nhe I/XhoI). Both fragments were then cleaned from the agarose gel, as described above and about 30 μg of DNA was used for transfection.

6.1.15.3. Southern Blot

For detection of the correct integration of "expression - selection" cassette in the *Tph2* locus an additional SpeI restriction site was used, that was created after the correct homologous recombination of the targeting construct. The 298bp Southern probe used to

detect homologous recombination was amplified by PCR with the primers TPH2 South 5' (5' CAG GAA GCG CTG GAT CTC C 3') and TPH2 South 3' (5' CCA AGC ACG TTT ATG ACT CAG 3') and labeled with radioactive CTP (α-^{32}P dCTP (Amersham, Braunschweig)) using Prime-It RmT Random Primer Labeling Kit (Stratagene). The probe was cleaned on the Quick Spin Columns (Roche) and the radioactivity measured on the scintillation counter. The quantity of the labeled DNA containing 1 x 10^6 cpm was mixed with 150µl of salmon sperm DNA and denaturated 5min on 95°C before applied on the previously prepared nylon membrane in the hybridization solution containing dextrane sulfate to condensate the probe. The membrane was, then, incubated over night at 65°C.

The membrane, containing capillary blotted (Sambrook et al. 1989) and UV fixed SpeI digested DNA, was previously treated 2h at 65°C with prehybridization solution.

After the hybridization, the membrane was washed and exposed, first 1h and then, if necessary, up to 3 days, and the signal was detected on BAS2000 Phosphoimager (Fuji, Tokyo).

Prehybridization solution:

20 x SSC 3ml

50 x Denhardt´s solution 1ml

Salmon sperm (10mg/ml) 0.1ml

2M Sodium phosphate, pH6.5 0.125ml

Formamide 5ml

H_2O up to 10ml

Hybridization solution:

20 x SSC 3ml

50 x Denhardt´s solution 1ml

Salmon sperm (10mg/ml) 0.1ml

2M Sodium phosphate, pH6.5 0.1ml

10% Dextrane sulfate in formamide 5ml

H_2O up to 10ml

Washing solution:

0.2 x SET 0.2 % SDS 3 x 45min

or

2 x SSC/0.1%SDS	2 x 15 min
0.1xSSC/0.1%SDS	1x 15 min

6.2 Proteins

6.2.1 Isolation of the proteins from cells and organs

To isolate the proteins from the cells, the cell culture was washed 2 times with cold PBS, trypsinized, centrifuged (3000g, 3min) and resuspended in Protein Lysis buffer. The suspension was kept on ice and homogenized using Ultrasonicator for 10 sec with max strength. The cell debris was precipitated by centrifugation (13000g, 15min, 4^{o}C) and the supernatant was kept at – 80^{o}C.

Protein Lysis buffer

Tris-HCl pH 7.4 20mM

NaCl 150mM

EDTA 1mM

EGTA 1mM

Triton x-100 0.5%

PMSF 1mM

Mini Complete protease inhibitor cocktail 1 tablet /10ml

For the isolation of proteins from organs, for each 100mg of the organ, 1 ml of the protein lysis buffer was used and the organs were first homogenized using Potter-Homogenizer and then sonicated with the ultrasound. The debris was then centrifugated as for the cells, and the supernatant kept at – 80^{o}C before measuring the concentration.

6.2.2 Determining the protein concentration

The protein concentration was determined using the Bradford reagent and according Bradford method (1976). For every measurement, a standard curve was premeasured, using the known BSA concentrations prepared in PBS. The probes were also diluted in PBS before the measurement and 5µl of each probe as well as standards were mixed with 250µl of Bradford reagent. After 10 min incubation at the RT, the absorbance was determined on the photometer at 595nm. The protein concentration was then recalculated from the standard curve, using Microsoft Excel preset pattern.

6.2.3 Western blot

6.2.3.1 The separation of the proteins by SDS Polyacrylamide gel electrophoresis (SDS PAGE)

For the separation of the proteins using SDS-PAGE, 20µg of the proteins are mixed with 4x Roti-Load SDS-loading buffer and denatured 5min at 95^{o}C.

The proteins are first collected through the collecting gel for 15min at 80V, and then let run for 1-2h at 120V. The running gel was 10% acrylamide-/bisacrylamide 37.5:1 in 375 mM Tris pH 8.8. The collecting gel 5% in 125mM Tris pH. The gels were made in specially designed chambers.

SDS PAGE running buffer

Tris 25mM

Glycin 200mM

SDS 0.1%

6.2.3.2 Protein blotting

The proteins were blotted on the nitrocellulose membrane. The membrane is incubated previously for 10 min in ddH_2O and then in the transfer buffer. The blotting was performed for 2h in a chamber with ice, at 100V.

Transfer buffer

Tris 25mM

Glycin 200mM

Methanol 20%

6.2.3.3 Blocking of the membrane

The blocking is performed to prevent the binding of the antibodies to the unspecific sites of the proteins bounded on the mebrane. The membrane was blocked for 1-2h in TBST in 5% milk powder.

TBST

Tris 50mM

NaCl 150mM

PH 7.5

Tween20 0.5%

6.2.3.4 Incubation with the primary antibodies

The primary antibodies were incubated over night at 4^{o}C on the roller mixer in appropriate plastic dishes in TBST with 5% milk powder. The membrane was then washed 6 times for 5min with TBST.

6.2.3.5 Incubation with the secondary antibodies

The secondary antibodies were incubated for 2h at RT on the roller mixer in TBST with 5% milk powder. The membrane was then washed 6 times for 5 min with TBST.

6.2.3.6 ECL reaction

SuperSignaling West Dura Extended Duration Substrate was used to detect bound secondary antibodies by ECL reaction. After 5 min incubation, the luminescence was detected on a x-ray film for 5 sec up to 1h of exposure and the film developed.

6.2.4 Immunocytochemistry

Immunocytochemistry was performed on the cells in culture growing on glass slides on gelatine coated culture dishes, during the differentiation protocols aiming to obtain serotonergic neurons.

The cells were washed two times with PBS, fixed for 10 min with 4% PFA and then perforated with 0.1% Triton X, and then washed again two times with PBS.

Blocking was performed with 1.5% BSA for 1h at the RT.

The first antibody was used in the concentration previously established (1:1000 5-HT, 1:500 Tuj1) and incubated in 1.5% BSA in PBST for 1h at RT or over night at 4^{o}C. The first antibody was washed out three times with PBST.

The secondary antibody was incubated for 1h at RT in 1.5% BSA in PBST and the unbounded antibody was washed out three time for 5 min.

Dapi (Hoechst) (5μg/ml) staining was performed for 10 min at RT, and the rest washed out by three times washing with PBST.

The fluorescence was preserved by mounting with Dako mounting medium.

6.2.5 Fluorescent Activated Cell Sorting (FACS)

The cells were harvested at different time points of differentiation and resuspended in PBS supplemented with 0.1%EDTA. Fluorescent protein expression was analyzed on a

FACSCalibur (BD Biosciences, San Jose, CA, USA) using argon-ion laser excitation (488nm). EGFP and EYFP were detected using the FL1 parameter (emission filter 530±15nm) and dsRed using FL3 parameter (emission filter 650nm). Dead cells and debris were excluded from the analysis by gating for intact cells using the forward and sideward scatter and by propidium iodide (PI) labeling (the cells were suspended in 5μg/ml PI in 1ml PBS and stored in the dark). Untransfected cell line (E14) was always included as a control to detect autofluorescence. Data acquisition was carried out by analyzing 10 000 events/sample using CellQuest Software (BD Biosciences). FACS data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

6.3 ESC maintenance

6.3.1 Growing feeder free ESC (E14Tg2a (129/Ola mouse line))

E14Tg2a ESC are grown without the requirement for feeder cells. For maintaining pluripotency, β mercaptoethanol and LIF are added in the ES medium (see recipes). For better attachment, the cell culture dishes are covered with 0.1% gelatine.

6.3.2 Coating cell culture dishes with 0.1 % gelatine

25 ml of 2% bovine gelatine solution was added to 500ml of PBS and stored at 4^{o}C. To coat the cell culture dishes, cold 0.1% gelatine solution was pured on the dishes until the surface is covered, then left at 37^{o}C to polymerize for at least 15min. Before plating the cells, the surplus of gelatine solution was aspirated, and the suspension of cells added to the medium. After several hours, the cells would attach to the gelatinized surface.

6.3.3 Thawing ESC

ESC are frozen in medium containing 10% DMSO. Since DMSO can induce the differentiation of ES cells, the cells were thawed late in the day, and the medium changed the following morning, to minimize the effects of residual DMSO.

15ml falcon tubes were filled with 10ml of ES medium and pre-warmed at 37^{o}C. The frozen vials of cells were taken out from liquid nitrogen (1 x 10^6 cells/ml) and placed in the warm water bath (37^{o}C) until just thawed. The thawed cell suspension was transferred in the 10ml of pre-warmed medium in the sterile atmosphere and spinned for 3 min at 800rpm. The cell medium was then aspirated, the cells resuspended in 1ml of pre-warmed FFES medium, transferred on the prepared 25cm^2 flasks and grown in the incubator (37^{o}C, 5% CO_2).

6.3.4 Passage and expansion of ESC

ES cells were routinely passaged every second day and the medium changed on alternate days. Feeder independent ES cells grow rapidly and quickly acidify the medium, turning it yellow. Acid medium causes cells to undergo crisis, triggering excess differentiation and cell death, after which the totipotency cannot be guaranteed. Plating cells at low density, insufficient dispersion of cells during passage, or uneven plating can cause similar problems, as the cells will form large clumps before reaching confluence and the cells within those clumps will differentiate or die. Germline transmission is a significantly reduced in cells that have been mistreated, even when they appear healthy at the time of injection.

The cells were passaged at 60-70% of confluence. The medium was aspirated and the cells washed twice with 1xPBS. 1ml of Tryple Express was added to the dish, rocked until the cells are covered and the rest of Tryple Express aspirated. The cells were then left in the incubator 1-2min, or until the detachment. The side of the dishes were tapped to dislodge the cells, and the cells resuspended in the ES medium until a single cell suspension to minimize spontaneous differentiation. The cells were spinned (3min, 800rpm), resuspended again, counted and the cells plated at density 1 x 10^5/cm^2.

6.3.5 Freezing ESC

The freezing medium was 10% DMSO in ES medium (v/v), filter sterilized and made fresh before use. This cryoconservation medium was kept on ice for 30min together with cryo tubes. The cells were trypsinized at 60-80% confluence, collected and pelleted for 3min at 800rpm. The cells were resuspended in 1 x PBS and the small aliquot was taken for counting. The cells were spinned again, and resuspended in the defined volume of ice cold freezing medium to obtain a suspension 1 x 10^6 cell/ml. The cells were then aliquoted in the pre-cooled cryo-tubes, frozen at – 80^{o}C overnight and transferred to liquid nitrogen for long-term storage.

6.3.6 Electroporation and selection of ESC

6.3.6.1 Preparation of DNA for electroporation

DNA vector was linearized by overnight digestion at 37^{o}C (150μg for "knock-in" construct (B52); 30μg for transgenic cell lines (ePet3kb; ePet40kb). DNA was precipitated with 2V of absolute ethanol on ice for 5 min, spinned down in a table microcentrifuge and washed several times with 70% ethanol. The remains of the ethanol were left to evaporate in a sterile laminar flow hood, leaving the tube open. DNA was then resuspended in 0.1ml of sterile PBS and left shaking at 55^{o}C for 4 hours vortexing occasionally to ensure that DNA is in solution.

6.3.6.2. Electroporation

For optimal results, ESC were around 80% of confluency on the day of electroporation and the medium is changed 2 hours before the cells were harvested. 2 x 15 cm dishes of 80% confluency were trypsinized, the cells were collected and pelleted in sterile PBS. The cells were then counted and resuspended at a concentration of 1 x 10^8 cells/0.7ml of PBS. 0.7ml of this cell suspension was quickly mixed with 0.1ml of DNA solution, and transferred to 0.4cm electroporation cuvette. The cells were electroporated at 0.24V and 500μF, recovered for 20min at room temperature and transferred to 20ml of pre-warmed FFES medium. 5 x 10^6 – 2 x 10^6 cells were plated on pre-gelatinized 10cm culture dishes. The following day, the medium was changed to the medium with appropriate antibiotic. The medium was changed every day, and the colonies of antibiotic resistance cells were visible between 6-8 days, and ready to be picked.

6.3.6.3. Picking colonies

The colonies were picked in pre-gelatinized 96 well plate. The colonies were picked up in 50μl PBS using a pipetman (P200) and added too 200μl of FFES medium in each well. The colonies were replated every second day until reaching confluency, and then the plates were replated in two identical replicas using a multi pipette - one of the plates were frozen, and another one used for DNA preparation. DNA was prepared for analysis and detection of positive clone.

6.3.6.4. DNA preparation

40µl of the trypsinized cell suspension were mixed with 20 µl of PK-H_2O (EC: 1mg/ml), incubated at 55°C for 30min, 95°C for 10min and cooled down on ice. 100µl of TE/RNase was added in the mixture and 2 – 5µl taken for PCR.

Proteinase K

10mg/ml in ddH2O

RNase A, stock

4mg/ml in ddH2O

TE – buffer (1x)

10mM tris – HCl (pH 8.0)

1mM EDTA

TE/RNase-buffer

20µg/ml RNase

PK-H_2O

3mg/ml PK

6.4 ESC differentiation

To perform a successful and comparable ESC differentiation, a set of protocols was obtained as a part of the FunGenES cooperation. The protocols (Figure 5.1) used were already established in the laboratories of the FunGenES partners, and included monolayer differentiation, EBs differentiation and the differentiation on the layer of PA6 cells (kindly provided by the Stem Cell Sciences, Edinburgh, UK).

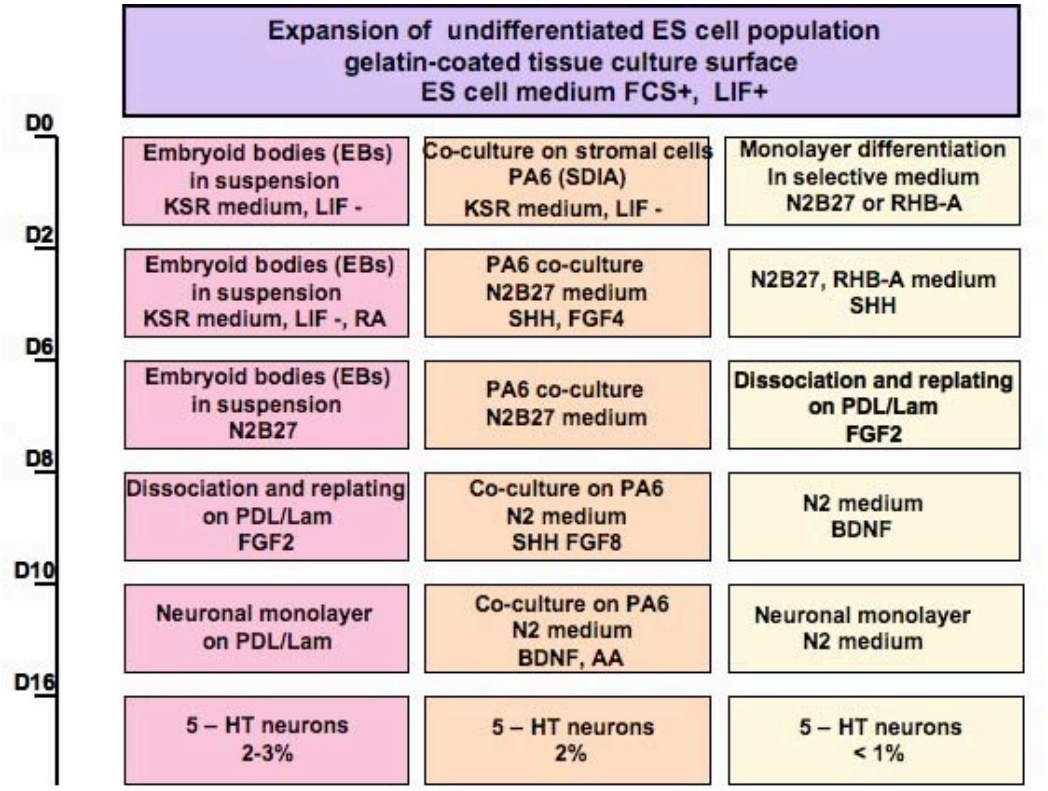


Figure 5.1. Scheme of differentiation protocols obtained as a part of the FunGenES cooperation. FCS - fetal calf serum, LIF - leukemia inhibitory factor, KSR - knock-out serum replacement, RA - retinoic acid, AA - ascorbic acid, BDNF - brain derived neurotrophic factor, FGF2 (bFGF) - fibroblast growth factor 2, SHH - sonic hedghog, FGF4 - fibroblast growth factor 4, N2B27, RHB-A - cell culture media, N2 - cell culture media supllement.

6.4.1 Embryoid bodies (EBs)

The basic retinoic acid (RA) protocol was used as a starting point, then, several changes were performed that showed better yield of serotonergic neurons in culture.

RA protocol (4-/2+/2-)

The cells were passaged 24h before starting the differentiation and the differentiation was started at 70% of cell confluency. The cells were grown in FFES medium.

The cells were then pelleted and resuspended in KSR medium (LIF-). 6 x 10^6 cells were then seeded in 10cm bacterial dish in 10ml of KSR medium. On Day 2 (D2) after starting the

differentiation the medium was changed to KSR, on D4 RA was added (1μM, final concentration) in a new KO medium. On D6 the medium was changed to N2B27.

Replating of EBs was done on D8. The cells were washed with PBS, trypsinized with Tryple express, counted and seeded on poly-D-lysine/laminin (PDL/Lam) coated cell culture dishes. 0.5 - 1 x 10^6 cells were coated per one well of the 6-well plate, and the number was adjusted accordingly if plated on the smaller surface. The cells were plated in N2B27 with bFGF added (10ng/ml). The medium with bFGF was changed on the first day post-plating (Dpp1), and, on the second day bFGF was removed (Dpp2). Since, the medium was changed every second day. The differentiation was kept in culture as long as viable neurons were visible.

Day	***Cells***	***Medium***	***Substance (final concentration)***
D0	Seed 5 x 10^6 cells/	KSR	
	10cm bacterial dish		
D2	Change the medium	KSR	
D4	Change the medium	KSR	RA (1μM)
D6	Change the medium	KSR	
D8	Replating on PDL/Lam	N2B27	bFGF (FGF2) (10ng/ml)
	0.25 – 5 x 10^5 cells/cm^2		
Dpp1	Change the medium	N2B27	bFGF (10ng/ml)
Dpp2	Change the medium	N2B27	-
Dpp4	Change the medium	N2B27	-
Dpp6	Change the medium	N2	-
Dpp8	Change the medium	N2	-
Dpp10	Change the medium	N2	-
Dpp12	Change the medium	N2	-
Dpp14	Fix the cells	4% PFA, 10min	

FGF4/SHH/AA protocol

In order to obtain better yield of serotonergic neurons in culture, we exchanged RA with FGF4/SHH cocktail that is known to promote the serotonergic differentiation *in vivo*. Thus, instead of adding RA on D2, FGF4/SHH (100ng/ml, each) was added and then continued as described above. After replating on D8, ascorbic acid (AA) was added (100μM) on Dpp2,

after withdrawal of bFGF, and kept in the medium usually until the end of the differentiation, or minimum for the first week, before the neurons with developed axons were observed.

Day	***Cells***	***Medium***	***Substances (final concentration)***
D0	Seed 5 x 10^6 cells/ 10cm bacterial dish	KSR	
D2	Change the medium and transfer to 5cm dish	KSR	FGF4 (100ng/ml)
D4	Change the medium	KSR	SHH (100ng/ml)
D6	Change the medium	KSR	
D8	Replating on PDL/Lam 0.25 - 5 x 10^5 cells/cm^2	N2B27	bFGF (FGF2) (10ng/ml); AA (10ng/ml)
Dpp1	Change the medium	N2B27	bFGF (10ng/ml); AA (10ng/ml)
Dpp2	Change the medium	N2B27	BDNF (100ng/ml)
Dpp4	Change the medium	N2B27	BDNF (100ng/ml)
Dpp6	Change the medium	N2	BDNF (100ng/ml)
Dpp8	Change the medium	N2	BDNF (100ng/ml)
Dpp10	Change the medium	N2	BDNF (100ng/ml)
Dpp12	Change the medium	N2	BDNF (100ng/ml)
Dpp14	Fix the cells	4%PFA, 10min	

6.4.2 PA6 protocol

PA6 cell were received from one of the FunGenES partners (Stem Cell Sciences, Edinburgh, UK) and maintained in culture on gelatine by replating when confluent in a PA6 medium.

Thawing, freezing and maintainance of PA6 cells was performed as for E14Tg2a cell line. Before the differentiation was started PA6 cells were cleavage inactivated with mitomycine C (1μg/ml final conc in PA6 medium) for 2.5h, counted, and seeded in the tissue culture dishes as a monolayer (1/3 of 15cm dish of confluent PA6 cells was evenly distributed on the 24 well or 6 well tissue culture dish) and left to attach for 24h. The next day a clonal density of E14 cells (5 x 10^3 - 10^4 cells/6 well) was seeded above the monolayer in N2B27 medium and the medium was changed every second day. Adding FGF4/SHH cocktail (100ng/ml each) on the second day of differentiation usually produced more serotonin neurons in culture.

Day	*Cells*	*Medium*	*Substances (final concentration)*
D0	Seed PA6 cells	PA6	
D1	Seed	N2B27	
	0.25 – 5 x 10^3 cells/cm^2		
D3	Change the medium	N2B27	FGF4 (100ng/ml)
D5	Change the medium	N2B27	SHH (100ng/ml)
D7	Change the medium	N2B27	
D9	Change the medium	N2B27	AA (10ng/ml)
D11	Change the medium	N2B27	BDNF (100ng/ml)
D13	Change the medium	N2B27	
D15	Fix the cells	4% PFA	

6.4.4. Monolayer protocol

The cells were replated 24h before the differentiation and let to grow to 70% confluence. The cells were trypsinized, counted and diluted in N2B27 medium. A gradient of the cell number was seeded in gelatinized tissue culture plates (10^4 - 10^5 cells/6well plate) and let differentiate. To promote the growth of neurons, the monolayer was replated every fourth day of differentiation, but the yield of the serotonergic neurons stayed very low, even after the stimulation with FGF4/SHH cocktail at different time points.

Day	*Cells*	*Medium*	*Substances (final concentration)*
D0	Seed 0.5-3x10^4 cells/cm^2	N2B27	
D2	Change the medium	N2B27	FGF4 (100ng/ml) + SHH (100ng/ml)
D4	Replate 0.5-1x10^3 cells/cm^2	N2B27	bFGF (10ng/ml)
D6	Change the medium	N2B27	
D8	Change the medium	N2B27	add AA (200mM) + BDNF (20ng/ml)
D10	Change the medium	N2B27	
D12	Change the medium	N2B27	
D14	Fix the cells	4% PFA	

6.5 The animals

6.5.1 Maintaining the mouse stock

The experiments during the course of this work were performed in adult (12-16 weeks old) *Tph2* deficient mice, 4 generation backcross to FVB/N, using +/+ and +/- littermates as a control.

The mice were kept in small groups (3-6 animals) in an air-conditioned room with 12-12h light dark cycle, and free access to the laboratory chow and tap water.

6.5.2 Superovulation of the blastocyst - donor females

All the methods for mouse embryo manipulation were performed by Larissa Villianovitch as described by Hogan et al. 1986.

The superovulation of the female mice was performed by the intraperitoneal (i.p.) injection of PMS (Pregnant Mare Serum; Intergonan, Vemie, Kempten, Germany) (7IE in 500µl), followed, after 24h, by the injection of HCG (Human Chorionic Gonadotropin, Predalon, Organon GmbH) (7IE in 500µl). To this purpose, young female mice (4-6 weeks) were used. The females were mated the next day (third day after the first injection), and the next morning they were checked for the presence of the vaginal plaque (VP). VP consists of the coagulated proteins of the sealing liquid, and is an indicator of successful copulation. The mice containing VP were taken for the isolation of the blastocysts.

6.5.3 Isolation of blastocysts

The blastocysts are isolated 3.5 days after VP (VP counted as day 1). Mice were kiled by cervical dislocation and the uteri collected in PBS. Each uterus was cut at the cartilagous part of the vagina, and put in a culture dish. The uterus was then held under the microscope with the help of a microdissection pipette at the oviduct end, and washed with 500µl of M16 medium with a 27-gauge needle. The blastocysts collected during the procedure were washed with fresh M16 medium and then transferred to M2 medium, and kept in the incubator until injection (37^oC, 5% CO_2). M2 and M16 were purchased from Sigma, Steinheim, Germany.

6.5.4 Microinjection of blastocysts

For the injection of ESC into host blastocysts a micromanipulator (Leitz), a microscope (Axiovert 35, Zeiss) and a pressure regulator was used as a microinjector, all as described by Hogan et al, 1986. ESC were separated with trypsin/EDTA and 8-12 cells were sucked in the injection needle, and injected in the cavity of the blastocysts. The blastocysts were hold on the embryoblast side to protect the trophoectoderm.

6.5.5 Preparation of foster mothers and retransfer of embryos

The evening before the embryo transfer adult female mice were mated with vasectomized males. The vasectomized males have interrupted vas deferens, but are still able to transmit seminal liquid and the successful copulation can be checked by VP the next morning. The pseudopregnant females are then used as foster mothers.

The manipulated embryos are retransferred directly in the uterus of the foster mother through a small puncture made with a 27-gauge needle. The mice were first narcotized with 50ml Ketavet and 50 ml Rompun per 10mg of weight i.p., and the skin opened at the back and front of the ovaries. The back opening was used to keep the ovaries from slipping backwards with a help of an operation clamp. The ovaries were taken out through the stomach opening and manipulated with forceps. To prevent bleeding the ovaries were treated with a drop of Suprarenin (Hoechst AG) that contracts the blood vessels. After the transfer, the wound was clamped, and sprinkled with antibiotic powder (Leukase, Smith & Kline, Becham).

The pups were born 3 weeks after the manipulation, and the colours of chimeras could been recognized between 3 - 4 weeks of age. The male chimeras were bred with C57Bl/6 mouse females to produce heterozygous offspring if the germ line transmission of the manipulated ESC into host blastocysts was successful, and the heterozygous offspring was bred to produce homozygous, *Tph2* deficient animals.

6.5.6 Organ collection

To collect organs for RT-PCR, gene expression and high performance liquid chromatography (HPLC) analysis, animals were anesthetized i.p. with ketamin (100mg/kg) and xylazine (100mg/kg) and perfused with PBS to wash out blood. Organs were isolated and immediately snap frozen in the liquid nitrogen.

6.5.7 High performance liquid chromatography (HPLC)

For the determination of monoamines and their metabolites, GABA, and glutamate, frozen tissues were homogenized in lysis buffer containing 10μM ascorbic acid and 2.4% perchloric acid, centrifuged for 30 min at 20 000 g and the supernatant was used for the HPLC measurement. Heike Hörtnagl, Reinhardt Sohr and Katja Tenner performed the measurements. The brain tissue levels of serotonin, 5-hydroxyindoleacetic acid, 3,4-dihydroxyphenylacetic acid and homovanilic acid (HV) were analyzed as described previously using HPLC with electrochemical detection (HPLC-ECD) (Sperk 1982). Dopamine and noradrenaline were measured by HPLC-ECD technique after extraction to alumina, according to Felice et al. with minor modifications (Felice et al. 1978, Sperk et al. 1981). For determination of γ-aminobutyric acid (GABA) and glutamate tissue levels, amino acids were precolumn derivatized with o-phthalaldehyde/2-mercaptoethanol using pre-cooled autoinjector and then separated on a HPLC column (ProntoSil C 18 ace-EPS, 50 mm x 3 mm i.d.) at a flow rate of 0.6 ml/min and a column temperature of 40^{o}C. The mobile phase was 50mM sodium acetate pH 5.7 in a linear gradient from 5% to 21% acetonitril. Derivatized amino acids were detected by their fluorescence at 450nm after excitation at 330nm (Piepponen et al. 2001).

6.5.8 ELISA: IGF-I, FSH and serotonin measurement

For the evaluation of IGF-I and FSH levels in sera, blood was taken periorbitally, left coagulate at RT and centrifuged 10min at 6000rpm (table Centrifuge Sorvall RC 5C, Haereus, Germany). 25μl of serum was used for each measurement using commercially available IGF-I EIA Kit (DSL-10-29000, Diagnostic System Laboratories, Webster, TX), Follicle Stimulating Hormone (FSH) (Rat) ELISA (29-AE-R004, Alpco Immunoassays, Salem, NH). For measurement of serotonin in the cells supernatant RayBio^{R} Serotonin EIA Kit for Serum, Plasma, Culture Supernatant and Cell Lysates (Raybiotech, Inc., Norcross, GA) was used following manufacturer´s instructions.

6.5.9 Immunohistochemistry and in-situ hybridization

For in situ hybridization and immunohistochemical analysis animals were anesthetized as above and transcardially perfused with 1x PBS - Heparin (5000I.E/1L) and

then with buffered 4% paraformaldehyde (PFA). Brains were removed and postfixed in the same buffered 4% PFA overnight at 4°C.

PFA fixed brains were incubated in 30% sucrose solution and cryosectioned at 20μm. The sections were dried after mounting on SuperFrost Plus (Menzel, Braunschweig, Germany) slides and directly used for immunohistochemistry, as described in Muller et al. 2000. Fluorescent images wre collected using Axioplan2 imaging microscope and sensicam 12BIT camera (Zeiss, Jena, Germany)

For the in-situ hybridisation PFA fixed brains were embedded in paraffin and sectioned at 8μm. The sections were deparaffinised, rehydrated and treated with proteinase K (Roche, Mannheim, Germany). Hybridisation was performed as previously described (Dony et al. 1987, Schmidt-Ulrich et al. 2006) using dioxigenin – UTP (Qiagen) – labeled mouse *Tph2* and *SERT* antisense – RNA probes. Pictures were taken with Axioplan2 Imaging microscope / Axiophoto camera (Zeiss).

6.5.10 Telemetry

The telemetric techniques were performed as described in Xu et al, 2008. Briefly, PhysioTel PA – C20 pressure transmitter (DSI, ST.Paul, MN) were implanted into the femoral artery of 3 months old female mice and recordings of blood pressure (BP), heart rate (HR) and locomotor activity were started 10 days after surgery. Respiration rate (RR) was calculated from the telemetric blood pressure data using the respiRATE module of the Dataquest A.R.T software (DSI). Data for day/nighttime BP, HR, RR and sleeping time were averaged from 7 am to 7 pm and vice versa of 96 hours recording time.

To perform the body temperature measurement PhysioTel TA – F20 transmitters (DSI) were implanted into the peritoneal cavity of 4 months old female mice and 10 days after surgery temperatures were recorded. After 2 days of baseline recordings at room temperature (22°C) animals were subjected to cold room (4°C) for 4hr and then returned back to ambient temperature.

6.5.11. Calcium, phosphor, glucose and insulin measurement

The measurement of Calcium (Ca^{2+}), Phosphor (P), glucose and insulin was done in sera of *Tph2* deficient and WT animals, by Lab28, Berlin, Germany.

6.5.12. Metabolic cages

For measuring water intake and urine volume, the mice were kept for 2 days in metabolic cages with free access to drinking bottles containing water. The bottles were refilled each morning. After conditioning for 24 h, in the next 48h water drinking volumes and urine volume was measured (2 times – every 24h).

6.6 Statistics

Results are expressed as mean ± SEM, or mean ± SD, as stated when presenting the data. Tests of significance (PRISM, GraphPad, San Diego, CA) were conducted by paired or unpaired Student's t-test, one way ANOVA, 2-way ANOVA followed by Tukey´s post-hoc test as stated for each data set separately.

The survival of animals was analyzed using Kaplan/Meier method followed by Logrank test.

III RESULTS

7. GENERATION OF TPH2 DEFICIENT ANIMALS

7.1. Tph2 „expression - selection" cassette

A Tph2 expression - selection cassette was created as described in the methods section of this work. Shortly, the homologous arms of the *Tph2* gene were cloned from a commercially available BAC containing *Tph2* sequence (BAC RP23-226H2) and introduced in the backbone of a targeting vector by BAC recombineering (Gene Bridges, Dresden, Germany) (Figure 7.1). The homology region of 10kb and 6kb at the 5' and 3' end, respectively, was used to introduce the linearized (I-Sce I) targeting construct at the beginning of *Tph2* gene and an additional SpeI restriction site was used for detection of the correctly integrated targeting construct (Figure 7.2).

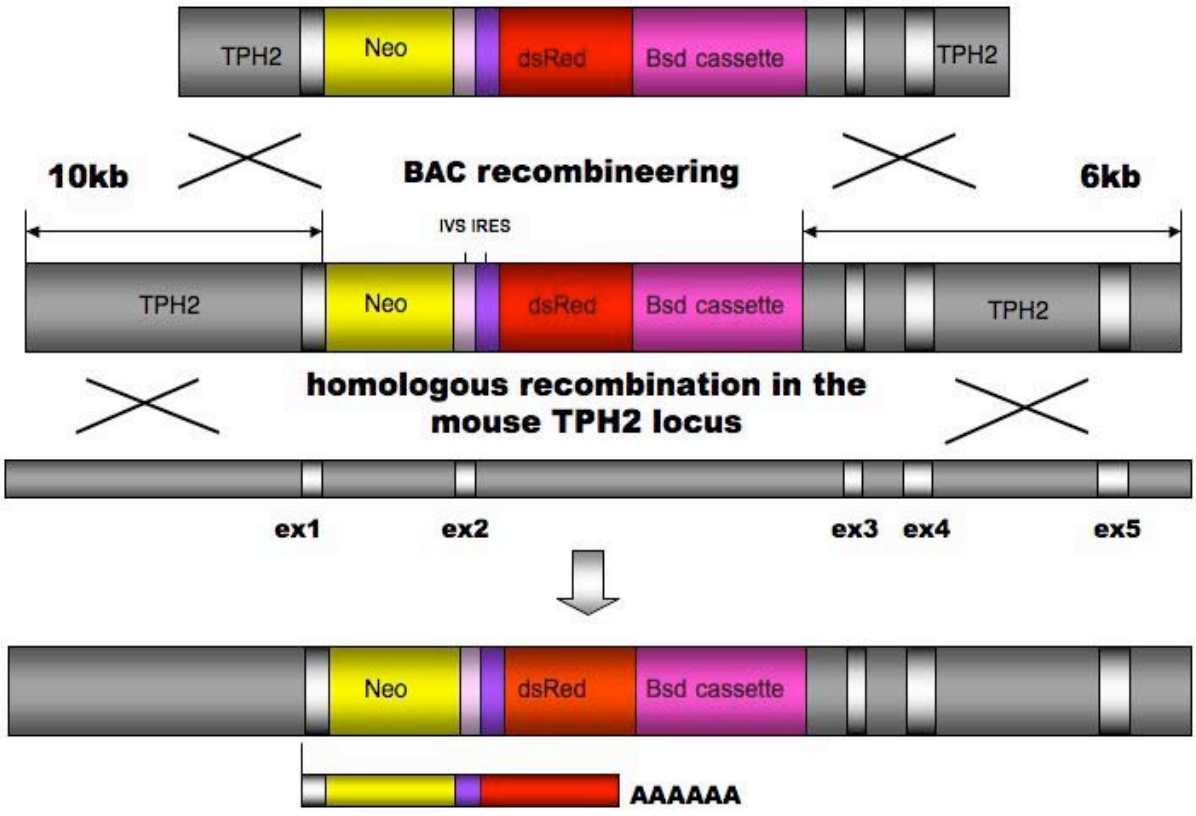


Figure 7.1. *Tph2* targeting construct. For details see the text and methods 6.1.14.1.

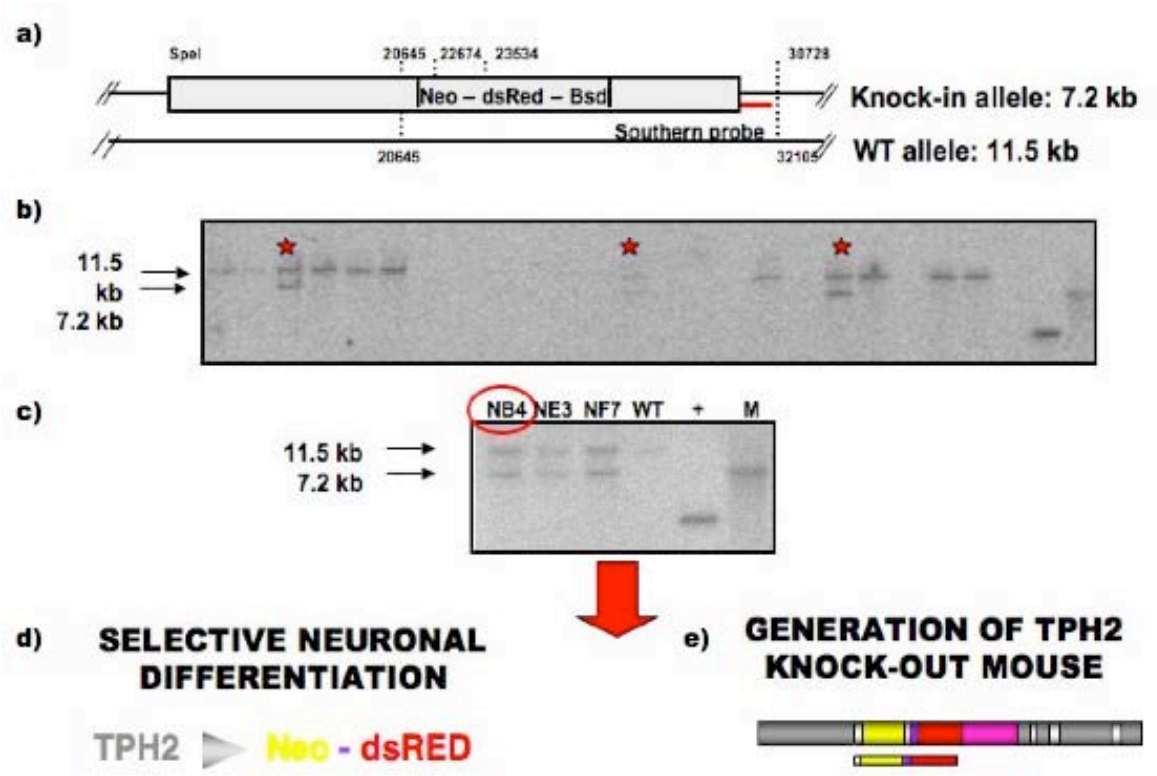


Figure 7.2. a) Southern blot scheme with additional SpeI site, b) Southern blot showing several positive and negative clones, grown under Bsd selection c) Southern blot showing several positive clones grown under Bsd selection confirming the correct integration. The same clones were amplified further - NF7 gave chimeras and founders for *Tph2*$^{-/-}$ mice (e) while clone NB4 was successfully used for *in vitro* differentiation (d).

After the correct integration, the expression of neoR linked by an IRES with dsRed gene was supposed to be driven by the *Tph2* promoter (Figure 7.1, 7.2). At the same time, the exclusion of exon 1 and exon 2 by the knock-in cassette including an independent stop codon and polyA signal (pA), interrupted the expression of the endogenous Tph2 allele, creating a knock-out allele in the ES cells. A ratio of 6% of positive clones was detected. Several positive clones were amplified, selectively, using an additional Bsd cassette expressed in ES cells for the positive selection of knocked-in cells (see methods 6.1.15.1). After the amplification both, NB4 and NF7 clones were used for creation of chimeras, but only NF7 cells successfully transmitted to the germ line and gave *Tph2*$^{-/-}$ founders. NB4 was used for *in vitro* differentiation.

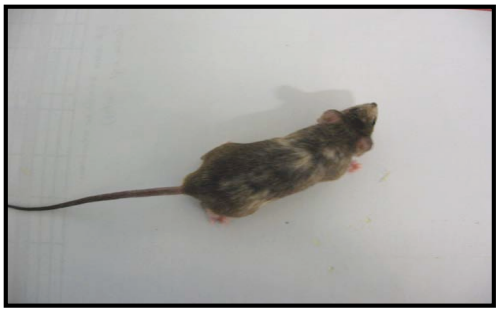
7.2. Creation of chimeras

Two positive ES cell clones (NB4, NF7) were injected into host blastocysts, and after retransfer of the blastocysts in the foster mother, 15 chimeras were born. The chimerism of the animals was optically determined, whereby 100% chimerism was considered to result in agouti fur colour and 0% in black fur colour of the animal. 2 male NB4 chimeras and 5 male NF7 chimeras were mated with 20 Bl6 females in total, during several months (Figure 7.3a). Out of 35 animals obtained, 12 were heterozygote for the *Tph2* allele. 2 males from the same NF7 chimeric father were chosen to be the founders, and mated further to obtain *Tph2*$^{-/-}$ animals (Figure 7.3 c, e).

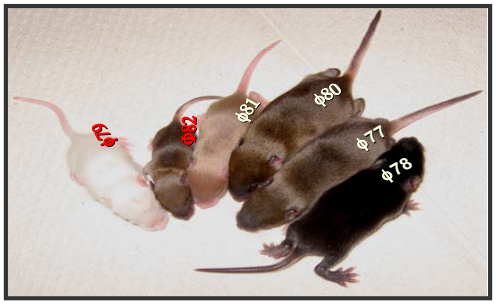
a)

Chimeras	*Ch40 (NB4)*	*Ch41 (NB4)*	*Ch68 (NF7)*	*Ch70 (NF7)*	*Ch72 (NF7)*	*Ch74 (NF7)*	*Ch75 (NF7)*	*Ch76 (NF7)*
Offspring	12	49	7	1	1	17	3	6
Heterozygote	0	0	5	0	0	7	0	0
Founder						2		

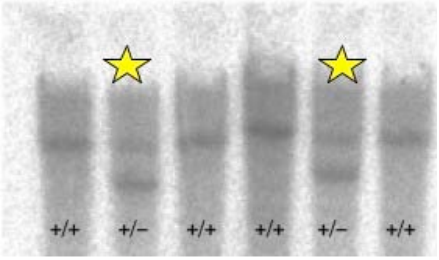
b)



c)



d)



e)

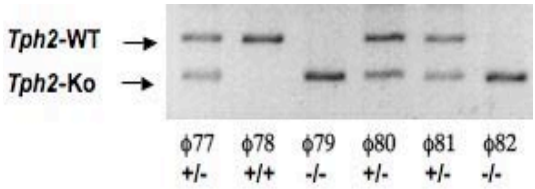


Figure 7.3. a) Table of *Tph2* chimeras and their offspring from breeding with Bl6 animals b) one of *Tph2* chimeras c) the first *Tph2* $^{-/-}$ offspring d) Southern blot of F1 animals e) PCR genotyping of *Tph2*$^{-/-}$ animals shown in c).

For detection of Tph2 heterozygous and homozygous animals PCR was used with specifically designed primers and under optimized conditions (Neo3, TPH54, TPH34, see methods 6.1.13.1 and Figure 7.3e). The animals are further backcrossed to FVB/N and C57Bl/6 mouse background.

8. PHENOTYPING OF Tph2 DEFICIENT ANIMALS

8.1. Serotonin (5-HT) and its metabolites in Tph2 deficient animals

8.1.1. RT-PCR

After the first Tph2 deficient animals were born, the first steps were to determine the absence of TPH2 and of serotonin in the brain, as well as of its metabolites. To this purpose, two sets of primers were designed (see methods), used to detect the absence of the 5′ and the 3′ end of *Tph2* mRNA (Figure 8.1). cDNA was synthesized from the total RNA extracted from the brains of the adult *Tph2* deficient and wild typ (WT) animals.

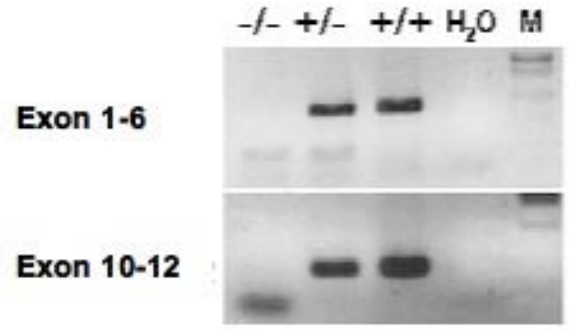


Figure 8.1. Expression of *Tph2* detected by RT-PCR. Brain cDNA of $Tph2^{+/+}$, $Tph2^{+/-}$ and $Tph2^{-/-}$ animals was used, M – ϕX174 DNA marker, H_2O - water (negative PCR control).

8.1.2. HPLC

HPLC was performed to confirm the absence of serotonin and its metabolites, first in the raphé nuclei (Fig. 8.2), and then in other parts of the brain (Table 8.1) The results showed expected deficiency in serotonin levels in the brain of *Tph2* deficient animals. The residual serotonin (~1%) might present the leftovers of the serotonin present in the circulation during the preparation of the samples, or it can be contributed to the basal activity of *Tph1*. The possibility that some other enzyme from the family of hydroxylases is complementing for

the loss of TPH2 and takes over the synthesis of serotonin cannot be excluded, and remains to be elucidated (Renson et al. 1962).

A slight augmentation of tryptophan, the serotonin precursor and the direct substrate of *Tph2* was observed in KO animals, suggesting the physiologically buffered accumulation of the substrate in the lack of the enzyme. However, the difference showed no statistical significance, and may present a normal physiological variation. The absence of the serotonin metabolite 5-HIAA is the consequence of the lack of serotonin.

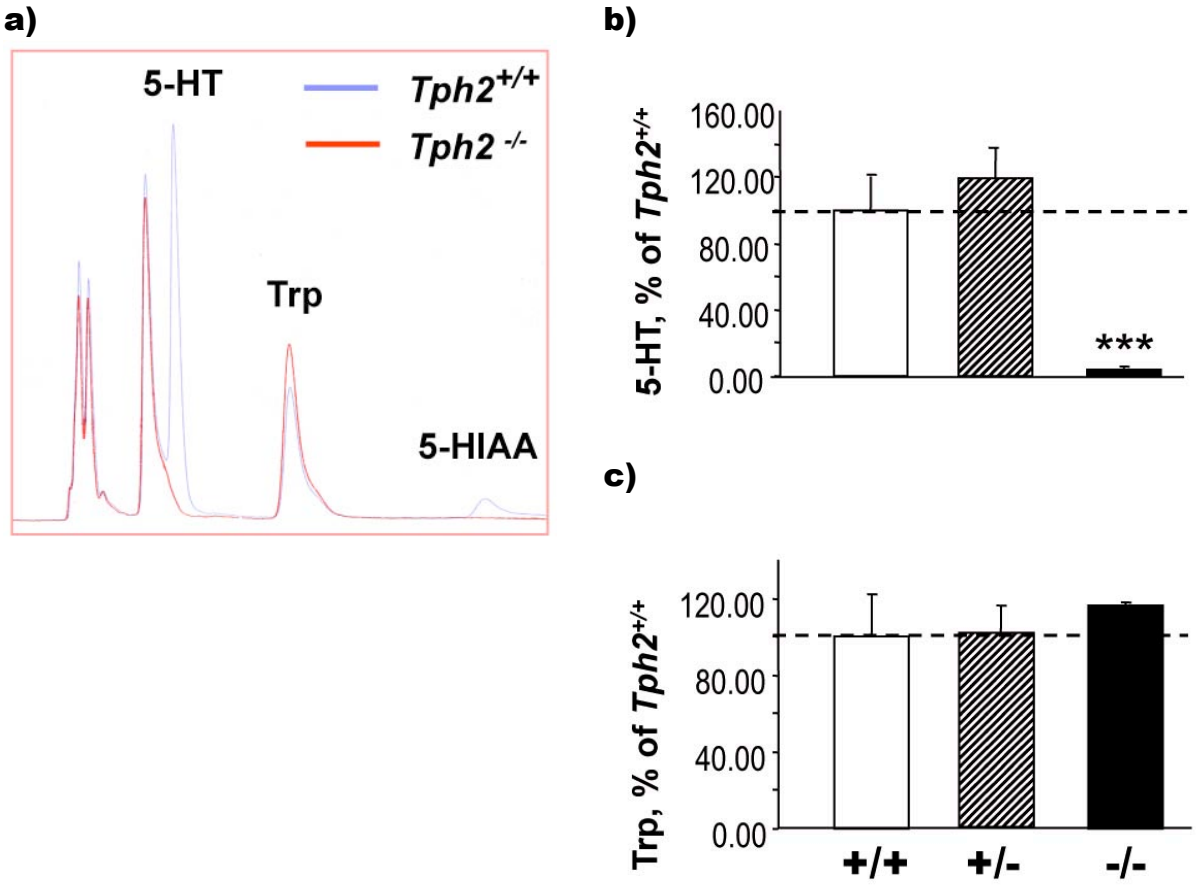


Figure 8.2. a) HPLC of 5-HT and metabolites with extract from dorsal raphé of the first *Tph2*$^{-/-}$ and WT animals b) 5-HT levels normalized to the values of *Tph2*$^{+/+}$ animals (+/+ = WT, +/- = heterozygote, -/- = *Tph2*$^{-/-}$ animal) c) tryptophan levels normalized to the values of *Tph2*$^{+/+}$ animal. ***p<0.001 *Tph2*$^{-/-}$ *vs.* *Tph2*$^{+/+}$ and *Tph2*$^{+/-}$, Student´s t test.

The serotonin levels in other parts of the brain were determined by HPLC. The total absence of serotonin in the brain was confirmed, highlighting *Tph2* as a single gene responsible for its synthesis in all brain regions (Table 8.1). The absence of serotonin

synthesis by TPH2 in organs other then brain was confirmed, as no significant difference of the serotonin level was observed in duodenum, spleen, liver or blood of *Tph2*$^{-/-}$ and WT animals (Table 8.2).

	Striatum		***Frontal cortex***		***Hippocampus***		***Hypothalamus***	
	5-HT	***5-HIAA***	***5-HT***	***5-HIAA***	***5-HT***	***5-HIAA***	***5-HT***	***5-HIAA***
***Tph2*$^{-/-}$**	7.67 ± 2.56 ***	n.d.	10.78 ± 1.12 ***	n.d.	7.54 ± 1.95 ***	n.d.	7.13 ± 4.76 ***	n.d.
***Tph2*$^{+/-}$**	718.98 ± 23.24	286.27 ± 9.36 ##	858.94 ± 38.09	159.18 ± 7.78	1152.21 ± 30.09	316.22 ±10.98 #	1738.09 ± 50.28	306.87 ± 8.26 ###
***Tph2* $^{+/+}$**	623.68 ± 68.44	345.92 ± 17.31	840.75 ± 34.85	180.22 ± 10.85	1048.17 ± 68.55	379.69 ± 36.44	1932.18 ± 74.18	423.30 ± 23.56

Table 8.1. Levels of 5-HT and its metabolite 5-HIAA in brain regions of *Tph2* deficient mice. Serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) levels were measured by HPLC in lysates of brain regions from *Tph2*$^{-/-}$ (n=5), *Tph2*$^{+/-}$ (n=11) and *Tph2*$^{+/+}$ (n=6) mice (mixed background). Data are presented in pg per mg of wet tissue as mean ± SEM; n.d. = not detectable. ***p<0.001 *vs. Tph2* $^{+/+}$ and *Tph2* $^{+/-}$ one way ANOVA, followed by Tukey's post hoc test; #, ## and ### p < 0.05, 0.01 and 0.001, respectively, *vs. Tph2* $^{+/+}$; Student's t-test

	Duodenum (pg)		***Liver (pg)***		***Blood (ng/ml)***		***Spleen (pg)***	
	5-HT	***5-HIAA***	***5-HT***	***5-HIAA***	***5-HT***	***5-HIAA***	***5-HT,***	***5-HIAA***
***Tph2*$^{-/-}$**	7053.50 ± 701.78	2547.10 ± 349.66	70.58 ± 5.51	n.d.	4138.0 ± 426.4	n.d.	3208.03 ± 443.72	n.d.
***Tph2*$^{+/-}$**	8609.05	3166.91	86.69	n.d.	4161.3	n.d.	4180.70	n.d.

	± 605.91	±292.54	± 14.60		± 664.2		± 300.04	
Tph2 $^{+/+}$	6374.44	2851.19	95.81	n.d.	3887.9	n.d.	3033.81	n.d.
	± 483.94	± 546.21	± 14.37		± 444.8		± 432.07	

Table 8.2. Levels of 5-HT and its metabolite 5-HIAA in peripheral tissues and blood of *Tph2*-deficient mice. Serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) levels were measured by HPLC in tissue lysates and in whole blood of *Tph2*$^{-/-}$ (n=5), *Tph2*$^{+/-}$ (n=4) and *Tph2*$^{+/+}$ (n=6) mice (FVB/N-F7 genetic background). For duodenum, liver, and spleen data are presented in pg per mg of wet tissue as mean ± SEM; n.d. = not detectable. No significant difference was observed between genotypes.

8.2. Immunohistochemistry and in situ hybridization

To further confirm the absence of serotonin in the raphé nuclei of *Tph2* deficient animals immunochistochemical studies were performed with 5-HT antibody (Sigma Aldrich), and the results were confirmed on the expression (mRNA) level using *in situ* hybridization technique (see methods). No serotonin (5-HT immunostaining) or *Tph2* mRNA (*in situ* hybridization) were detected in *Tph2*$^{-/-}$ animals (Fig. 8.3).

To address the question of the presence of serotonergic neurons despite the absence of serotonin, *in situ* hybridization of serotonin transporter (SERT) was performed. SERT is highly expressed in the serotonergic neurons and present all over the axonal connection, where it is involved in reuptake of serotonin from the synaptic cleft. *In situ* staining showed unchanged pattern in the brain stem region (raphé nuclei). The analysis of other brain regions innervated by serotonin afferents are still pending.

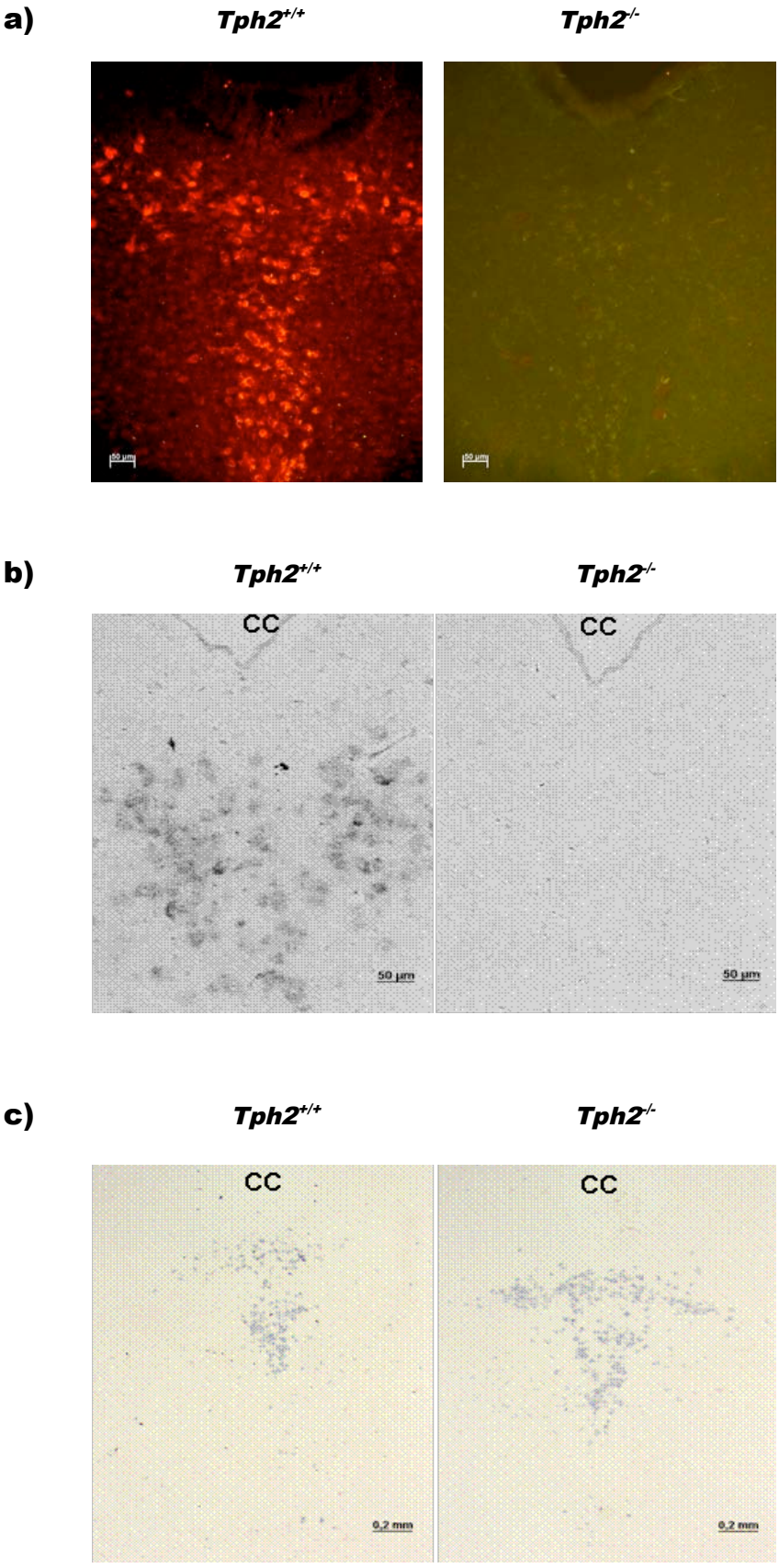
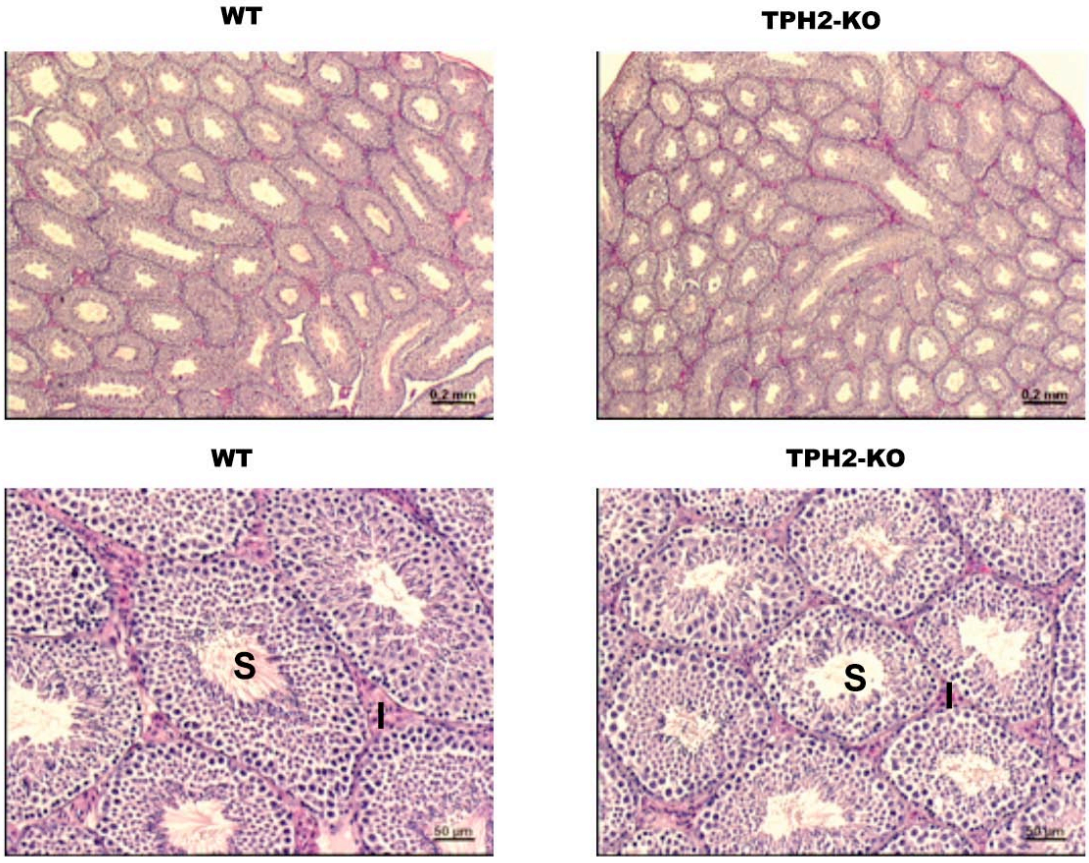


Figure 8.3. a) 5-HT immunostaining and b) Tph2 and c) SERT *in situ* hybridization in raphé nuclei (see materials and methods for details), cc – central canal.

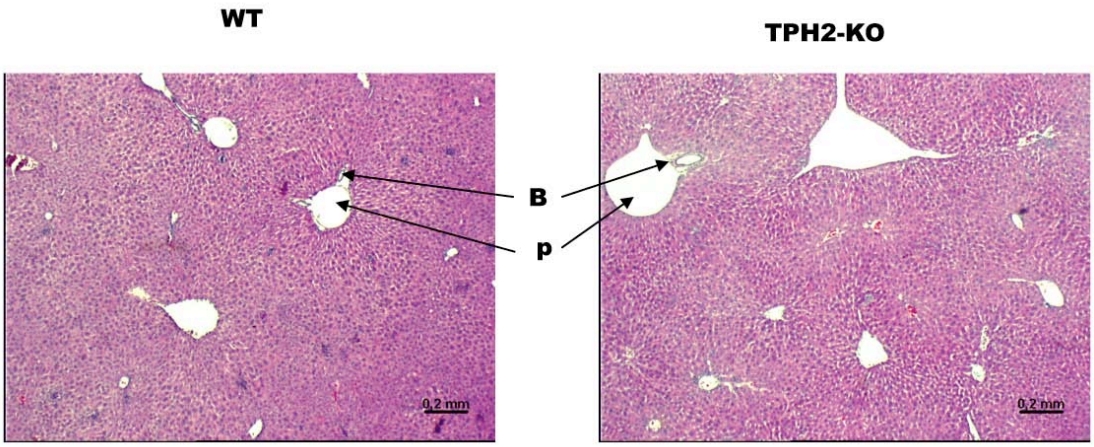
8.3. Histological analysis of organs

To further characterize Tph2 deficient animals histological analysis of organs was performed using the common method of HE staining (see methods). No gross histological changes were observed. The lungs showed normal architecture and bronchioli with normal epithelium. The bronchial wall showed a very mild chronical infiltration. Some alveolar groups seemed to be extended. No bigger emphysema is observed – but probably the beginning of centrilobar emphysema and a mild bronchial inflammation. There is an observed hyperemia in the blood vessels. In the liver there were no pathological changes. Neither heart nor kidney tissue showed any pathological changes or inflammation. We were not able to say if those changes were caused by the lack of serotonin directly, or they were secondary effects: *Tph2* deficient animals showed a lower breathing rate in further experiments, which might cause emphysema.

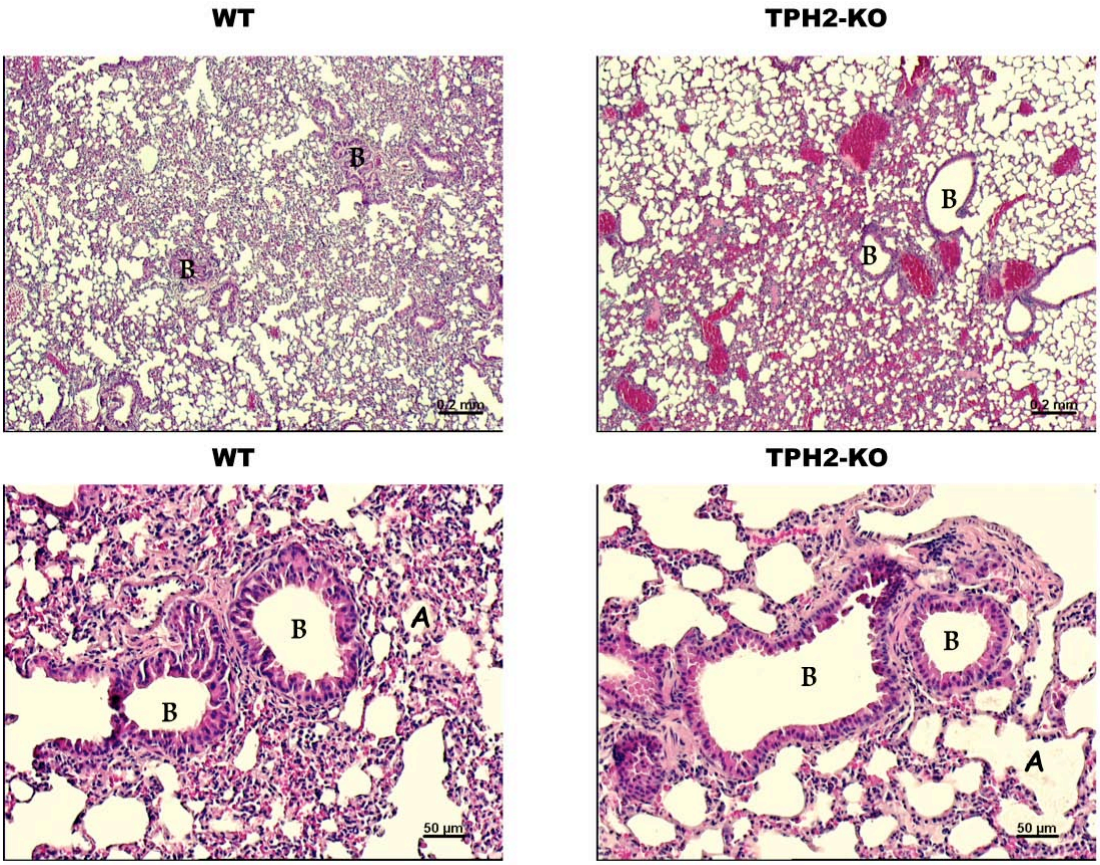
a)



b)


WT
TPH2-KO
B
p
0,2 mm
0,2 mm

c)


WT
TPH2-KO
B
B
B
B
0,2 mm
0,2 nm
WT
TPH2-KO
B
B
A
B
B
A
50 µm
50 µm

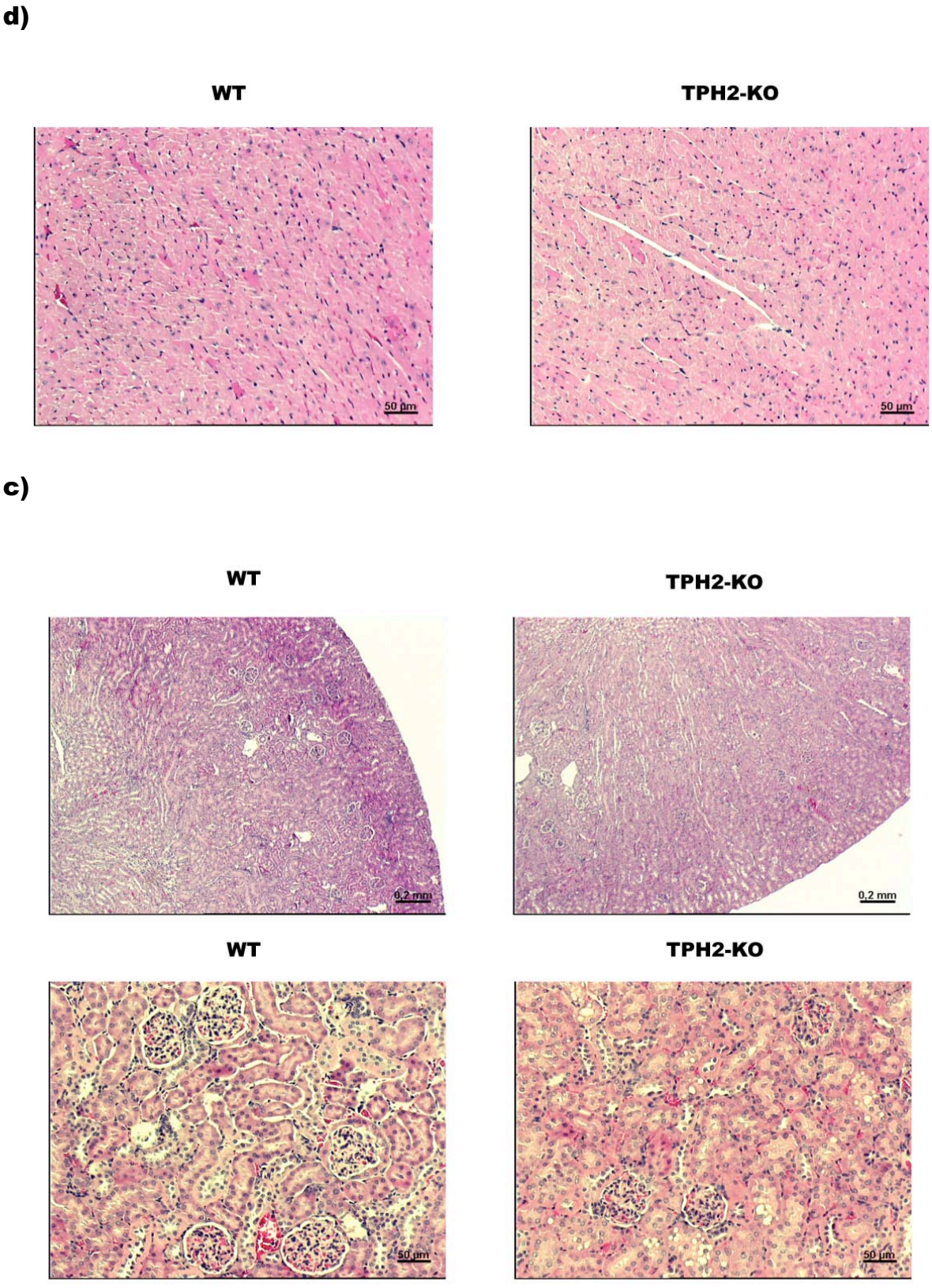


Figure 8.3. HE staining of a) testis, I - interstitial cells (Lydig-Cells), S - seminiferous tubule b) liver, B - bile duct, P - portal vein branch c) lung, A - alveolar sac, B - bronchiole d) heart and e) kidney of *Tph2*$^{-/-}$ and control animals. For details see text.

8.4. Survival and growth

8.4.1. Growth retardation of Tph2 deficient animals

8.4.1.1. Body weight

The first observation regarding the phenotypic characteristics of the animals deficient in serotonin synthesis was, strikingly, not of neuronal nature. *Tph2* deficient animals are born in Mendelian ratio and at birth showed no visible abnormalities. The growth of the animals was, however, impaired. Already three days after birth $Tph^{-/-}$ mice were visibly smaller, had softer skin and appeared weaker than their WT littermates. The difference was aggravating in the next 3 weeks - the length and the weight of $Tph^{-/-}$ animals at 15 days of age was one third of their WT littermates (Figure 8.5). The growth retardation did not allow the weaning of $Tph^{-/-}$ animals at three weeks of age but only 2 weeks later. After weaning, however, they started a catch-up growth and reached normal size at 4 months of age.

a) **b)**

c)

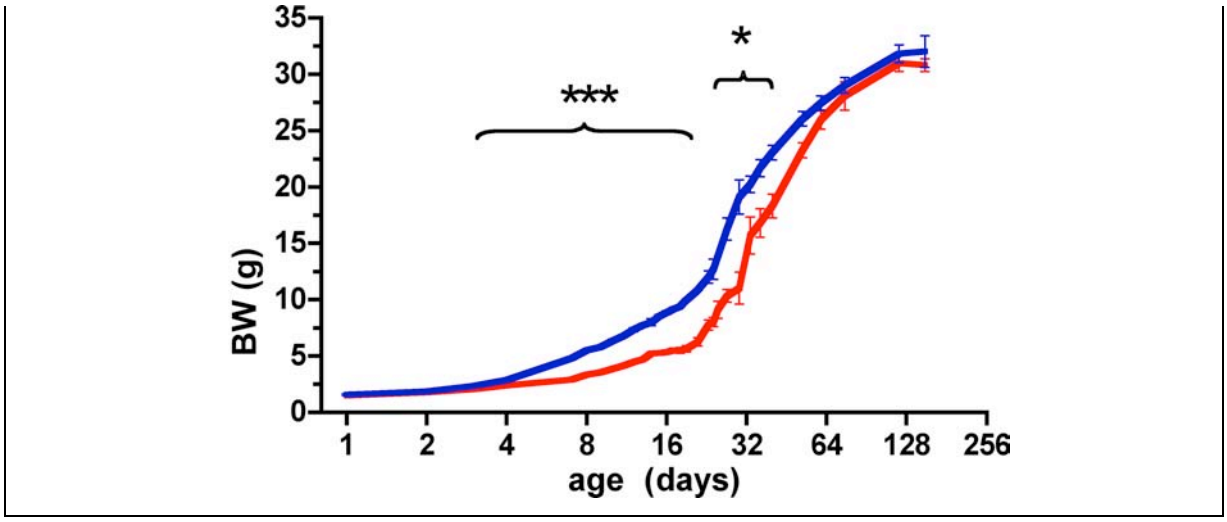
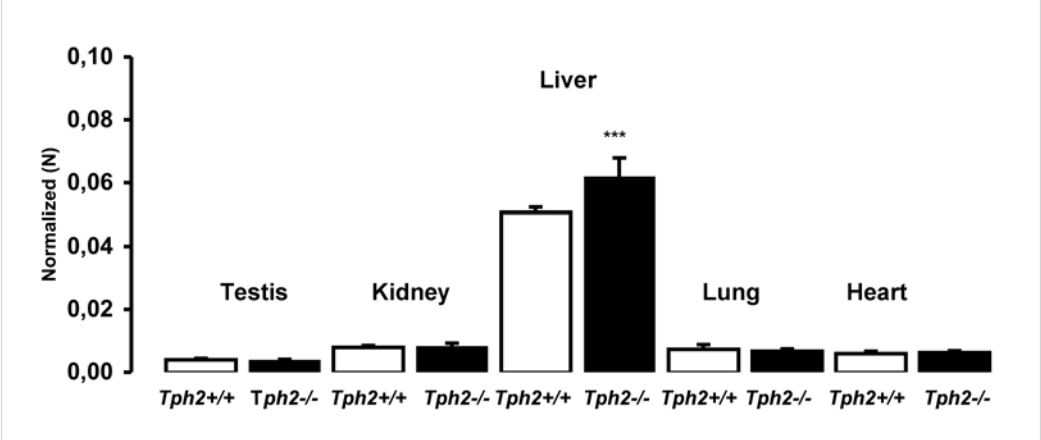


Figure 8.5. a) Representative photographs of 15 days old FVB/N-*Tph2*-deficient and WT animal b) and 21 days old C57Bl/6 *Tph2* deficient mice c) body weight curve of *Tph2*$^{-/-}$ and WT animals - the growth retardation shows the most profound difference between 10 - 15 days after the birth. *p<0.05, ***p<0.001 *Tph2*$^{+/+}$ *vs. Tph2*$^{+/-}$ Student´s t-test.

8.4.1.2. Organ weight

The weight of organs was measured several times during organ collection but no big differences were observed. However, the anesthetized animals showed a slight augmentation in liver size (Fig. 8.6a), while the non-anesthetized animals showed slightly bigger testis (Figure 8.6b). We speculated that increased liver size after anesthesia is due to the increased blood flow as a consequence of an impaired sympathetic/parasympathetic response caused by the lack of serotonin (see discussion).

a)



b)

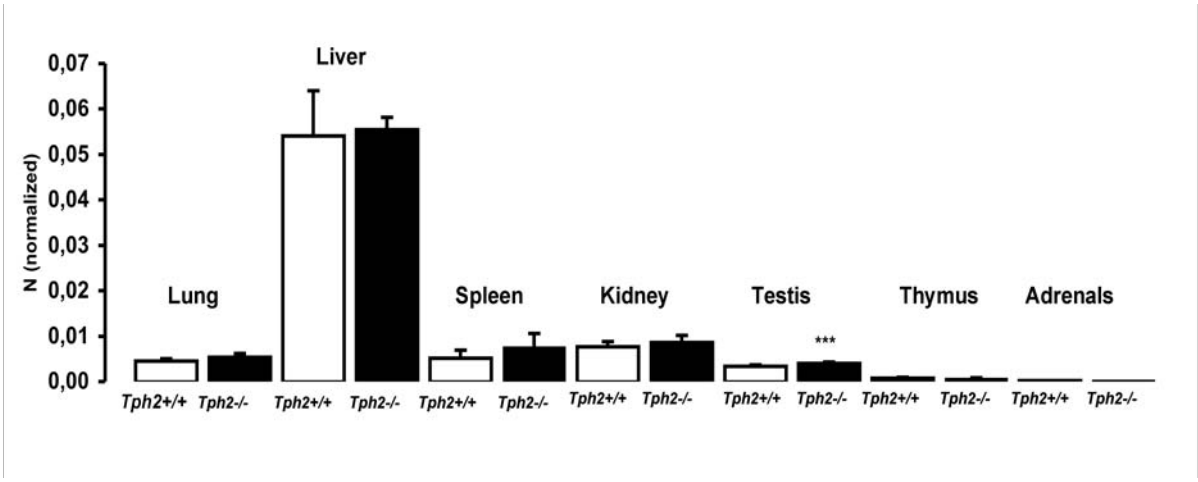


Figure 8.6. a) weight of different organs normalized to the body weight when animals were anesthetized with ketamine/xylazine before the organ collection b) when animals were sacrificed by cervical dislocation. ***p<0.001 *Tph2* $^{+/+}$ *vs. Tph2* $^{+/-}$, Student´s t-test, males (n=6), FVB/N F4 backcross, 4 months of age.

8.4.2. IGF-I concentration in the blood of Tph2 deficient animals

The lack of difference in organ weight when normalized to the body weight of the whole animal pointed to the simultaneous growth of both *Tph2*$^{-/-}$ and WT animals. Thus, the observed growth retardation is most probably caused by the systemic deficit in the hormones responsible for growth and its effective molecules. This led us to investigate the concentration of insulin-like growth factor I (IGF-I) in the blood as it is known that it stimulates growth of organs under the influence of growth hormone (GH).

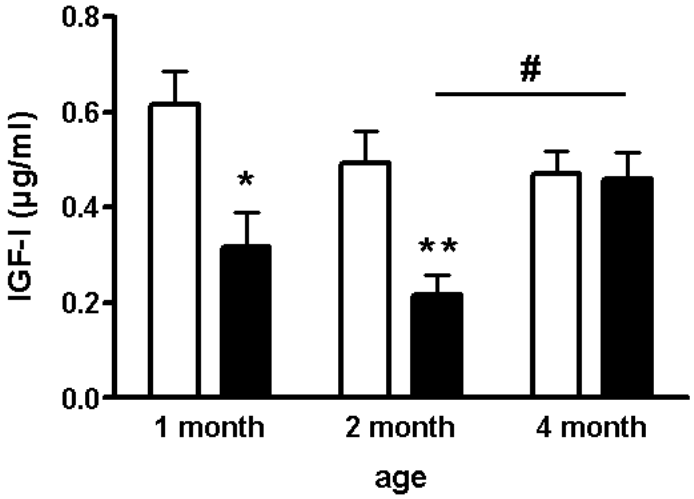


Figure 8.7. IGF-I concentration (µg/ml) in the serum of the *Tph2* deficient animals (FVB/N-F4 genetic background) at different ages. *p<0.05, **p<0.01 *Tph2* $^{+/+}$ *vs. Tph2* $^{+/-}$, Student´s t-test. # p<0.05 (t-test) 4 month old *vs.* 2 month old *Tph2*$^{-/-}$ mice.

Serum levels of IGF-I were markedly lower in *Tph2*$^{-/-}$ mice until two months of age but afterwards reached the levels of control animals, when the animals had attained normal size (Figure 8.7),.

8.4.3. Hypothalamo-Hypophyseal Axis

After the observation that IGF-I level is decreased during the development of *Tph2* deficient animals, we were interested in the transcriptional levels of other hormones of the hypothalamo-hypophyseal axis. The relative expression of the genes involved in the synthesis of growth hormone (GH) - somatotropine (Smtrpn), follicle-stimulating hormone (FSH), thyreostimulating hormone (TSHb) and prolactine (Prln) were measured by qPCR in the hypothalamus, hypophysis and testis of the male FVB/N-F4 animals (the results were normalized to the expression of β actin). The dynamics of growth was observed in hormonal levels - the highest difference in expression of FSH is present in mice just after the weaning (1 months old males) and before they start the catch-up growth (Fig. 8.8). The difference is still detectable in 2m old mice, but 4m old *Tph2*$^{-/-}$ animals had the same transcriptional level of FSH as their WT littermates (Fig. 8.9).

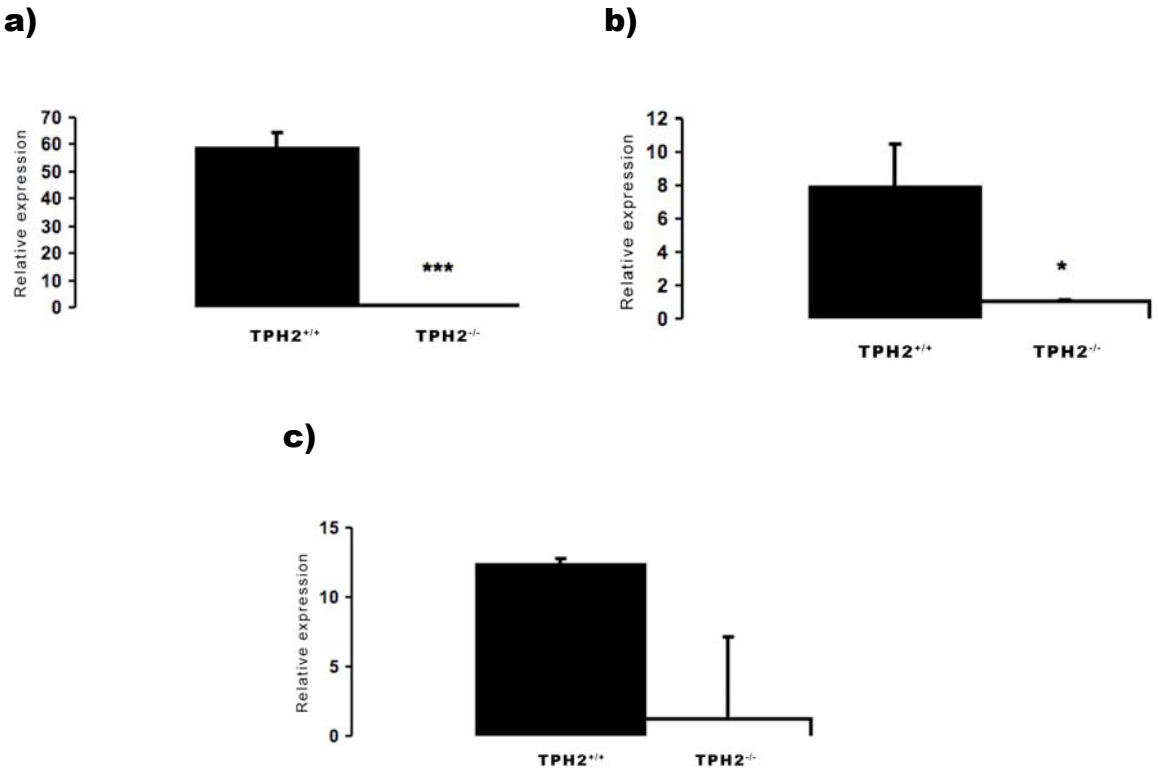


Figure 8.8. FSH mRNA levels in different organs of 1 month (1m) old male FVB/N-F4 *Tph2*$^{+/+}$ and *Tph2*$^{-/-}$ mice (n=3) as measured by qPCR a) hypothalamus b) hypophysis c) testis. Level 1 of relative expression denotes the lowest expressing animal. *p<0.05, ***p<0.001 *Tph2*$^{+/+}$ *vs. Tph2*$^{+/-}$, unpaired Student´s t-test.

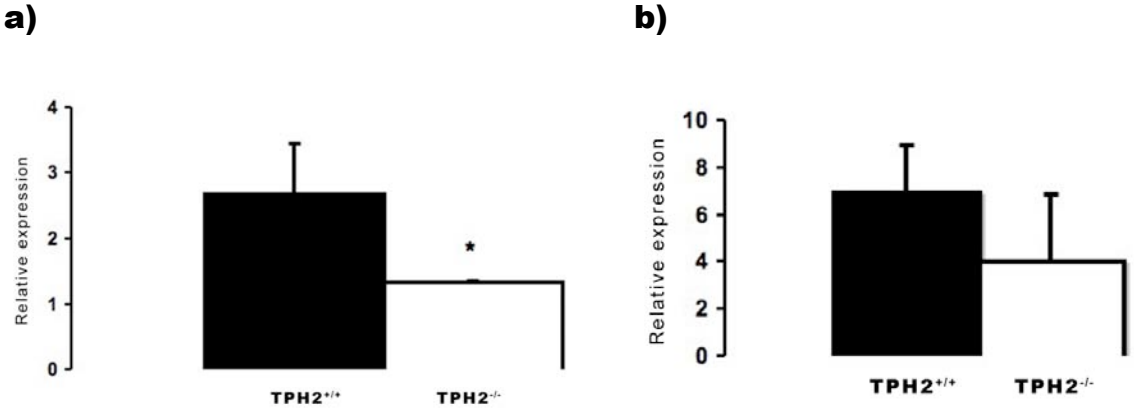


Figure 8.9. FSH mRNA levels in the hypophysis of a) 2m and b) 4m old FVB/N-F4 male *Tph2*$^{+/+}$ and *Tph2*$^{-/-}$ mice (n=3) as measured by qPCR. Level 1 of relative expression denotes the lowest expressing animal. *p<0.05 *Tph2*$^{+/+}$ *vs. Tph2*$^{+/-}$, unpaired Student´s t-test.

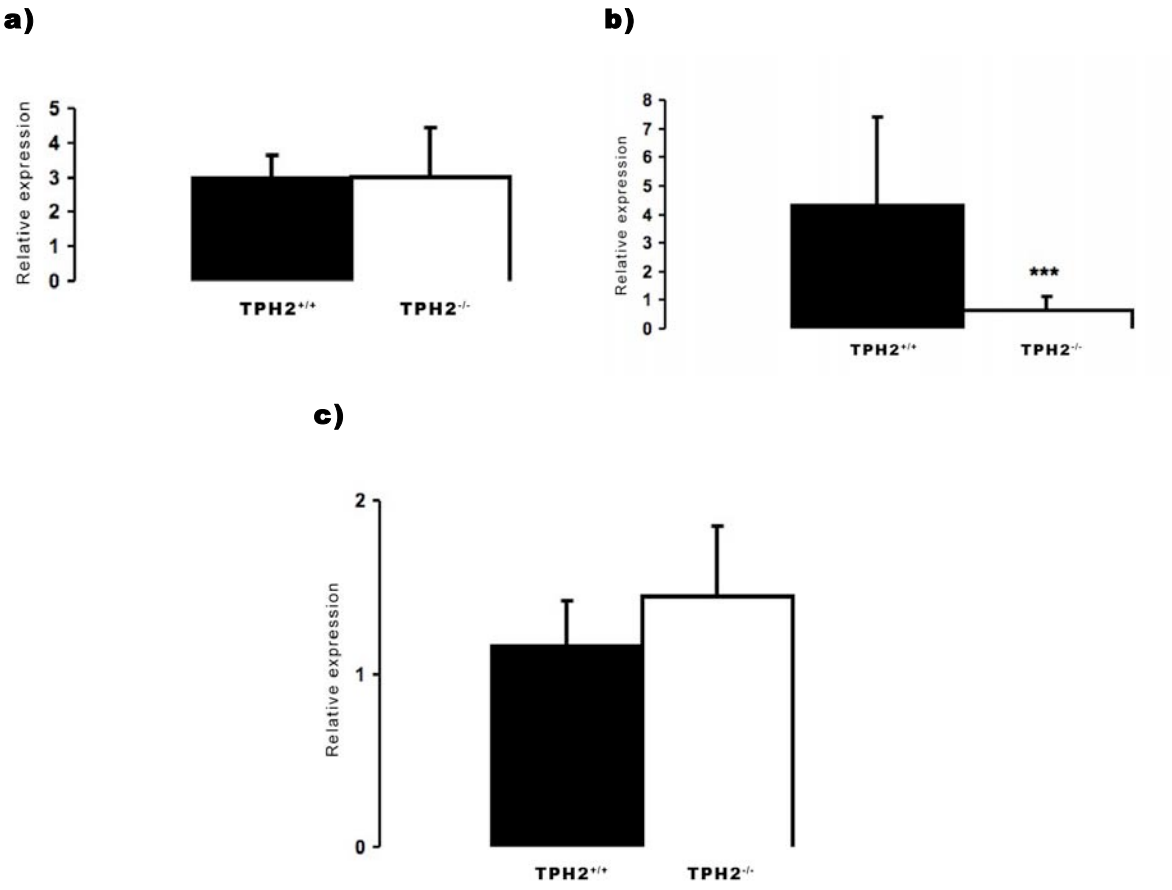


Figure 8.10. Prl (a), Smtrpn (b) and TSHb (c) mRNA levels in the hypophysis of 2m old FVB/N-F4 male *Tph2*$^{+/+}$ and *Tph2*$^{-/-}$ mice (n=3) as measured by qPCR. Smtrpn, the precursor of GH, is still significantly reduced. Level 1 of relative expression denotes the lowest expressing animal. ***p<0.001 *Tph2* $^{+/+}$ *vs. Tph2* $^{+/-}$, unpaired Student´s t-test.

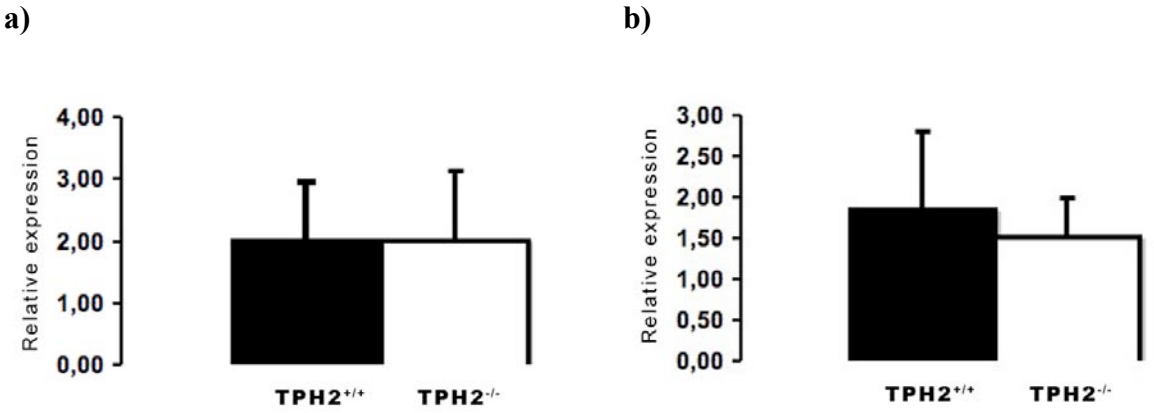


Figure 8.11. Smtrpn mRNA levels in the a) hypophysis and b) testis of 4m old FVB/N-F4 male *Tph2*$^{+/+}$ and *Tph2*$^{-/-}$ mice (n=3) as measured by qPCR. Level 1 of relative expression denotes the lowest expressing animal. There was no difference between the groups observed after the catch-up growth.

We used the collected organs during the basic characterization of *Tph2*$^{-/-}$ animals to perform one more qPCR analysis in liver, where IGF-I synthesis together with it carrier protein acid-labile subunit (ALS) is extensive, and constant. Transcriptional levels of growth hormone receptor (GH-R) were also measured and no difference was observed (Figure 8.12).

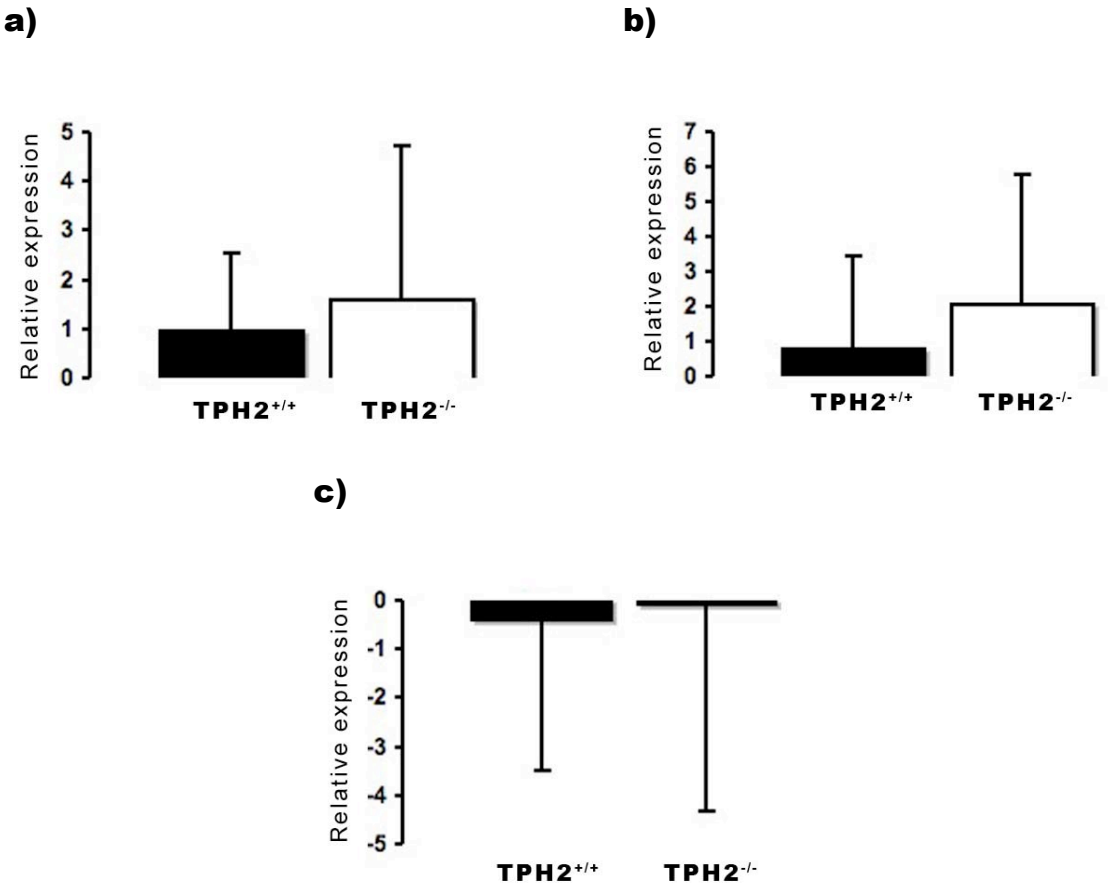


Figure 8.12. a) ALS b) GHR and c) IGF-I mRNA levels in liver of FVB/N-F4 *Tph2*$^{+/+}$ and *Tph2*$^{-/-}$ mice (n=3)) as measured by qPCR. No significant transcriptional changes were observed.

8.4.4. FSH in the serum

After the observed decreased expression of FSH/LH mRNA, the level of FSH was measured in the sera of WT and *Tph2*$^{-/-}$ males. To our surprise, higher levels of FSH were observed in the sera of 4m old *Tph2*$^{-/-}$ animals. Up to now, we could not find any reasonable explanation for this phenomenon, except that it is in accordance with slightly higher weight of testis observed previously in *Tph2* deficient animals (see 8.4.1.2 and Figure 8.13). If the lower

concentration of FSH in younger age is influencing testis to complement for the lack of hormone is to be elucidated.

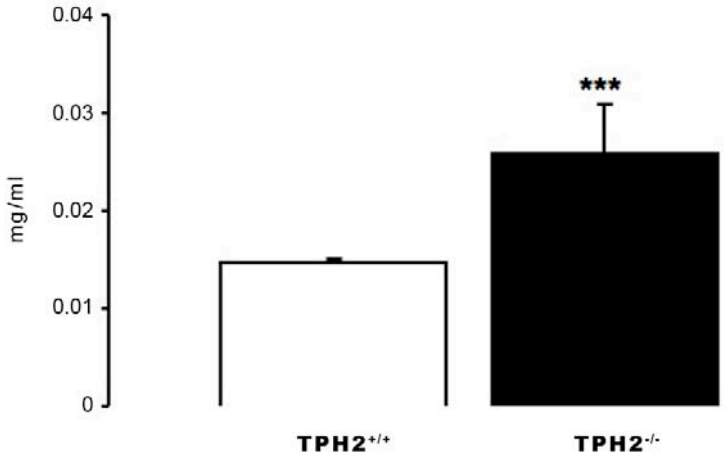


Figure 8.13. FSH concentration in sera of *Tph2*$^{+/+}$ and *Tph2*$^{-/-}$ deficient animals (n=6), ***p<0.001 *Tph2*$^{+/+}$ *vs. Tph2*$^{+/-}$, Student´s t-test.

8.4.5. Survival

Tph2$^{-/-}$ *mice on the mixed background showed considerable lethality* in the first 4 weeks of life (27%). Backcross to FVB/N background improved the survival rate (88.6%), whereas after backcross to the C57Bl/6 strain the lethality got more pronounced (Figure 8.14) and about half of the *Tph2*$^{-/-}$ pups were lost (52.6% survival). We still do not know the reason for increased mortality of *Tph2-/-* pups in the first weeks of life (see discussion).

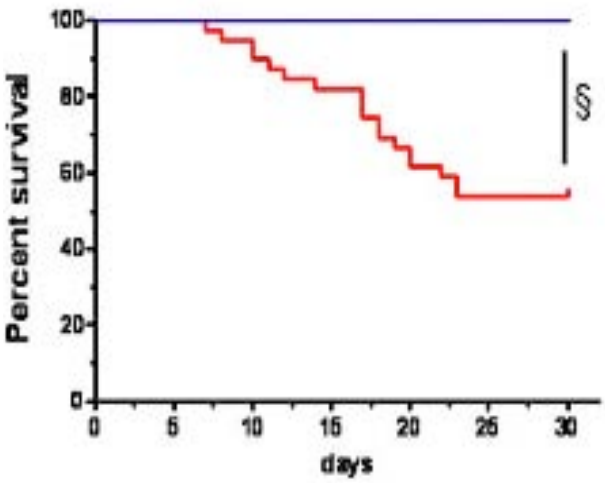


Figure 8.14. Kaplan Meier curve of survival of *Tph2*$^{-/-}$ animals (C57Bl/6 genetic background). Red line: *Tph2*-/-; blue line: control mice. §p<0.0001 (Logrank test)

8.5. The search for complementation

The mild behavioral and survival changes of Tph2$^{-/-}$ *animals,* presented until, came as a surprise. The viability and reproductive fitness of brain serotonin deficient animal is a hallmark of this work. By the role serotonin exerts during embryogenesis and in adults (Gaspar et al. 2003), we expected far more sever phenotypes. Other monoaminergic neurotransmitters, like dopamine or catecholamines, exerted a lethal phenotype *in utero* when ablated (Thomas et al. 1995, Kobayashi et al. 1995, Zhou et al. 1995).

Monoaminergic neurotransmitters are biochemically very similar, and involved in the control of the same physiological processes (introduction). They also influence the synthesis and the effects of each another. Excretion of dopamine is known to be able to be modulated by serotonin (Barnes and Sharp 1999, Herman 2003) and to exert affinity for some of the serotonergic receptors, and the mice deficient in MAO-A accumulate serotonin in atypical locations in the vicinity of serotonin, dopamine and norepinephrine transporters (Cases et al. 1998). Several experiments were done during the course of this work to investigate the possible complementation by other monoaminergic neurotransmitters, notably dopamine. These experiments, however, did not show any expected upregulation of any of the genes involved in any other pathway. More strikingly, our results show no influence of serotonin on transcriptional level of any of its receptors. The possible further complementational analysis will be discussed in the discussion chapter of this work.

8.5.1. Levels of neurotransmitters in the brain

First, we measured the concentration of other neurotransmitters: dopamine, γ-aminobutyric acid, norepinephrine, and glutamate, and their metabolites: 5-HIAA and HVA, in the brain of *Tph2*$^{-/-}$ and WT animals by HPLC (see methods 6.5.7 and Table 8.3).

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

Table 8.3. Levels of neurotransmitters and their metabolites in the brain of *Tph2*$^{-/-}$ and control animals. Serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), norephinephrine (NA), Glutamate, γ-aminobutyric acid (GABA), dopamine (DA), dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) levels were measured in whole brain lysates per mg of wet tissue of *Tph2*$^{-/-}$ (n=4) and control (n=6) mice (mixed background). Data are presented as mean ± SEM; n.d. = not detectable. ***p<0.001 *vs.* control group, one way ANOVA, followed by Tukey's post hoc test.

8.5.2. Affymetrix analysis

We, next, studied the effects on gene expression in the brain of the near complete lack of serotonin. Total mRNA of the whole brains of 2m old WT and *Tph2*$^{-/-}$ FVBN/F4 animals was sent to microarray analysis, expecting the differences in transcriptional levels not only of the enzymes and receptors of other monoaminergic systems, but also of serotonin receptors regulated by serotonin. About 500 genes showed significant alterations in expression, but only in six cases these changes exceeded a 1.7 fold up or downregulation compared to controls. However, these genes (*Pctk2*, *Dmt2*, *Ccdc53*, *Tmem4*, *Scyl2*, and an unknown one) are located on chromosome 10 in proximity of the *tph2* locus and may therefore still be of 129/OlaHsd origin in our backcross (Jansen and Nap 2001). To our surprise the level of no other transmitter was altered in comparison to control animals. Thus, we could not detect any compensatory mechanisms in the brain of mice drastically deficient in serotonin.

When the threshold of the change was moved to 1.3 fold, we were able to detect around 30 genes located outside of chromosome 10 (Appendix 3). Using The Database for Annotation, Visualization and Integrated Discovery (DAVID) 2008 (http://david.abcc.ncifcrf.gov/) we sorted the genes by their putative role (Table 8.4).

Negative regulation of cellular processes	*Negative regulation of cellular physiological processes*	*Negative regulation of physiological processes*	*Negative regulation of metabolism*	*Morphogenesis*	*Cell migration*	*Negative regulation of biological process*
Rgs9	Prkca	Prkca	Prkca	Smyd1	Prkca	Rgs9
Prkca	Smyd1	Smyd1	Smyd1	Gsk3b	Evl	Prkca
Smyd1	Gsk3b	Gsk3b	Evl	Evl	Slit2	Smyd1
Gsk3b	Evl	Evl		Crim1		Gsk3b
Evl				Slit2		Evl

Locomotion	*Localization of cell*	*Cell motility*	*Behavior*	*Macromolecule metabolism*	*Intracellular protein transport*
Prkca	Prkca	Prkca	Prkca	A530088I07Rik	Gsk3b
Evl	Evl	Evl	Grm5	Prkca	Eif2ak4
Slitt2	Slit2	Slit2	Slit2	Ptprt	Ywhaz
				Smyd1	Dnajb14
				Gsk3b	
				Evl	
				Cpe	
				Pabpn1	
				Ribosomal protein S6	
				Dnajb14	
				Kl	

Cell organization and biogenesis	*Protein targeting*	*Intracellular transport*
Smyd1	Gsk3b	Gsk3b
Gsk3b	Eif2ak4	Evl
Evl	Ywhaz	Eif2ak4
Crim1	Dnajb14	Ywhaz
Eif2ak4		Dnajb14
Ribosomal protein S6		
Ywhaz		
Dnajb14		
Slit2		

Table 8.4. Differentially expressed genes (1.3 – 1.5 fold by Affymetrix analysis) in *Tph2*$^{-/-}$ vs. control animals. All genes are significantly changed, p<0.05, except Rgs9, p<0.01, *Tph2*$^{-/-}$ vs. control group.

Due to complexity of function, we excluded the genes involved in multiple processes. Only five genes appeared slightly differentially expressed in the context of serotonin deficiency Rgs9 (Regulator of G-protein signaling), Grm5 (glutamate receptor metabotropic 5), Fgf14 (Fibroblast growth factor 14), Crim1 (Cystein rich motor neuron protein precursor 1) and Kl (Klotho) (www.ensembl.org).

We, then, checked the expression of these genes using qPCR in different parts of the brain, to explore the possibility of omitting the detection of higher mRNA changes in specific brain parts by using the whole brain mRNA samples, and, thus, diluting the net effect of change.

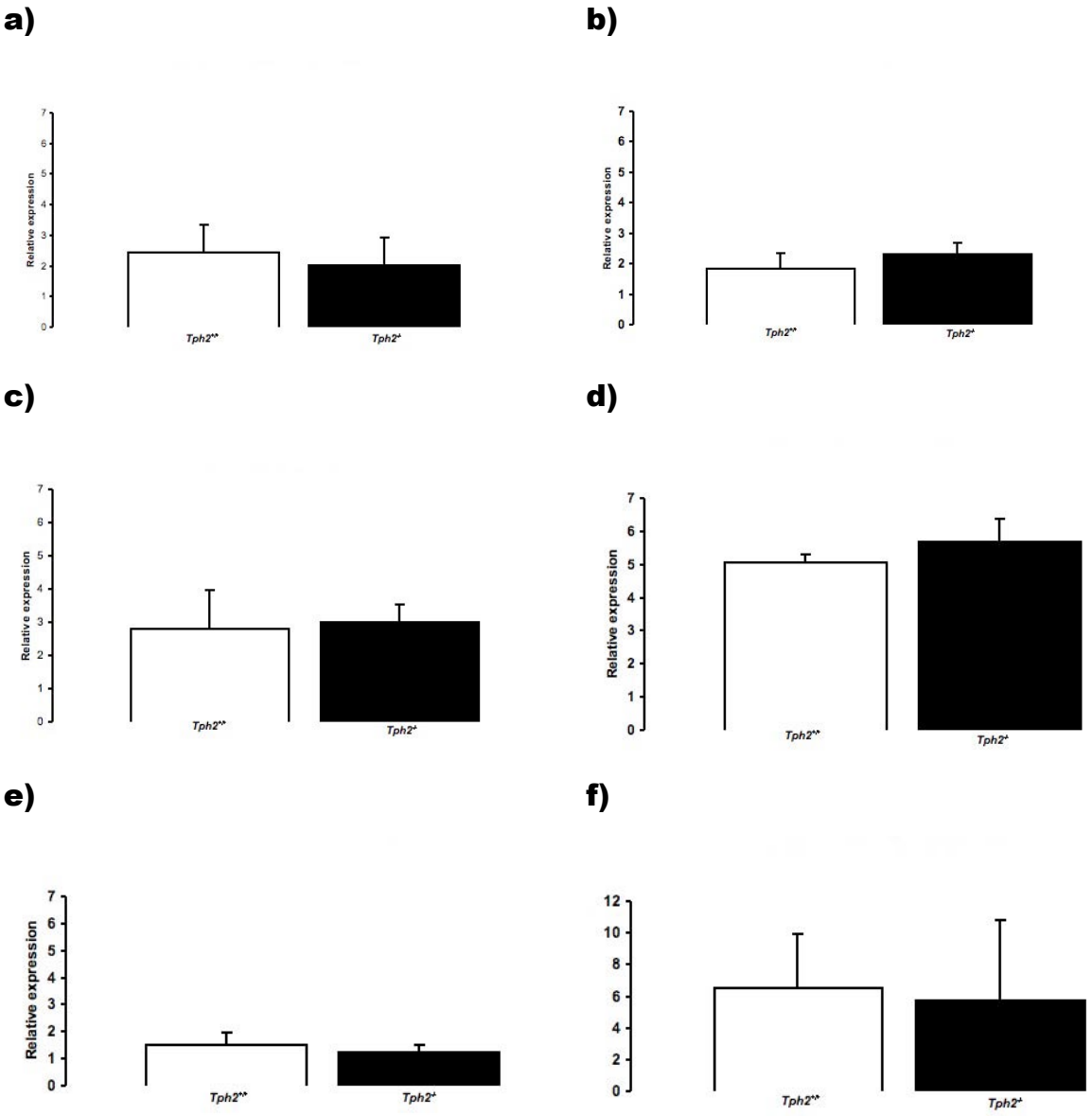


Figure 8.15. qPCR of differentially expressed genes in different brain parts of 1m old *Tph2*$^{-/-}$ and control animals a) Crim1 in cerebellum b) Crim1 in cortex c) Crim1 in brain stem d) Crim 1 in hippocampus e) Grm5 in cortex f) Grm5 in striatum. Data are presented as mean of relative expression level ± SD, where 1 denotes the animal with the lowest expression level.

qPCR did not reveal any significant changes in the expression of Crim1 and Grm5 (Fig 8.15), the genes we randomly chose to confirm the microarray data in different brain parts. We, thus, were not able to detect any significant change of higher fold in any of brain parts of any of monoaminergic enzymes or receptors, nor any other gene except those on Chromosome 10.

8.6. Klotho – a putative ageing gene

GH - IGF-I/insulin hormonal axis is, at least in mice, connected with ageing (for recent review see Russell and Kahn, 2007). The effect of simultaneous ageing is hormonally regulated. A novel gene, Klotho, was recently shown to be involved in this process, most probably by regulating calcium and phosphate homeostasis (Yoshida et al, 2002). Klotho deficient mice are IGF-I deficient (Mori et al, 2000; Utsugi et al, 2000) (for details see discussion 10.2.4). Surprisingly, after a microarray analysis of the brains of *Tph2*$^{+/+}$ and *Tph2*$^{-/-}$ animals was performed, Klotho appeared to be one of the rare significantly changed genes. Therefore, we performed the qPCR analysis of Klotho transcriptional levels, first in different brain parts (Fig 8.16), and then in hypophysis and testis of *Tph2*$^{+/+}$ and *Tph2*$^{-/-}$ mice (Figure 8.17), to explore a putative connection between impaired IGF-I secretion and eventual disturbed ageing process in serotonin deficient mice.

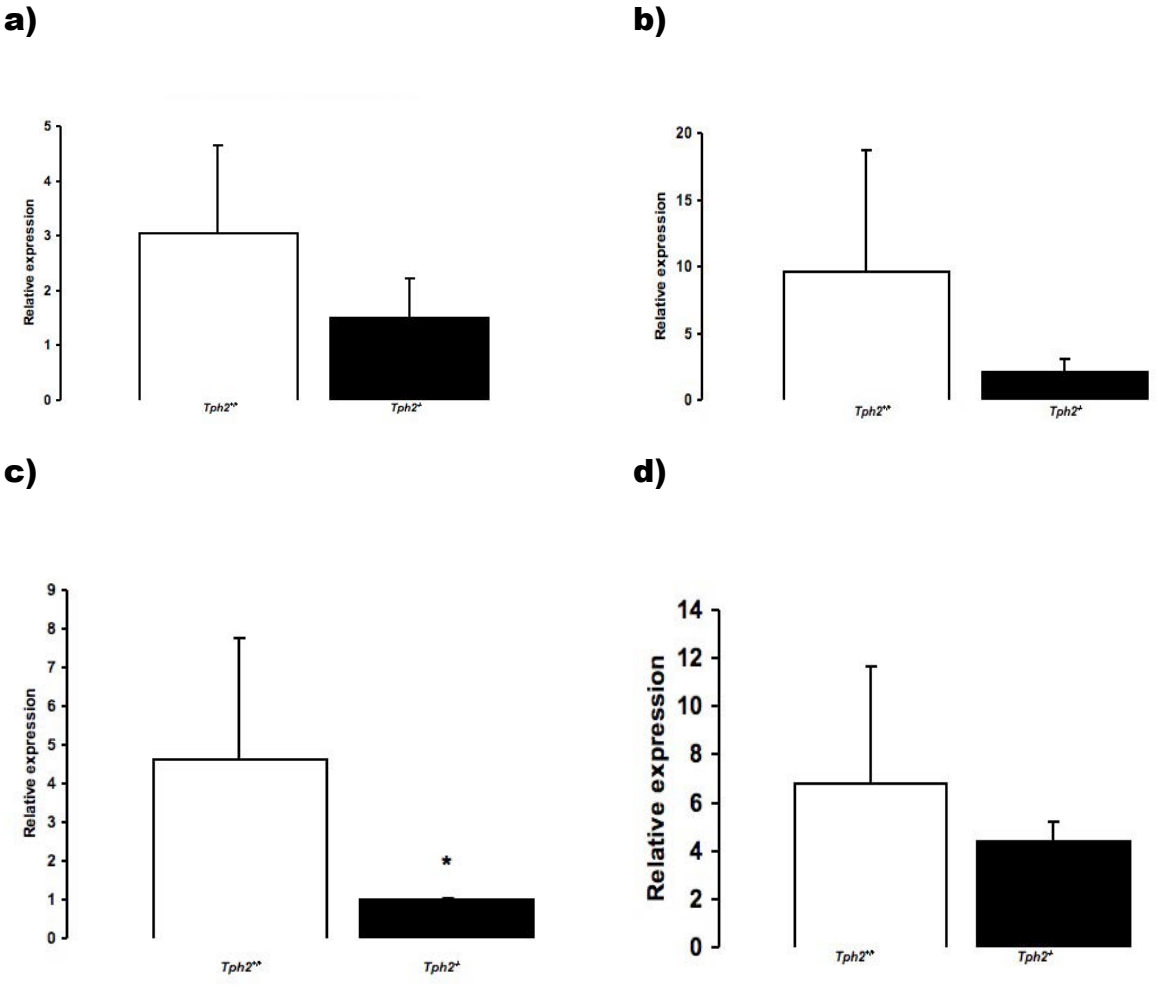


Figure 8.16. qPCR of Klotho (Kl) in different brain parts of 1m old *Tph2*$^{-/-}$ and control animals a) prefrontal cortex b) DAR c) brain stem d) cortex. Kl showed significant reduction in expression in brain stem, *p<0.05 *Tph2*$^{-/-}$ vs. control, unpaired t-test. Data are presented as mean of relative expression level ± SD, where 1 denotes the animal with the lowest expression level.

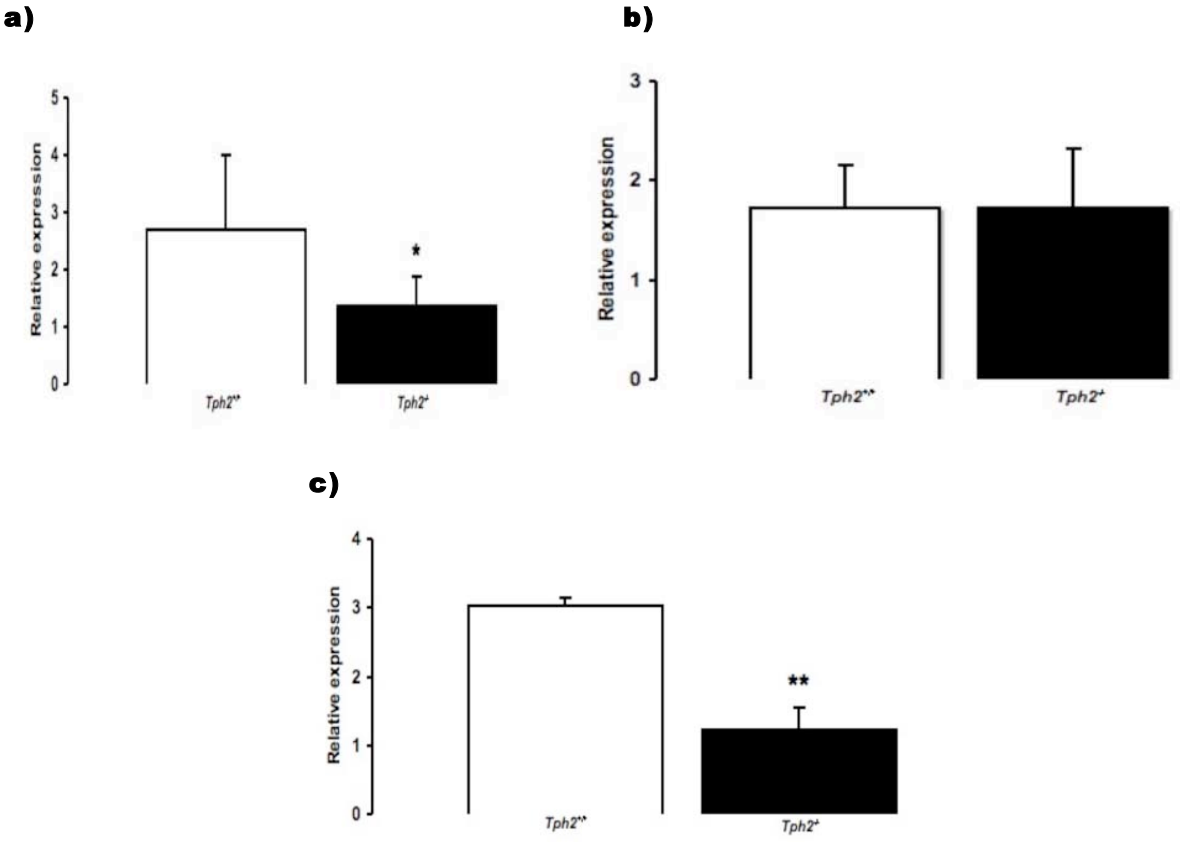


Figure 8.17. Klotho mRNA levels in the a) hypophysis of 2m old males b) hypophysis of 4m old males and c) testis of 1m old males (FVB/N-F4 *Tph2*$^{+/+}$ and *Tph2*$^{-/-}$ mice (n=3)) as measured by qPCR. The dynamic of downregulated Klotho expression follows the one observed for FSH and IGF-I. Level 1 of relative expression denotes the lowest expressing animal. *p<0.05, **p<0.01 *vs. Tph2* $^{+/+}$ and *Tph2* $^{+/-}$, unpaired t-test.

8.7. Metabolism of Tph2$^{-/-}$ *animals*

8.7.1. Metabolic cages and glucose

To describe basic metabolic functions of serotonin deficient animals, animals of different age were kept in metabolic cages for several days. The food intake, water intake, body weight changes and urine excretion were measured at the same time of each day.

Glucose concentration in the blood was determined in an independent experiment. The animals were subjected to fasting conditions and the glucose concentration was measured the same way. It is noteworthy that 20 days old animals after fasting showed significantly lower glucose levels in the blood, but they also had significantly lower body weight. When normalized to the body weight, glucose levels at this age appeared to be higher in normal conditions and after fasting, pointing to an impaired insulin/IGF-I signaling. IGF-I concentration in the blood of 1 months old animals is significantly lower (Fig 8.18), and its connection to metabolic homeostasis is to be determined.

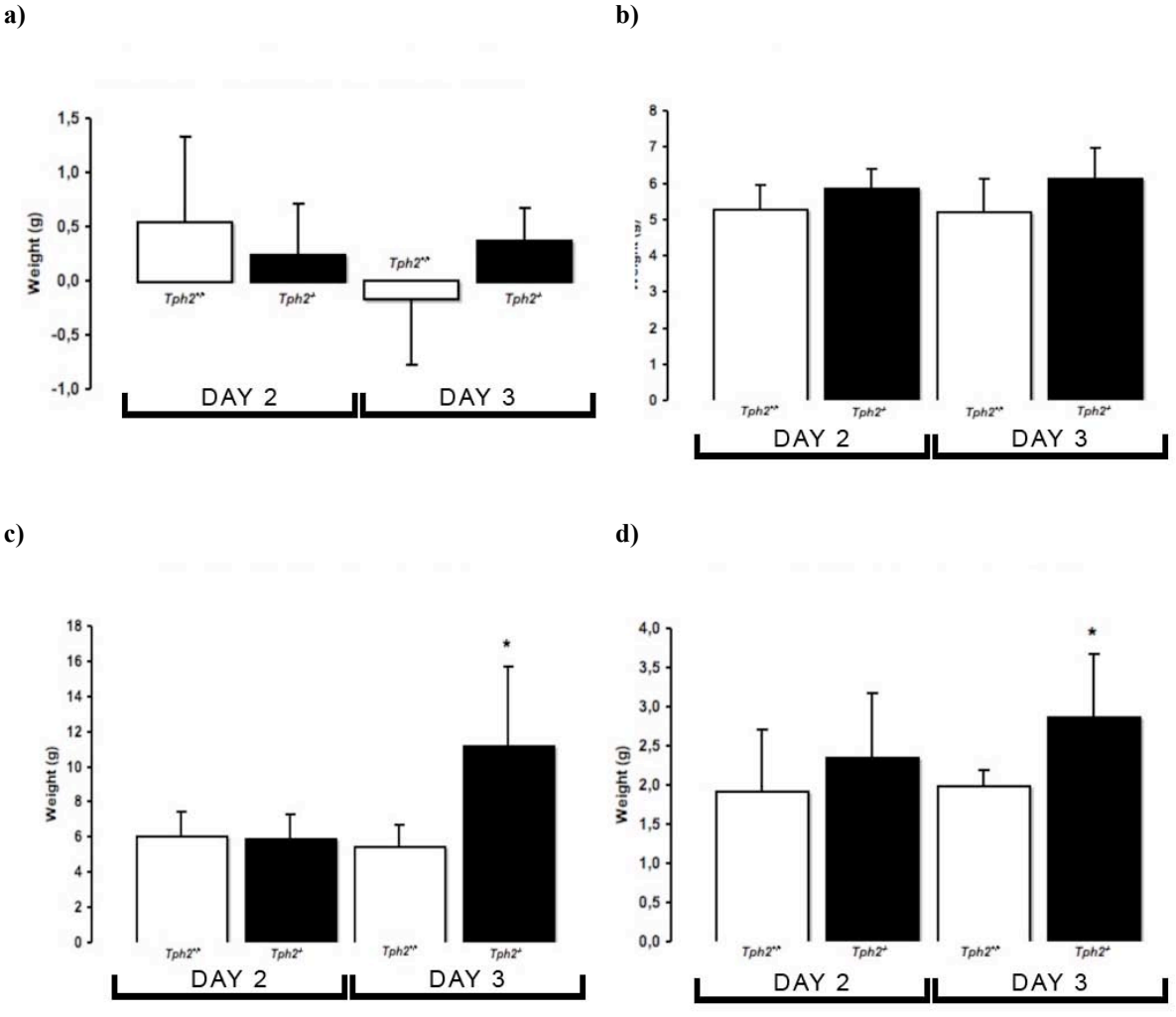
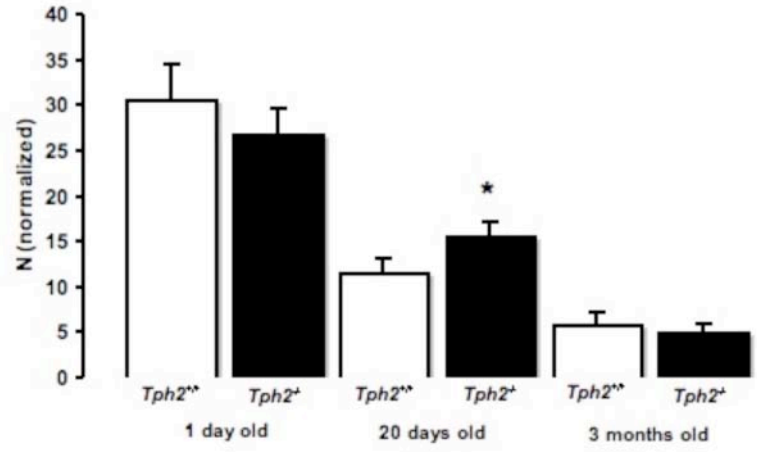


Figure 8.18. Metabolic cages. The animals were kept in metabolic cages for 3 days (72h). First 24h animals were left to accommodate. After that weight difference (a), food intake (b), water intake (c) and urine excretion (d) were measured at 48h (first two bars) and 72h (second two bars). *p<0.05 *vs. Tph2* $^{+/+}$ and *Tph2* $^{+/-}$, Student´s t-test.

a)



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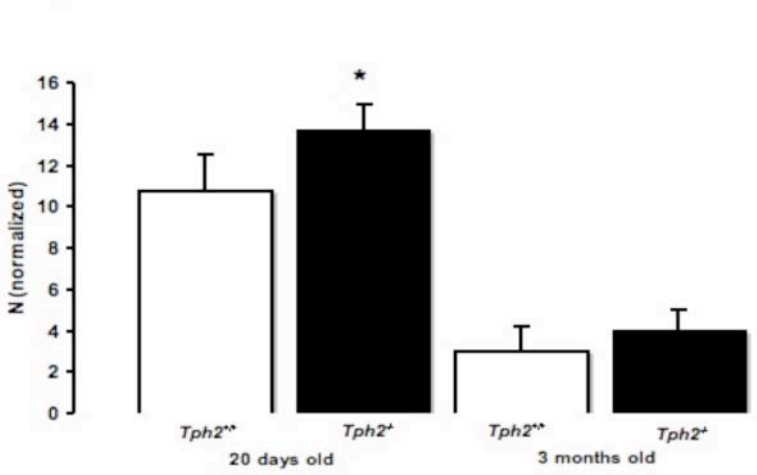


Figure 8.19. Glucose concentration in the blood of a) fed and b) fasted animals of different ages. For details see text. *p<0.05 *Tph2* $^{+/+}$ *vs. Tph2* $^{+/-}$, student´s t-test.

8.7.2. Levels of Ca^{2+}, PO_4^{3-}, Mg^{2+} and cholesterin

Klotho, a novel gene involved in the regulation of ageing through calcium/phosphate homeostasis, was shown to be significantly changed on the transcriptional level in the brain stem and in hypophysis of *Tph2*$^{-/-}$ animals (see paragraph 8.6 and discussion 10.4.2). To investigate the possibility that the lower Klotho concentration in the brain influence inorganic

homeostasis of the organism, we measured the level of Ca^{2+}, PO_4^{3-} and Mg^{2+} in the sera of *Tph2*$^{-/-}$ and WT animals at different ages.

One year old animals of a mixed background showed a significant difference in Ca^{2+} and PO_4^{3-} levels (Fig 8.20). In younger animals, we detected only impaired Ca^{2+} homeostasis (Figure 8.19). Moreover, the concentration of Ca^{2+} in the blood of 4m old animals was lower, whereas older *Tph2*$^{-/-}$ animals had higher Ca^{2+} concentrations.

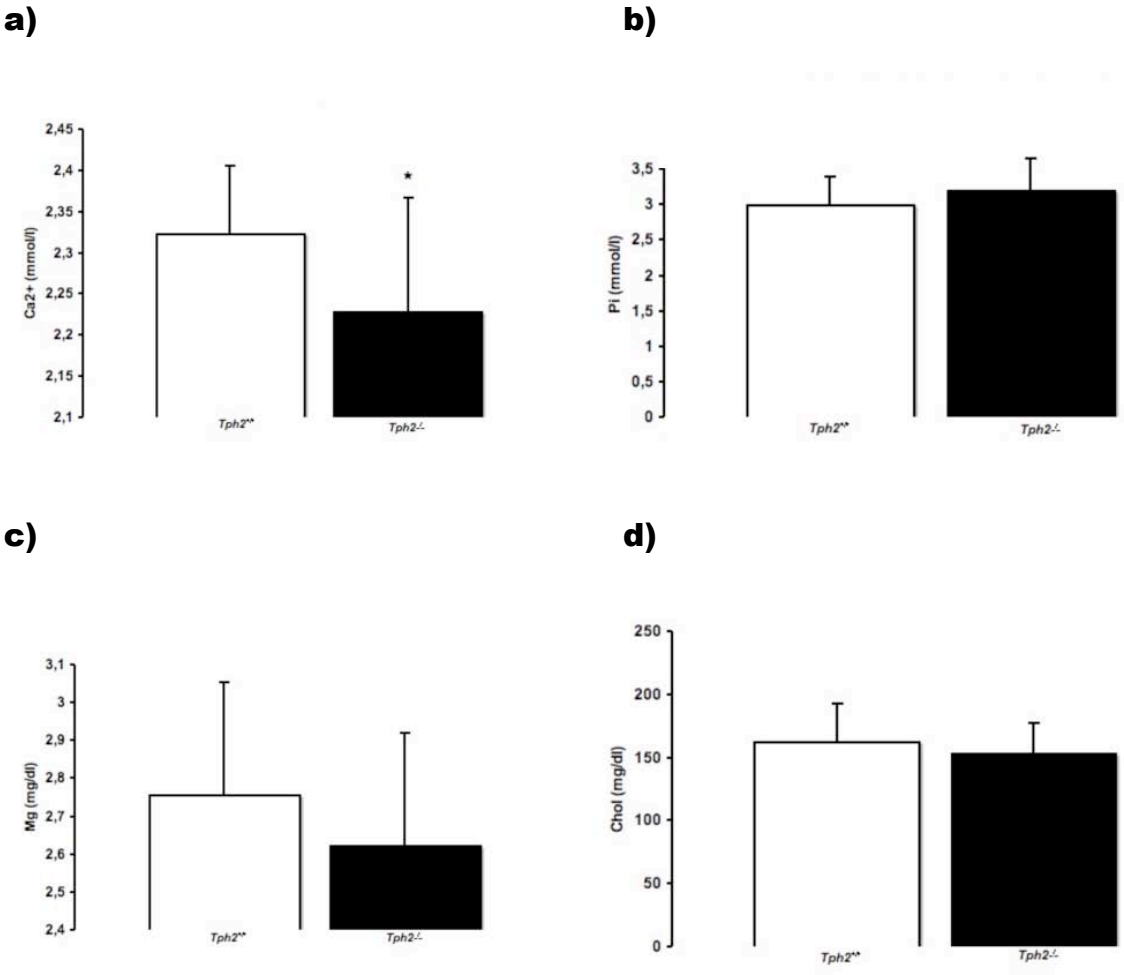


Figure 8.20. Ca^{2+}, PO_4^{3-}, Mg^{2+} and cholesterin in the blood of 4 months old animals. The homeostasis of Ca^{2+} is slightly impaired. *p<0.05 *vs. Tph2*$^{+/+}$ and *Tph2*$^{+/-}$, unpaired t-test.

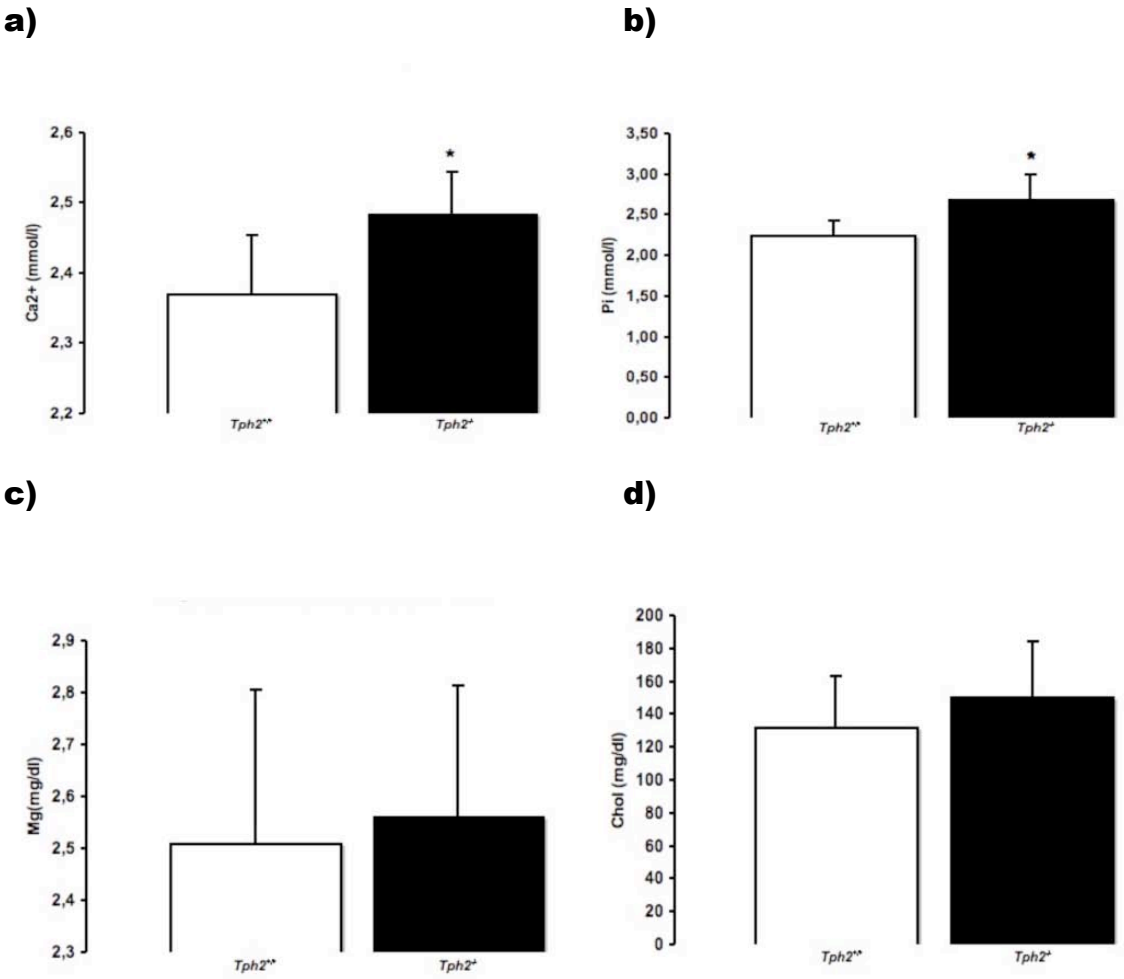


Figure 8.21. Ca^{2+},PO_4^{3-},Mg^{2+} and cholesterin in the blood of 11 months old animals. The homeostasis of Ca^{2+} and PO_4^{3-} is slightly impaired and the concentration is higher then in 4 months old animals. *p<0.05 *vs. Tph2* $^{+/+}$ and *Tph2* $^{+/-}$, unpaired t-test.

8.8. Autonomic Nervous System (ANS) and serotonin

Since serotonin is known to be an important modulator of autonomic functions (Jacobs 2002), we evaluated blood pressure, heart rate, respiration rate and the control of sleep and body temperature by telemetry.

8.8.1. Temperature regulation

The body temperature was determined using the telemetry system described in chapter 6.5.10 of this work.

Basic body temperature was normal in *Tph2*$^{-/-}$ mice (36.60 ± 0.04°C *vs.* 36.61 ± 0.22°C in controls). However, after a challenge by a cold environment (4°C), the observed drop in body temperature was more pronounced in serotonin deficient animals then in wild-type littermates (Figure 8.21). At the same time *Tph2*$^{-/-}$ animals exhibited hyperactivity in the first hour at 4°C, probably compensating by skeletal muscle movement for a stronger loss in body temperature. When after one hour the animals reduced their locomotion to the levels of control animals, their temperature started to fall more rapidly than in controls.

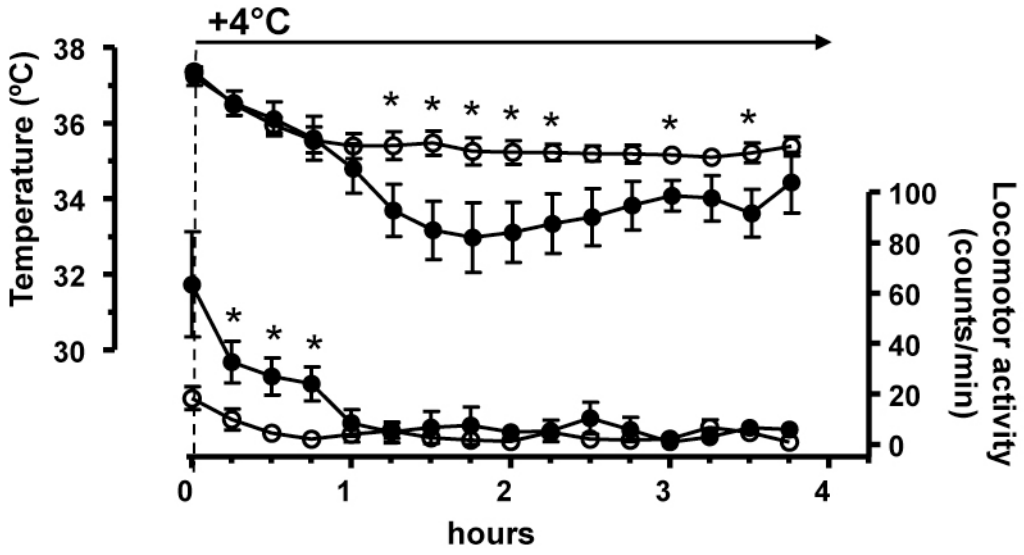


Figure 8.22. Changes in the body temperature and activity after a challenge by a cold (4°C) environment. Black circles - *Tph2*$^{-/-}$(n=7), open circles - control mice (n=7), *p<0.05 by 2-way ANOVA.

8.8.2. Cardiovascular parameters: heart rate and blood pressure

Serotonin in the brain has been shown to be of major importance for cardiovascular control (Jordan 2005). The effects it exerts on the sympathetic and parasympathetic tone are, however, unclear, and most probably dependent on the places of action and the type of receptors involved (see discussion 10.2.5).

Significant decreases in blood pressure and heart rate could be detected mainly in the late afternoon and in the night in *Tph2*$^{-/-}$ mice, (7 p.m – 7 a.m) during the active phase of the animal (Figure 8.22).

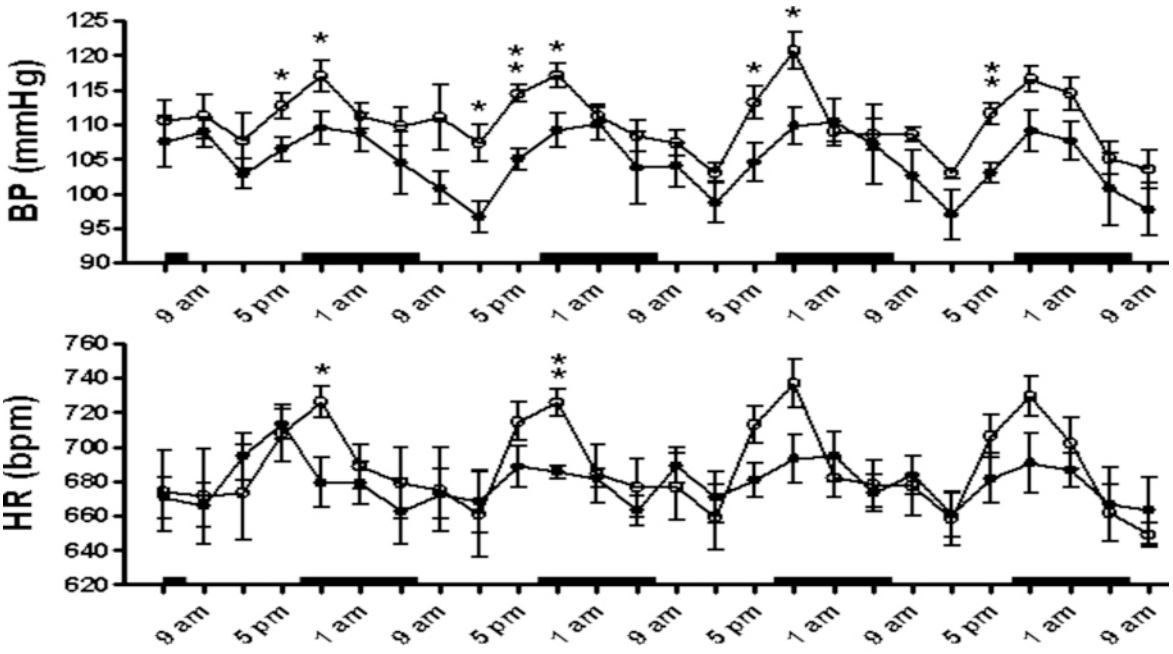


Figure 8.23. Circadian variations in mean arterial blood pressure (BP) (a) and heart rate in beats per minutes (bpm) (HR) (b) in *Tph2* deficient mice. Black circles - *Tph2*$^{-/-}$(n=5), open circles – control mice (n=5). *p<0.05, **p<0.01 by 2-way ANOVA.

8.8.3. Sleep

There is a long lasting debate on the role of serotonin in the regulation of sleep and wakefulness (see discussion 10.2.3). Telemetric recordings revealed marked disturbances in sleep of *Tph2*$^{-/-}$ mice (Figure 8.23). During daytime, these animals sleep more frequently and for longer periods of time than their control littermates (3.96 ± 0.78 30-min inactivity periods from 7 am to 7 pm in *Tph2*$^{-/-}$ *vs.* 1.04 ± 0.29 in control mice, $p<0.01$). In the night, sleeping periods were more rare in both strains and did not significantly differ in length and frequency.

Our model supports sleep promotion and a suppression of arousal as the net effect of a lack of serotonin in the brain. As with the heart rate and blood pressure, the change in the sleeping length and frequency showed circadian rhythm.

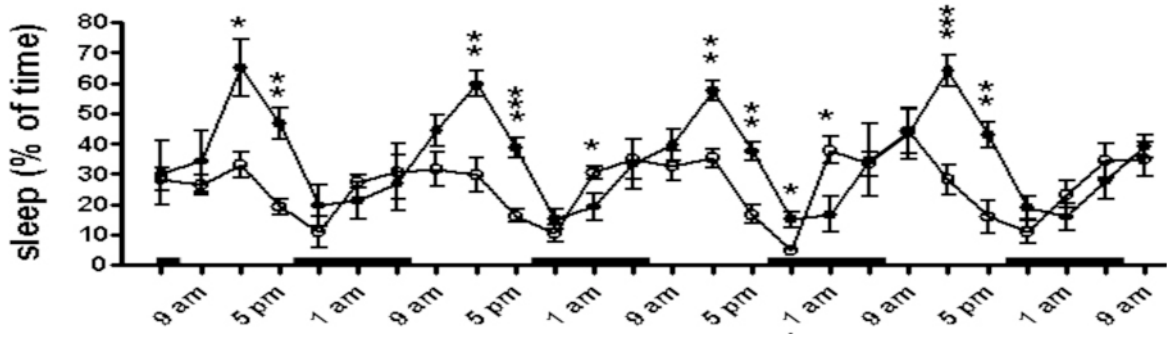


Figure 8.24. Circadian variations in sleep in *Tph2* deficient mice. Black circles - *Tph2*$^{-/-}$ (n=5), open circles – control mice (n=5). *p<0.05, **p<0.01, ***p<0.001 by 2-way ANOVA

8.8.4. Respiration

Previous pharmacologic and genetic studies and models of serotonin deficiency showed a prominent role of serotonin in breathing, but data was again contradictory. Both, dampening and excitatory role of serotonin in breathing was reported (reviewed in Richerson 2004).

The telemetric data revealed that respiration rate was significantly reduced in *Tph2*$^{-/-}$ mice (185.5 ± 9.7 min-1 in *Tph2*-/- *vs.* 231.5 ± 6.8 min-1 in WT, p<0.05) but with normal circadian variations (Figure 8.24).

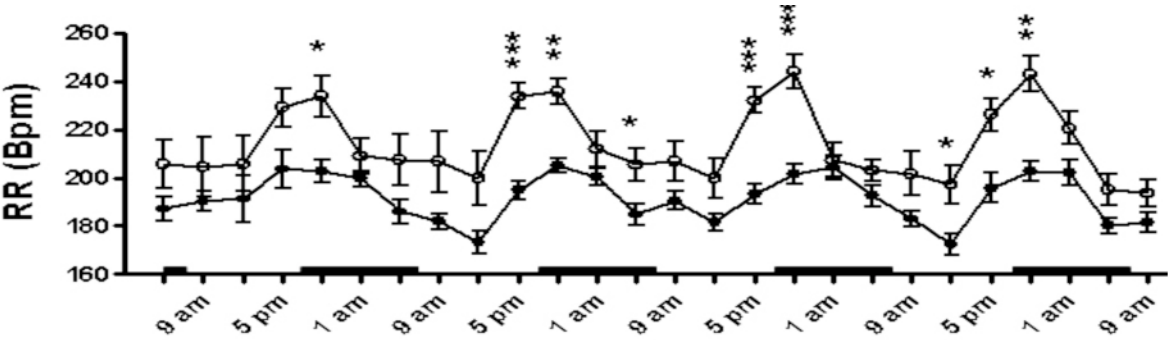


Figure 8.25. Circadian variations in respiration rate (breaths per minute (Bpm)) in *Tph2* deficient mice. Black circles - *Tph2*$^{-/-}$(n=5), open circles – control mice (n=5). *p<0.05, **p<0.01, ***p<0.001 by 2-way ANOVA.

8.9. Fertility and maternal neglect

Serotonin is known to influence mating behavior and aggressivity in mice (Popova 2002a, 2002b, Lerch-Haner 2008). By analyzing the breeding pattern of serotonin deficient animals we were looking for altered fertility, offspring number, offspring survival or parental behavior.

The number of pups per litter was normal even when both parents lacked *Tph2* (6.0 ± 1.5 in *Tph2*$^{-/-}$ vs. 5.9 ± 0.4 in controls; mixed genetic background). Thus, neither male nor female fertility was affected by the lack of brain serotonin. However, a high percentage of the dams did not take care of their pups. Of 10 litters born by *Tph2*$^{-/-}$ mothers bred with *Tph2*$^{+/-}$ fathers (FVB/N-F4 genetic background), only 4 survived for more then several days, compared to 8 litters of 9 born from couples of *Tph2*$^{+/-}$ mothers and *Tph2*$^{-/-}$ fathers. The same phenotype was observed when wild-type fathers were used for breeding and, thus, did not depend on the genotype of the offspring (Table 8.5). At the day of delivery (day0) *Tph2*$^{-/-}$ mothers showed normal milk production, were feeding pups (the stomach of newborns was visibly filled with milk a few hours after delivery), organizing and keeping a nest. However, in the following days pups of *Tph2*$^{-/-}$ were neglected and even often cannibalized by the mother in an aggressive manner. Consequently, most of the pups of *Tph2*$^{-/-}$ mothers were dead on day2 or 3 after birth (Fig 8. 25). The same results were obtained, when we performed cross-fostering experiments. *Tph2*$^{-/-}$ dams ate 50% of the pups (30% of whole litters) born by *Tph2*$^{+/+}$ mothers, while *Tph2*$^{+/+}$ mothers only cannibalized 5.6% of the pups (zero whole litter) from *Tph2*$^{-/-}$ mothers. This alteration in maternal instincts in *Tph2*$^{-/-}$ mothers was confirmed by the pups retrieval test (performed by Valentina Mosienko): on day1, 8 of 9 *Tph2*$^{-/-}$ dams were not able to collect their scattered pups within 30 minutes, whereas it took in average 3.9 ± 0.7 minutes for the control dams (n=6) to achieve this. However, there was no significant difference between females of each genotype in the time it took to find a hidden cookie (42.3 ± 15.5 sec in *Tph2*$^{-/-}$ vs. 29.7 ± 9.3 sec in control mothers) indicating that maternal neglect is

not caused by disturbed olfaction in *Tph2*$^{-/-}$ mice (test performed by Valentina Mosienko).

Mother genotype	*Number of dams*	*Litter size*	*Number of pups born*	*Number of pups survived until d5*	*% survival*	*Number of dead litters on day5*
Tph2$^{+/+}$	N=23	10.13±0.45	233	228	97.9%	0/23
Tph2$^{-/-}$	N=29	9.28±0.48	269	149	55.4%	11/29

Table 8.26. Breeding statistics of *Tph2*$^{-/-}$ (KO) and *Tph2*$^{+/-}$ (WT) mothers, mixed backcross. *Tph2*$^{-/-}$ and control mother show no difference in the breeding frequency, number of pups born, or the litter size. However, pups born by *Tph2*$^{-/-}$ mother show approx. 40% less survival rate, due to maternal neglect.

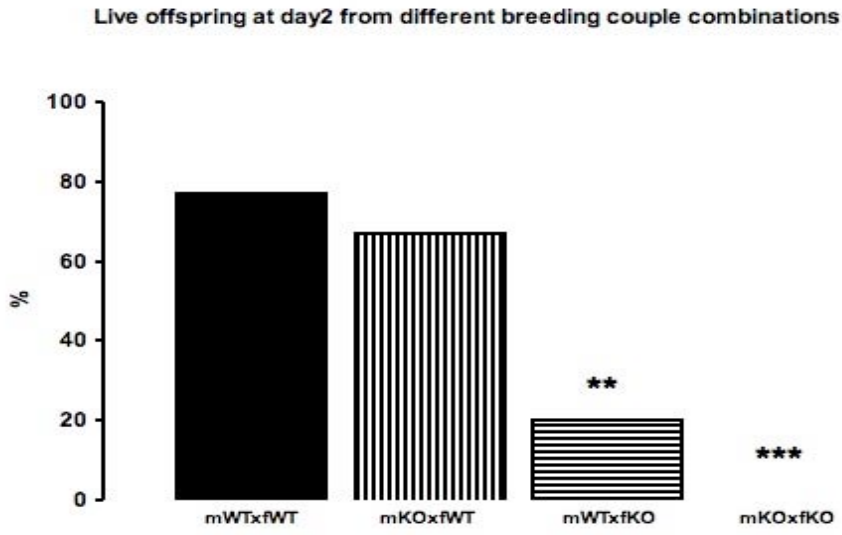


Figure 8. 27. Breeding statistics of *Tph2*$^{-/-}$ (KO) and *Tph2*$^{+/-}$ (WT) mice FVB/N F4 backcross. Minimum 3 breeding couples per group were observed for minimum 2 months time and the percentage of living offspring at day2 was determined in relation with total offspring born. **p<0.01, ***p<0.001 *vs.* mWTxfWT , Student´s t-test.

9. EMBRYONIC STEM CELLS AND in vitro DIFFERENTIATION

9.1. Tph2 "expression-selection cassette" – a marker for 5-HT neurons

The exclusive expression of Tph2 gene in 5-HT positive cells in the brain (introduction 1.5.2.2) enabled its use as a marker gene to select the serotonergic neurons in mice and *in vitro*. To this purpose we created the "expression-selection cassette" (results 7.1.), where, after the correct integration, the expression of neoR linked by an IRES with dsRed gene was supposed to be driven by *Tph2* promoter. By creating an ES cell line containing a heterozygous "knocked-in" *neoR-IRES-dsRed/Tph2* allele, we wanted to enable the selection of 5-HT neurons, in culture (neoR) and by FACS (dsRed).

9.2. Developing the protocol for efficient in vitro serotonergic differentiation

For the ESC differentiation project the basic protocols were distributed among the members of the FunGenES consortium (materials and methods 5.2.4.), with the aim to be able to compare the results of the gene expression patterns obtained during ESC differentiations towards particular cell types. To this purpose a common cell line was used (E14Tg2a), referred here as E14. Then, the protocols were modified in order to obtain higher percentage of serotonergic neurons in culture.

9.2.1. Monolayer protocol

The main purpose of developing the protocol with a high percentage of serotonergic neurons was to be able to select the serotonergic lineage by FACS sorting using dsRed fluorescence. The best protocol for this purpose would be obtaining the serotonergic neurons as a monolayer, as described previously (Ying et al, 2001, materials and methods 5.2.4.4), by

applying the morphogens involved in serotonergic differentiation in vivo, such as FGF4, FGF8, SHH, AA, 5-HT itself or BDNF.

We were testing the substances in different concentrations, at different time points and in different combinations, but we were not able to improve the already observed yield of serotonergic neurons (2%). We concluded that, most probably, in the late phase of the differentiation, to withdraw from the mitotic state, 5-HT neurons require an attachment to other cells, notably glial cells that differentiate in parallel.

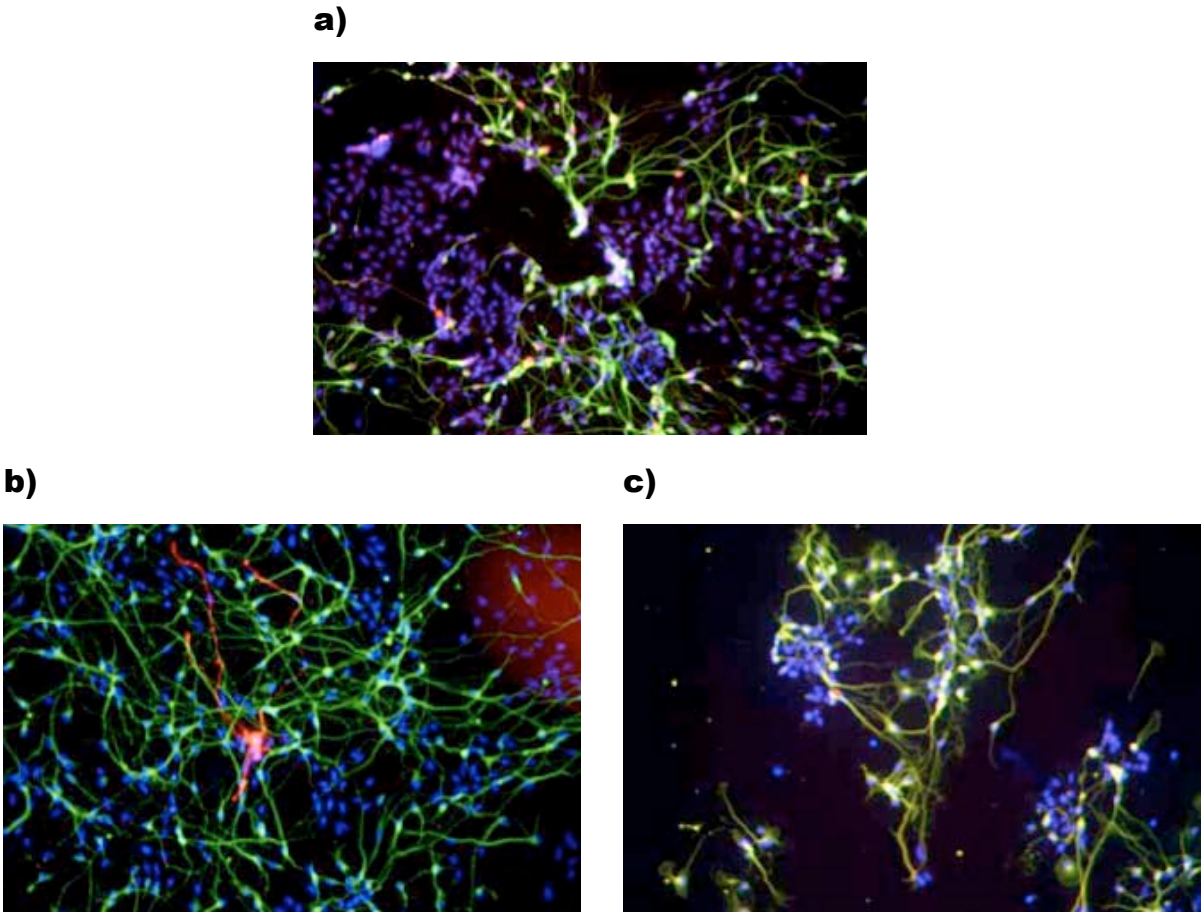


Figure 9.1. Monolayer protocol - cells were seeded at a density of $3x10^4/cm^2$ and stained at day 14 (D14) of differentiation on a gelatine surface a) Tuj1 – green, GABA – red, DAPI – blue, b) Tuj1 – green, TH – red, DAPI – blue c) Tuj1 – green, 5-HT – red, DAPI – blue. Tuj1 (neuronal class III b-tubulin) panneuronal marker, GABA (gamma-aminobutyric acid) marker for gabaergic neurons, 5-HT (serotonin) and TH (tyrosine hydroxylase) – marker for dopaminergic neurons, DAPI (4'-6-Diamidino-2-phenylindole) blue-fluorescent nuclei staining. Cy-2 (fluorescent green conjugated antibody), Cy-3 (fluorescent red conjugated antibody).

9.2.3. PA6 protocol

The most robust protocol for serotonergic differentiation appeared to be the PA6 protocol (for details see materials and methods 5.2.4.2). This ESC differentiation protocol was giving ICH detected 5-HT neurons, albeit at very low consistency. Until now, we were not able to understand the reasons for the high yield of 5-HT neurons during one differentiation and the low yield during another. We observed a pattern of the best 5-HT yield when high density of PA6 cells, and low density of ES cells were seeded. In the best cases, and with the application of FGF4/SHH cocktail at the D2 of differentiation, we were able to produce around 30% of serotonergic neurons.

a) **b)**

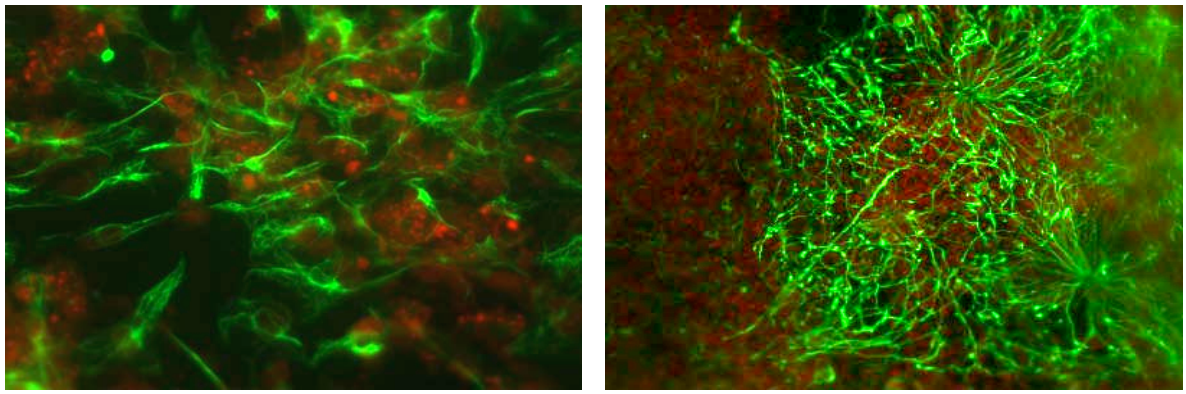


Figure 9.2. PA6 protocol, D10. DAPI - red, Nestin - green. Nestin, a marker for neuronal precursors, shows the precursors arranged as a layer on PA6 cells (unstained), or packed in so called rosettes. Rosettes are radial structures resembling neuronal tubes *in vivo* (Wilson PG, Stice SS, 2006).

a) **b)**

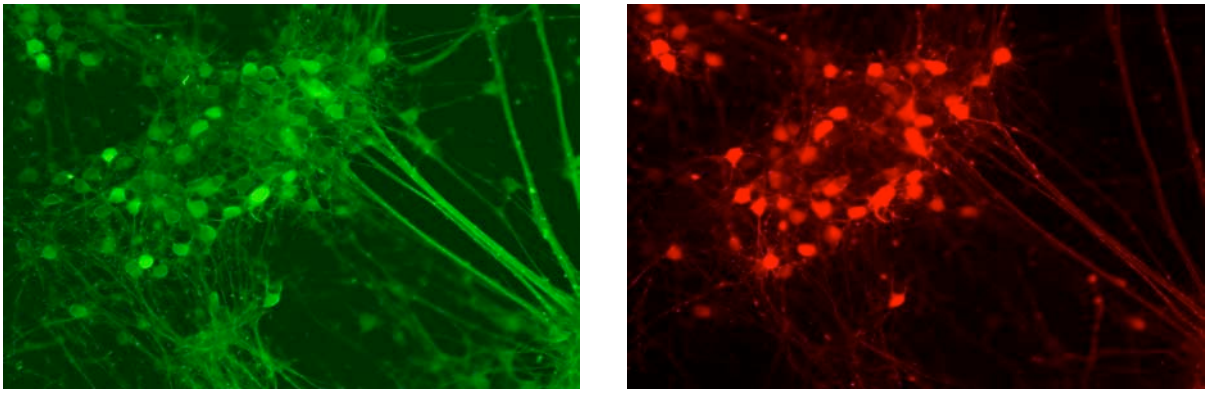


Figure 9.3. PA6 protocol, D14. The serotonergic neurons were abundant during one differentiation cycle, then low during another (not shown).

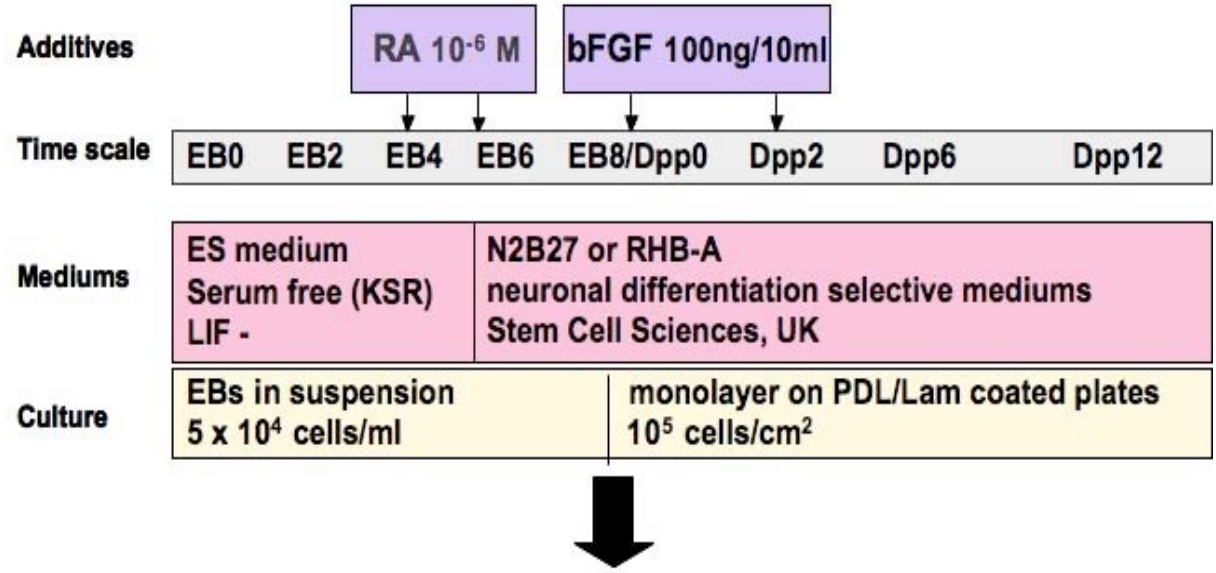
9.2.3. Embryoid Bodies (EBs) protocol

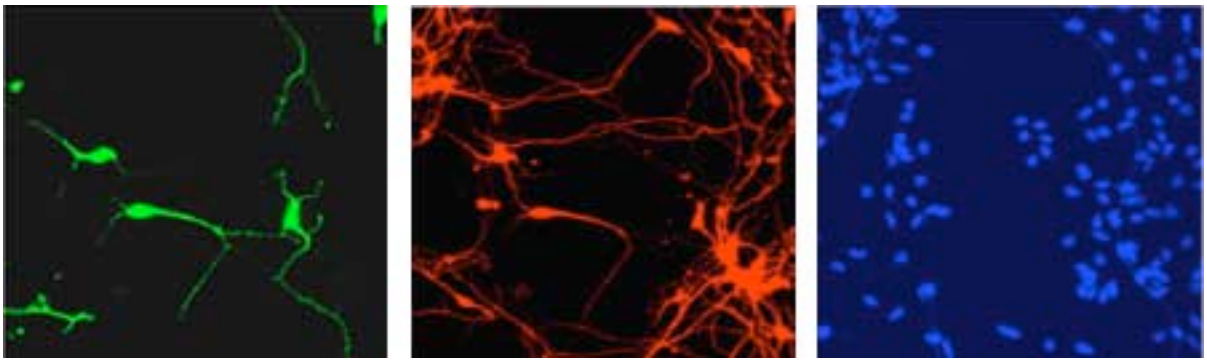
EB protocol has a robust start: the floating balls of cells contain the precursors of all three germ layers, and only subsequent stimulation, by replating the cells on a specific cell matrix and applying a cocktail of substances mimicking the developmental influences *in vivo*, will turn the outcome of EB differentiation towards a specific cell type. After replating, the neuronal precursors selected on PDL/Laminin extracellular matrix are grown as a monolayer of cells. Thus, the final outcome of the EB protocol is very similar to monolayer differentiation, containing mostly neuronal precursors, neurons and the glial cells as a monolayer. Despite the unknown influences at the first week of differentiation, the final outcome should be "clean" enough to enable efficient 5-HT selection by applying neoR, and further FACS using dsRed fluorescence.

We concentrated on this protocol as it did not include an additional layer of feeder cells, like PA6, and the 5-HT yield was, after application of discrete changes, quite high. In the best cases, we were able to produce up to 30% of 5-HT neurons out of Tuj1 positive cells in culture (Fig 9.4). Tuj1 positive cells present about 70% of all cells in culture, after EBs are replated on poly-D-lysin/laminine (PDL/Lam) surface (see methods 6.4.1).

High concentration of RA (1μM) used in the original protocol obtained as a part of FunGenES cooperation, was giving extremely low percentage of 5-HT neurons, even lower then the control without any treatment. There are evidences that RA treatment *in vivo* is blocking the synthesis of FGF4, FGF8 and SHH, morphogens that determine serotonergic differentiation. We proved this to be the case *in vitro*. RA, at the same time, promotes the transcription of retinoic acid receptor, thus, amplifying its own signaling (Figure 9.5).

a)





b) c) d)

Figure 9.4. Scheme of retinoic acid protocol (a), b) 5-HT staining (2%) c) Tuj1 staining (70%) d) DAPI staining (100%).

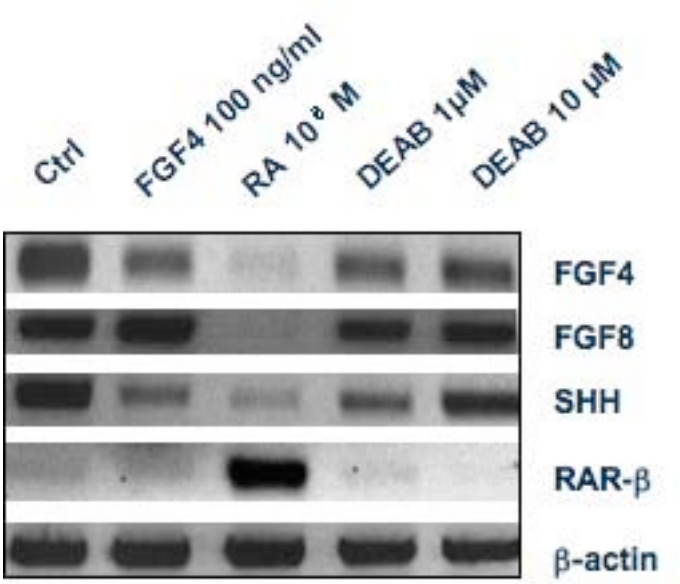


Figure 9.5. Effect of FGF4 (100 ng/ml) and RA (10^{-6}M) on gene expression of fibroblast growth factor 4 (Fgf4), fibroblast growth factor 8 (Fgf8) and sonic hedgehog (Shh) and retinoc acid receptor (RAR) as determined by semi-quantitive RT-PCR. Samples were collected on EB3 (24h after RA (1µM) admission). DEAB - diethylaminobenzaldehyde: inhibitor of endogenous RA synthesis.

Exchanging RA with the cocktail of FGF4/SHH (100ng/ml, each) on EB2 we were able to improve the 5-HT outcome to 15% (data not shown). We also noticed that concentrating the EBs in smaller dishes, influences the final outcome of differentiation, probably by promoting the interlayer signaling. After the transfer of the EBs on D2 in 6 cm radius Petri dish (instead of 10 cm radius Petri dish) we were able to reproduce the high 5-HT outcome of the differentiation for several passages of the cells (Figure 9.6). To select for neuronal precursors differentiated as a part of the ectodermal lineage in EBs containing all three germ layers, the cells were replated on PDL/Lam surface (simulating the extracellular matrix of the brain) on EB8 (see materials and methods). All consequent days were labeled as days post-plating (Dpp).

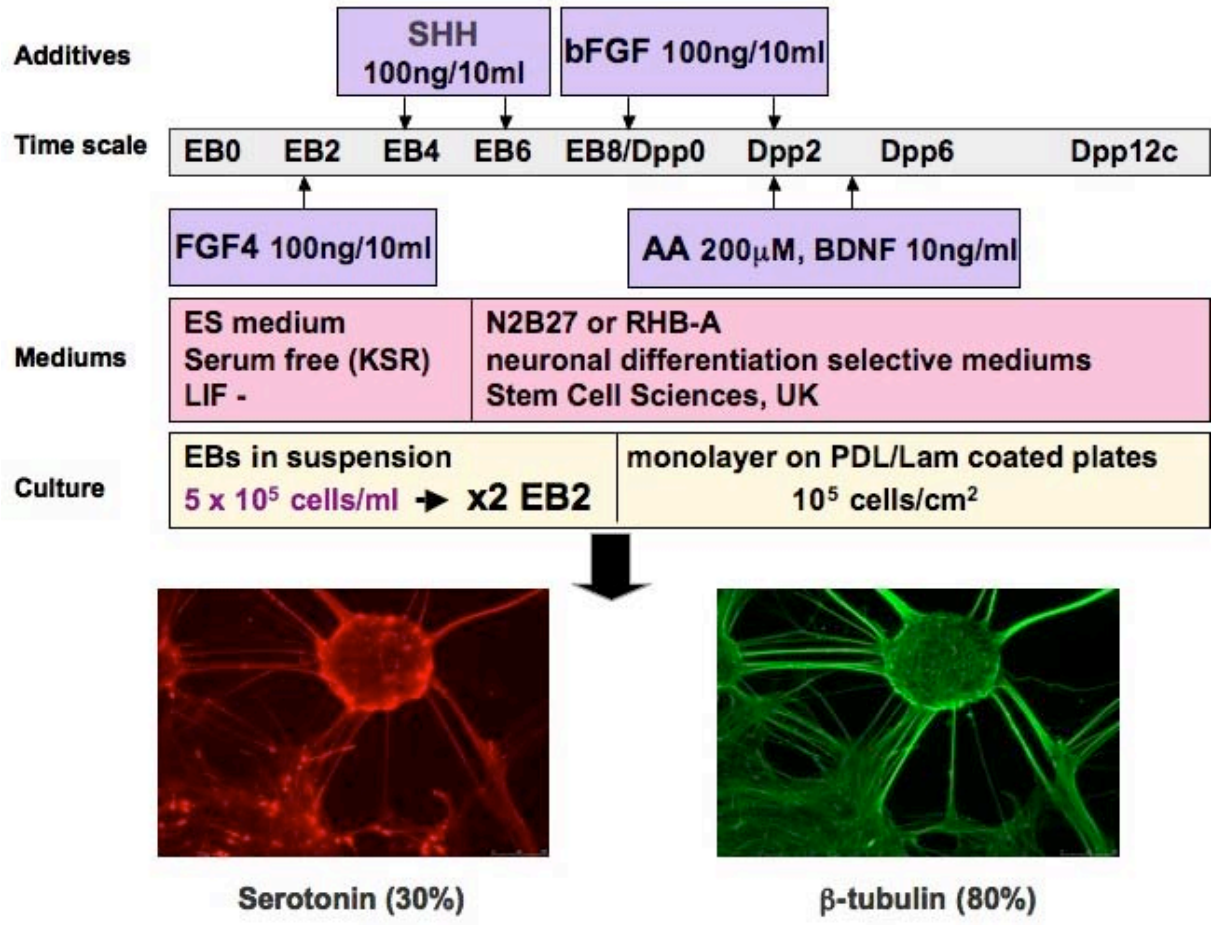


Figure 9.6. Scheme of final EBs protocol giving up to 30% of 5-HT$^+$ cells among Tuj1$^+$ (80%) cells. FGF4 (100ng/ml) is added before SHH (100ng/ml) to mimic the situation *in vivo.* Ascorbic acid (AA) and brain derived neurotrophic factor (BDNF) were added after fibroblast growth factor beta (bFGF) withdrawal, while the neuronal precursors were leaving mitotic stage.

To characterize the EBs protocol we were following the appearance of Nestin$^+$, Tuj1$^+$ and 5-HT$^+$ cells after replating on PDL/Lam matrix. The first neuronal precursors and single neurons are present from Dpp1 in culture, while the first 5-HT neurons are observed around Dpp3 and later (Figure 9.7). The final culture contains glial precursors scattered among neurons and marked by Alpha Beta fatty acid binding protein (Alpha Beta FABP) (Callahan et al. 2008).

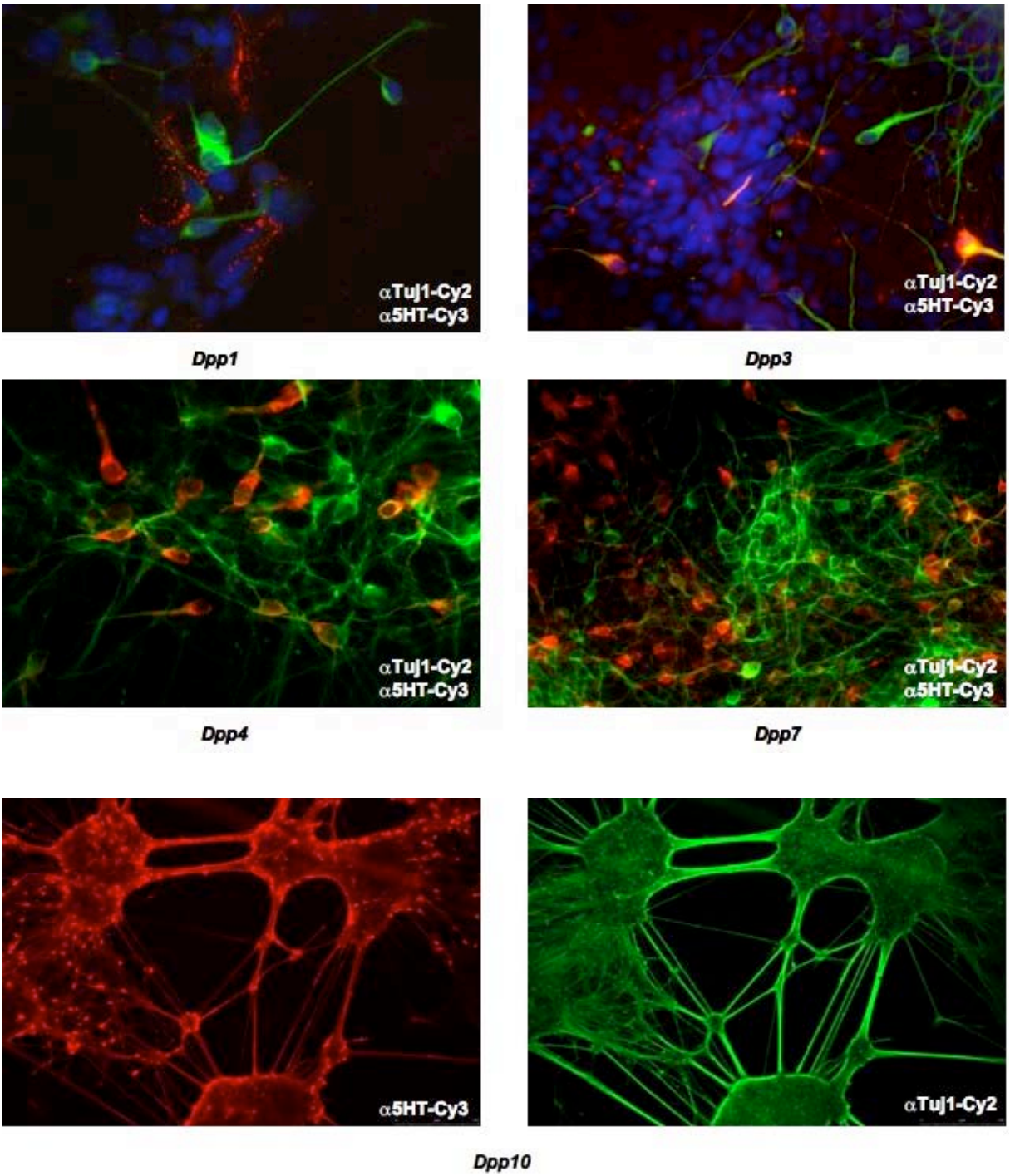


Figure 9. 7. Immunocytochemistry of EBs differentiation after replating on PDL/Lam surface (days post-plating (Dpp)). Already on Dpp1 the first neurons (Tuj1$^+$ cells) appear on PDL/Lam surface. First 5-HT$^+$ neurons are detectable 2 days later, around Dpp3. Tuj1-Cy2 – Tuj1 first antibody, Cy2 – secondary antibody, 5-HT-Cy3 – 5-HT first antibody, Cy3 – secondary antibody (see methods).

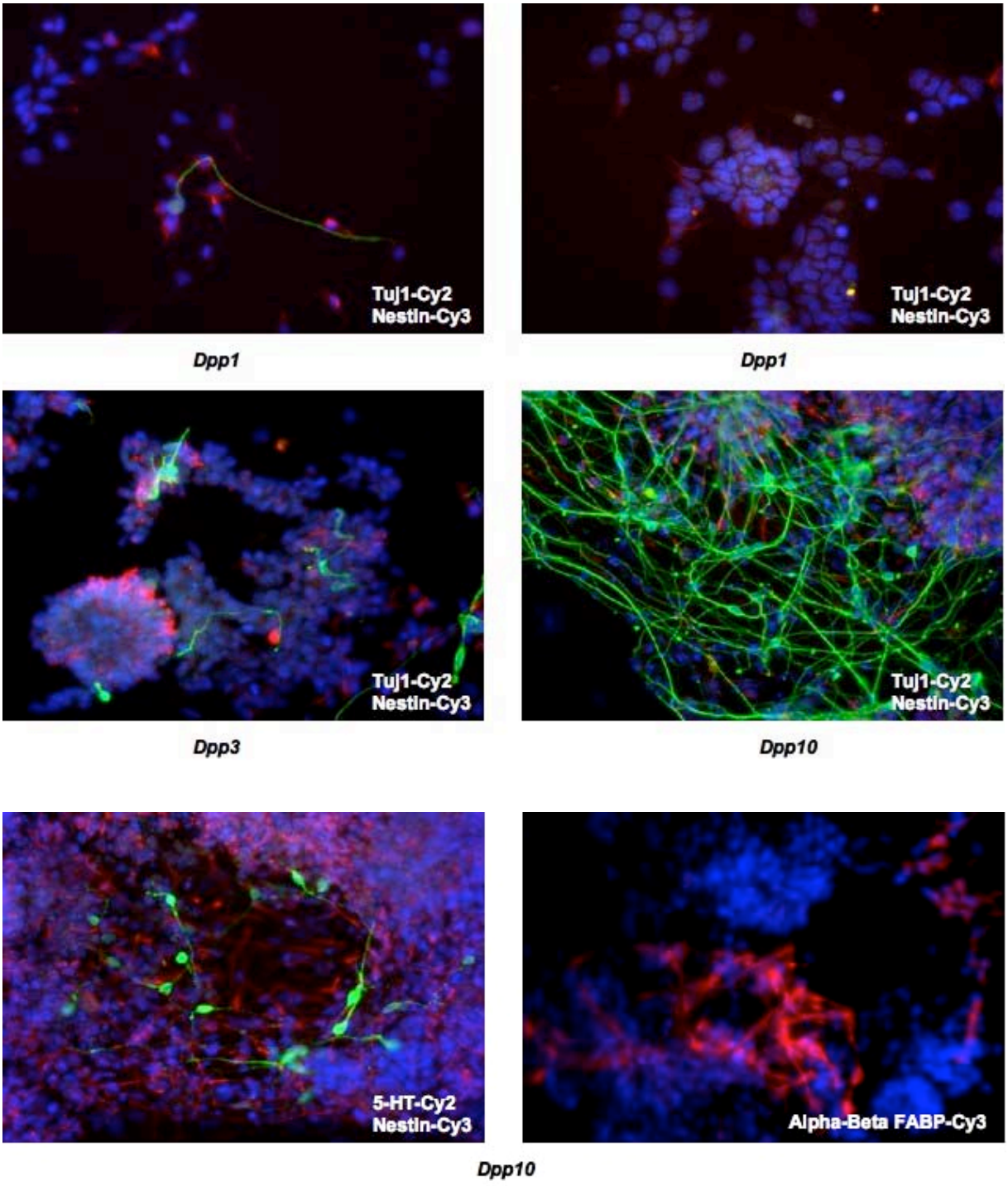
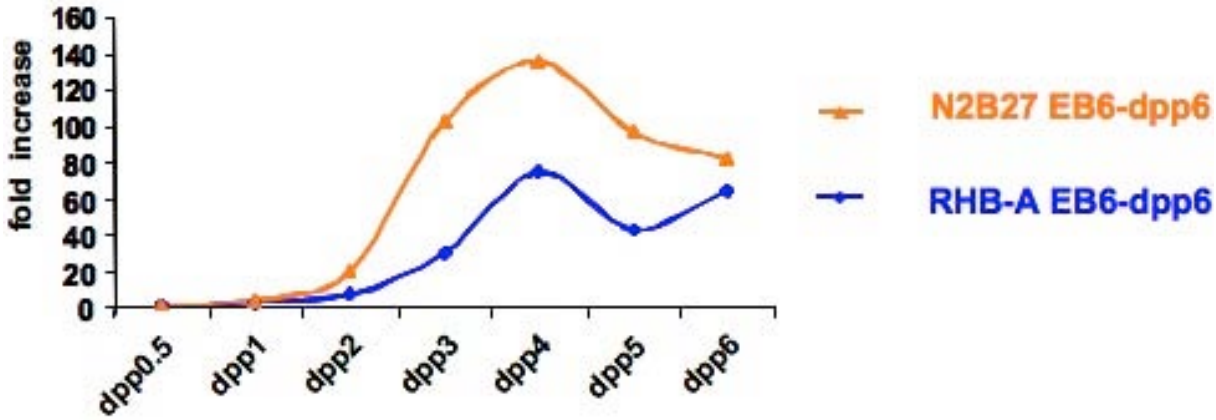
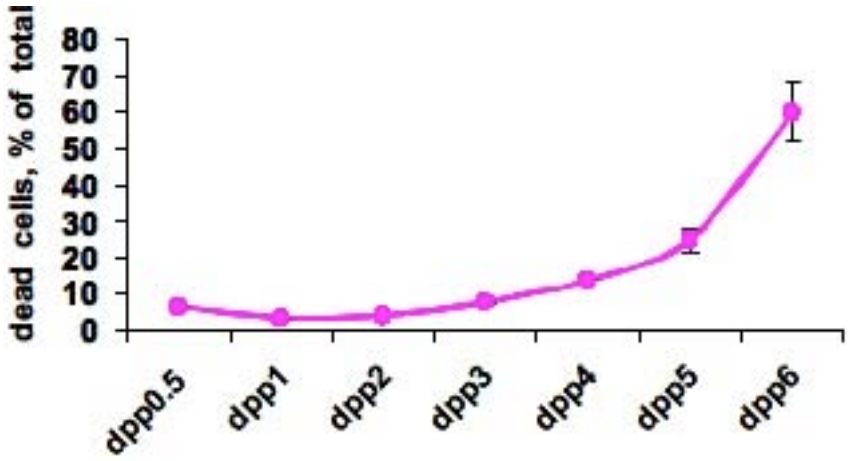


Figure 9.8. Immunocytochemistry of EBs differentiation after replating on PDL/Lam surface (days post-plating (Dpp)). The development of neurons *in vitro* is a dynamic process. Neuronal precursors (Nestin$^+$) are starting to form rosettes already from Dpp1, and are producing neurons throughout the whole culture period. On Dpp10 Tuj1$^+$ neurons are spreading from Nestin$^+$ rosettes. Some of Tuj1$^+$ cells are 5-HT$^+$. Next to the neurons glial precursors (Alpha Beta FABP$^+$) cells are always present in culture at all stages.

a)



b)



c)

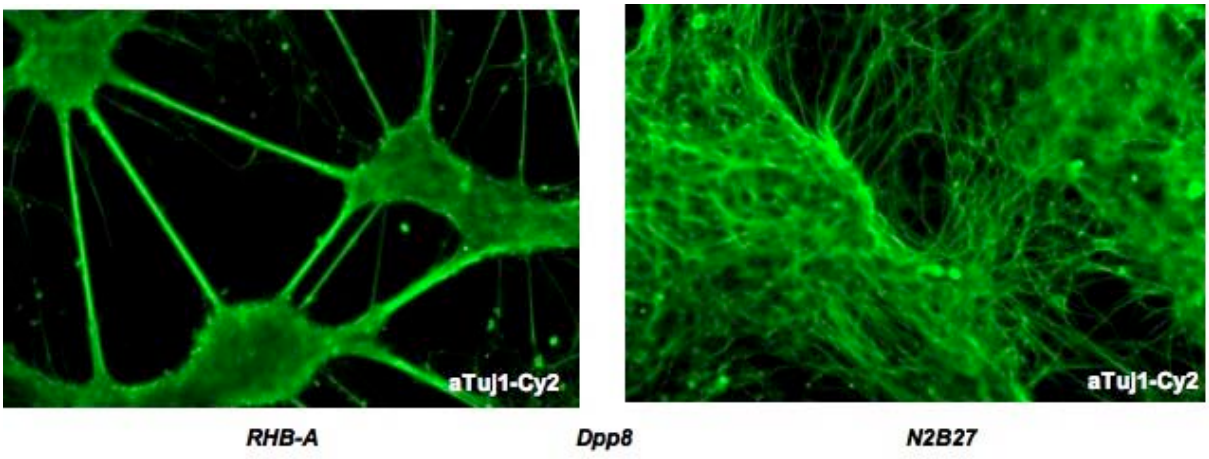


Figure 9.9. Comparison of two different media used for neuronal differentiation using EBs protocol. N2B27 promotes mitotic division of neuronal precursors a) and c), while RHB-A promotes differentiation

of neurons a) and c), b) survival of cells during postplating phase expressed as % of dead cells in total number of cells in culture.

After Dpp7 the cells are starting to die progressively, most probably due to increased density of cells. Usually, we followed neuronal differentiation up to Dpp14. 5-HT neurons were present in culture starting from Dpp3 and in abundance from Dpp7 to Dpp14 (data not shown). After Dpp14 the layers of neuronal precursors, neurons and dying cells would be piling up, preventing further culturing.

We measured the concentration of 5-HT in cell culture supernatant during differentiation (Figure 9.10) to quantify the amount of serotonergic neurons and their functionality *in vitro*. We observed steady increase in serotonin concentration in accordance with appearance of 5-HT neurons, as detected by immunocytochemistry. Thus, 5-HT neurons derived from ESC *in vitro* excrete serotonin and may have the possibility to be functional *in vivo*.

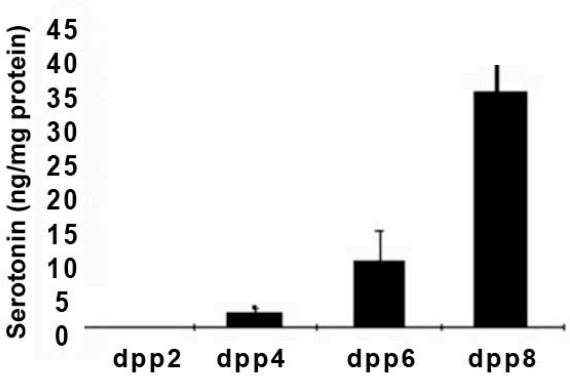


Figure 9.10. ELISA measurement of serotonin concentration in the supernatant of EB differentiation postplating (dpp) at different time points of 5-HT differentiation protocol.

EBs include all three germ layers, and the selection performed on extracellular matrix (PDL/Lam) is not excluding the possibility of the presence of precursors for cell layers. Furthermore, the neuronal population differentiated during the course of a differentiation is not homogenous: each time, it involves different percentages of glial cells, as well as different neuronal types. Obtaining a reproducible differentiation protocol for each neuronal type is one of the biggest hurdles of ES cells research. We performed a detailed RT-PCR analysis in order to see which cellular types are present at the end of a successful 5-HT differentiation (Figure 9.11). At the day 7 after replating (Dpp7), the culture was dynamic and geterogenous.

We could detect the presence of markers specific for pre-mitotic neuronal cells (SHH, Nestin), serotonergic precursors (Nkx2.2, Mash1, Gata2, En1), post-mitotic serotonergic neurons (Tph2), but also markers of other cellular lineages like motorneurons (Nkx6.1, Phox2b).

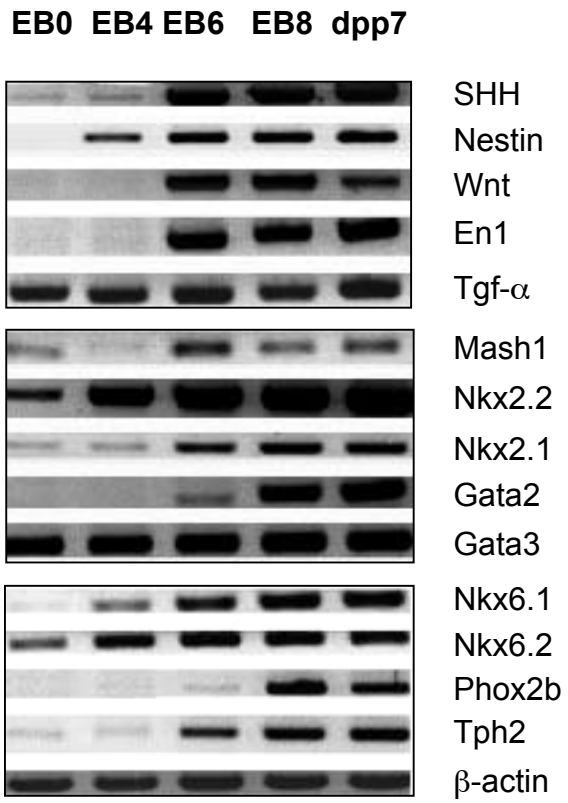


Figure 9.11. RT-PCR of different time points of a EBs 5-HT differentiation for Shh, Nestin, Wnt family genes involved in embryogenesis, engrailed 1 (En1), marker of r1 serotonergic lineage, transforming growth factor α (Tgf-α) that induces epithelial development, and the gene of serotonergic lineage (Mash1, Nkx family, Gata2 and Gata3, Phox2b and Tph2, for details see introduction 3.3).

As PA6 protocol, EB protocol showed high inconsistency in our hands, and we were not able to determine any pattern of the high or low yield of serotonergic neurons during differentiation. We could not achieve stably 30% of serotonergic neurons in the culture, and thus, it was not possible to perform a microarray analysis without selection as the mRNA of different neuronal lineages would cause high background preventing the discovery of the genes involved in defining the serotonergic lineage.

9.3. Tph2-neoR-IRES-dsRed selection

Introducing the Tph2-neoR-IRES-dsRed allele in ES cells and creating a heterozygous Tph2/neoR-IRES-dsRed cell line, we wanted to select 5-HT neurons exclusively during differentiation in the heterogeneous culture as described above. Tph2 is expressed starting from EB8 (eight day of differentiation – cells still as floating aggregates of all three germ layers, ready to be replated) (Figure 9.12).

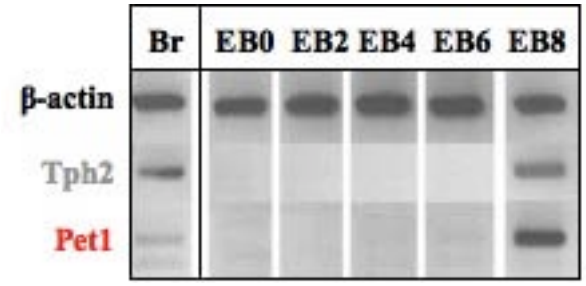


Figure 9.12. Expression of marker genes used to select 5-HT neurons during differentiation. Pet1 and Tph2 are detected at the day8 of EBs differentiation (EB8), just before replating.

The Tph2 "selection-expression cassette" described in details in methods section (6.1.15.1) is containing an artificial intron (intervening sequence - IVS), and, thus, the expressed mRNA can be detected in reverse transcription reaction as an autonomous band comparing to genomic DNA. We used cDNA synthesized from brains (B) and testis (T) of *Tph2*$^{-/-}$ animals, and NB4 cell line used for differentiation to detect the expression of the Tph2 cassette (Figure 9. 13). We were able to detect the spliced transcript in all three cases.

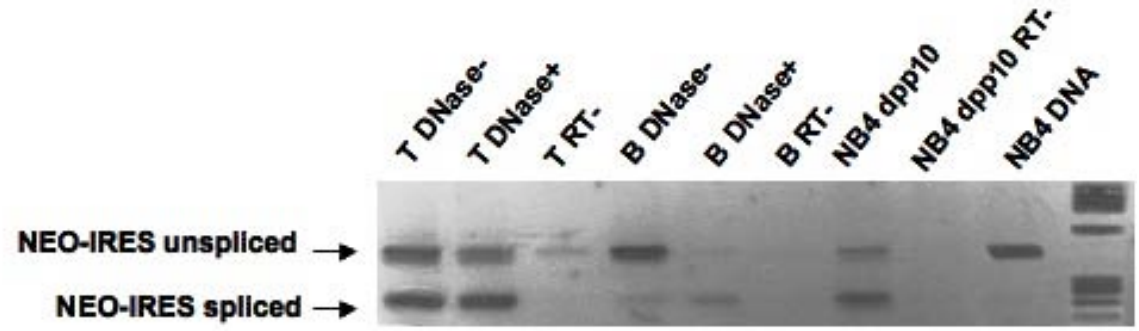


Figure 9.13. Expression of "selection-expression" cassette. RT-PCR of NB4 cell line (Tph2/neoR-IRES-dsRed) showing the spliced transcript of the integrated cassette. As a positive control testis (T) and brain (B) of *Tph2*$^{-/-}$ animals were used.

Neomycin resistance (neoR) is supposed to be active as soon as the *Tph2* promoter is activated during 5-HT differentiation (EB8). We tested different concentrations of neomycin in culture, and were not able to drive an efficient selection of 5-HT neurons (Figure 9.14).

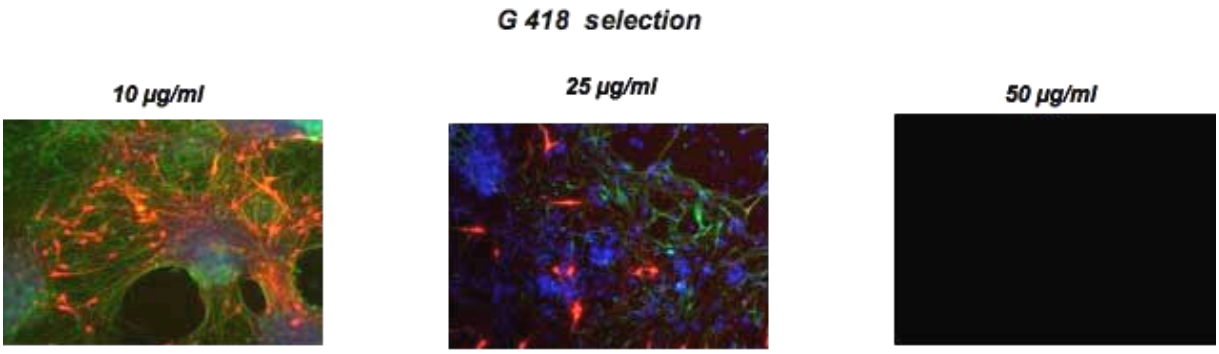


Figure 9.14. neoR selection during 5-HT differentiation. We were not able to detect the neoR expression in our culture. At low neomycin (G418) concentrations (10µg/ml) all neurons were surviving, independent of the presence or absence of the neoR cassette. At higher concentrations, 5-HT neurons were dying together with all other cells in culture.

Consistent with the failure of neoR selction, we could not detect dsRed fluorescence, neither by FACS of differentiated cells, nor in the brains or testis of *Tph2*$^{-/-}$ animals (immunofluorescence, data not shown). The reasons for the failure of the Tph2 "selection-expression" cassette are unknown (see discussion).

9.4. FunGenES cooperation – Sanofi-Aventis "Tph2 cluster"

Being a part of an international consortium allowed us to have a regular overview of the data produced in other labs. One of the FunGenES partners was Sanofi Aventis Pharma R&D, Vitry Research Center, France, from where we got the ESC line (E14Tg2a) and the original RA protocol for EBs differentiation. The same group did an Affymetrix analysis of

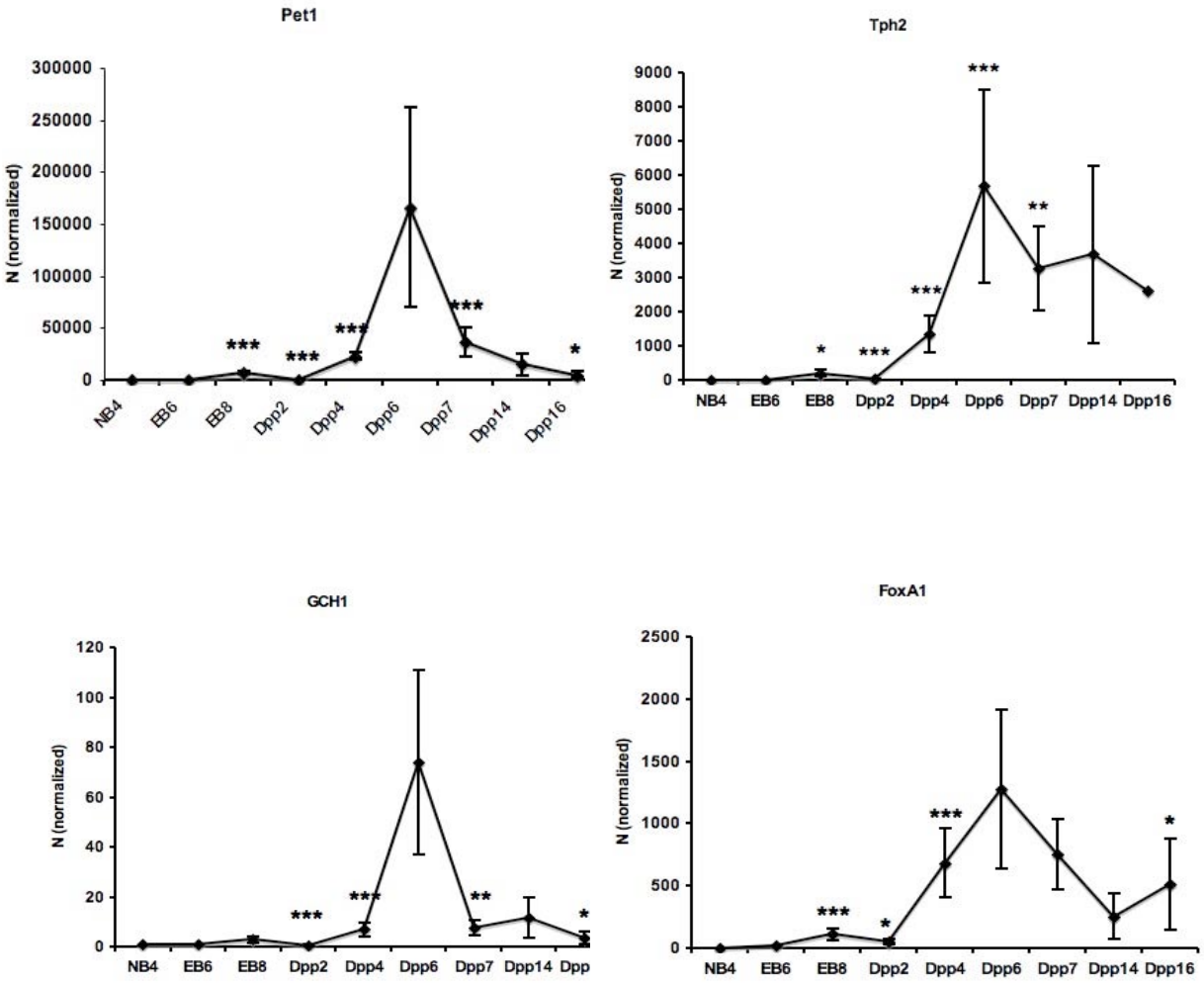
the successful neuronal differentiation in different time points of RA treated and control differentiation.

Surprisingly, by extracting the genes showing similar expression pattern as *Tph2* during differentiation, we were able to trace the so-called "Tph2 cluster", that included several known genes determining the serotonergic phenotype, like: Pet1 (FEV), VMAT2, and SERT (Figure 9.15).

1432556_A_AT	3100002J23RIK	RIKEN cDNA 3100002J23 gene
1455393_AT	CP	ceruloplasmin
1429113_AT	2900092E17RIK	RIKEN cDNA 2900092E17 gene
1418925_AT	CELSR1	cadherin EGF LAG seven-pass G-type receptor 1
1417150_AT	SLC6A4	solute carrier family 6 (neurotransmitter transporter, serotonin), member 4
1418317_AT	LHX2	LIM homeobox protein 2
1425382_A_AT	AQP4	aquaporin 4
1417999_AT	ITM2B	integral membrane protein 2B
1418876_AT	N/A	forkhead box D1 (Foxd1), mRNA
1454646_AT	TCP11L2	t-complex 11 (mouse) like 2
1425861_X_AT	CACNA2D1	calcium channel, voltage-dependent, alpha2/delta subunit 1
1434449_AT	AQP4	aquaporin 4
1419017_AT	CORIN	corin
1451691_AT	EDNRA	endothelin receptor type A
1427020_AT	SCARA3	scavenger receptor class A, member 3
1453310_AT	N/A	N/A
1450699_AT	SELENBP2	selenium binding protein 1
1428853_AT	PTCH1	patched homolog 1
1417495_X_AT	CP	ceruloplasmin
1460513_A_AT	EDNRA	endothelin receptor type A
1417533_A_AT	ITGB5	integrin beta 5
1449863_A_AT	DLX5	distal-less homeobox 5
1435292_AT	TBC1D4	TBC1 domain family, member 4
1454752_AT	RBM24	N/A
1449270_AT	PLXDC2	plexin domain containing 2
1452308_A_AT	ATP1A2	ATPase, Na+/K+ transporting, alpha 2 polypeptide
1418666_AT	PTX3	pentraxin related gene
1433532_A_AT	MBP	myelin basic protein
1423407_A_AT	FBLN2	fibulin 2
1419304_AT	T	brachyury
1427300_AT	LHX8	LIM homeobox protein 8
1418688_AT	CALCR	calcitonin receptor
1422410_AT	FERD3L	Fer3-like (Drosophila)
1450883_A_AT	CD36	CD36 antigen
1441107_AT	DMRTA2	doublesex and mab-3 related transcription factor like family A2
1417928_AT	PDLIM4	PDZ and LIM domain 4
1460500_AT	5033421C21RIK	RIKEN cDNA 5033421C21 gene
1433582_AT	1190002N15RIK	RIKEN cDNA 1190002N15 gene
1427252_AT	DMRTB1	DMRT-like family B with proline-rich C-terminal, 1
1435314_AT	TPH2	tryptophan hydroxylase 2
1448734_AT	CP	ceruloplasmin
1417494_A_AT	CP	ceruloplasmin
1419845_AT	DLX1	distal-less homeobox 1
1437079_AT	SLC18A2	solute carrier family 18 (vesicular monoamine), member 2
1449885_AT	TMEM47	transmembrane protein 47
1443823_S_AT	ATP1A2	ATPase, Na+/K+ transporting, alpha 2 polypeptide
1437113_S_AT	PLD1	phospholipase D1
1448669_AT	DKK3	dickkopf homolog 3 (Xenopus laevis)
1450699_AT	SELENBP1	selenium binding protein 1
1435148_AT	ATP1B2	ATPase, Na+/K+ transporting, beta 2 polypeptide
1456402_AT	N/A	N/A
1417312_AT	DKK3	dickkopf homolog 3 (Xenopus laevis)
1448735_AT	CP	ceruloplasmin
1455074_AT	EFCAB1	EF hand calcium binding domain 1
1439827_AT	ADAMTS12	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 12
1423825_AT	GPR177	G protein-coupled receptor 177
1418049_AT	LTBP3	latent transforming growth factor beta binding protein 3
1460546_AT	LGI3	leucine-rich repeat LGI family, member 3
1448154_AT	NDRG2	N-myc downstream regulated gene 2
1438799_AT	N/A	N/A
1450699_AT	SELENBP1	selenium binding protein 2
1420514_AT	TMEM47	transmembrane protein 47
1438312_S_AT	LTBP3	latent transforming growth factor beta binding protein 3
1450699_AT	SELENBP2	selenium binding protein 2
1437112_AT	PLD1	phospholipase D1
1434921_AT	NR2E1	nuclear receptor subfamily 2, group E, member 1
1436244_A_AT	TLE2	transducin-like enhancer of split 2, homolog of Drosophila E(spl)
1448147_AT	TNFRSF19	tumor necrosis factor receptor superfamily, member 19
1433919_AT	ASB4	ankyrin repeat and SOCS box-containing protein 4
1437434_A_AT	GPR177	G protein-coupled receptor 177
1455872_AT	BC065085	cDNA sequence BC065085
1422997_S_AT	ACOT2	acyl-CoA thioesterase 2
1425886_AT	FEV	FEV (ETS oncogene family)
1420926_AT	ARX	aristaless related homeobox gene (Drosophila)
1438651_A_AT	AGTRL1	angiotensin receptor-like 1
1454198_A_AT	EFCAB1	EF hand calcium binding domain 1
1427523_AT	SIX3	sine oculis-related homeobox 3 homolog (Drosophila)
1433667_AT	LGI3	leucine-rich repeat LGI family, member 3
1428221_AT	KLHDC8B	kelch domain containing 8B
1460003_AT	N/A	N/A
1434297_AT	E130304F04RIK	RIKEN cDNA E130304F04 gene
1424695_AT	2010011I20RIK	RIKEN cDNA 2010011I20 gene
1428549_AT	CCDC3	coiled-coil domain containing 3
1433581_AT	1190002N15RIK	RIKEN cDNA 1190002N15 gene
1449470_AT	DLX1	distal-less homeobox 1
1422009_AT	ATP1B2	ATPase, Na+/K+ transporting, beta 2 polypeptide
1434421_AT	ISLR2	immunoglobulin superfamily containing leucine-rich repeat 2
1423824_AT	GPR177	G protein-coupled receptor 177
1448877_AT	DLX2	distal-less homeobox 2
1455136_AT	ATP1A2	ATPase, Na+/K+ transporting, alpha 2 polypeptide
1428960_AT	4933434I06RIK	RIKEN cDNA 4933434I06 gene
1429692_S_AT	GCH1	GTP cyclohydrolase 1
1418496_AT	FOXA1	forkhead box A1
1449065_AT	ACOT2	acyl-CoA thioesterase 2
1418357_AT	FOXG1	forkhead box G1
1423422_AT	ASB4	ankyrin repeat and SOCS box-containing protein 4
1416992_AT	MFNG	manic fringe homolog (Drosophila)
1444517_AT	N/A	N/A

Figure 9.15. The genes of "Tph2 cluster". The genes of all cell lineages including serotonergic are present in the cluster. We checked the co-expression of all the genes and followed those whose pattern was similar to *Tph2* expression pattern (Allen Brain Atlas).

After our attempts to continuously reproduce an efficient serotonergic differentiation protocol and select serotonergic cells failed, we decided to analyze the genes discovered in Sanofi Aventis "Tph2 cluster", using RNA collected during one of our efficient differentiations (30% 5-HT). RNA was reversely transcribed to cDNA, and qPCR was done to quantify the specific gene expression normalized to the expression of a housekeeping gene (β-actin). To this purpose, several genes were chosen expected to be involved in maintaining or contributing to serotonergic phenotype by their co-expression with *Tph2* (Allen Brain Atlas: www.brain-map.org): ASB4, CACNA 2D, GCH1, FoxA1, Pdxl2, Egr2 and an unknown one 3100002J23RIK, together with several previously known genes that have exclusive pattern of expression (Nkx2.2, Nkx6.1), as well as *Tph2* and *Pet1* were chosen to verify the results (Figure 9.16).



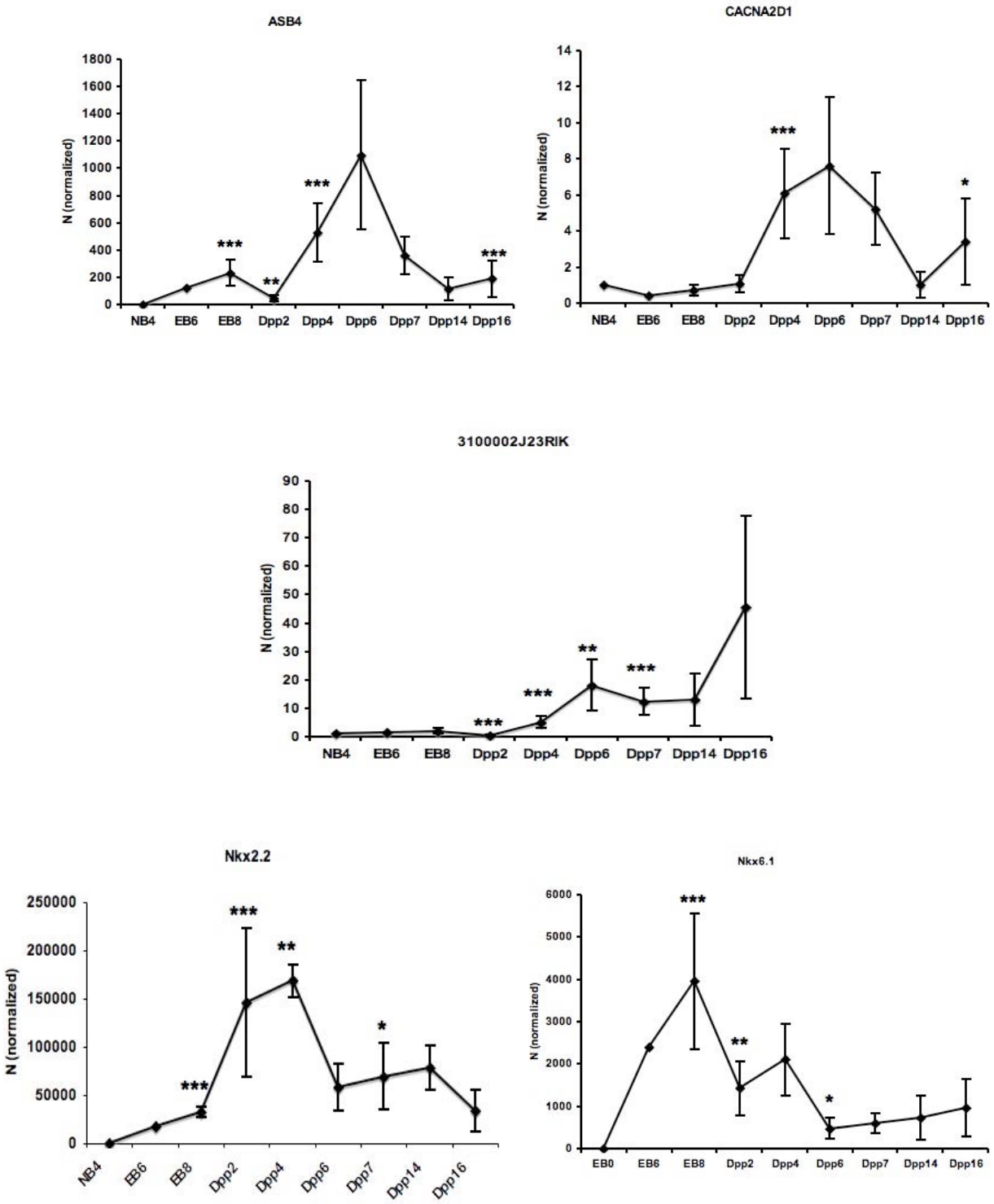


Figure 9.16. qPCR on the samples from minimum 4 different differentiations at each time point with ca. 30% 5-HT outcome. Most of the genes share a similar expression pattern with *Tph2* and *Pet1*. Only 3100002J23RIK probably belongs to a completely different cell lineage. ***p<0.001, **p<0.01, *p<0.05, *vs.* previous time point of differentiation, t-test. Data are presented as mean of relative expression level ± SEM, where 1 denotes the ESC state (NB4 clone before differentiation).

Our result showed simultaneity of expression of the investigated genes with *Tph2* expression. This result confirmed the microarray data of Sanofi-Aventis. Furthermore, the results extracted from one set of data could be confirmed independently from another by using the same protocol, the same cell line and the same materials (see materials and methods). The functional analysis of the "Tph2 cluster" genes is still to be performed and their putative role will be discussed in the discussion section.

9.5. An additional approach - ePet transgene

After the Tph2 "selection-expression cassette" failed we decided to mark and select 5-HT neurons in culture by applying a transgene construct with the Pet1 enhancer (ePet) driving GFP selection (Scott et al. 2005 and introduction 6.1.15.2). We tested both of ePet lengths reported: 3kb, and about 50kb (Figure 9.17). The last was kindly provided by E. Deneris, and modified to enable ES selection as described in the methods section.

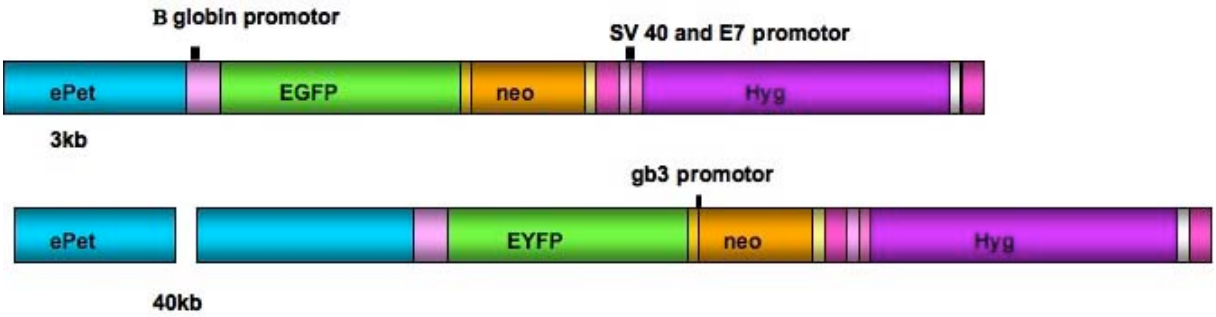
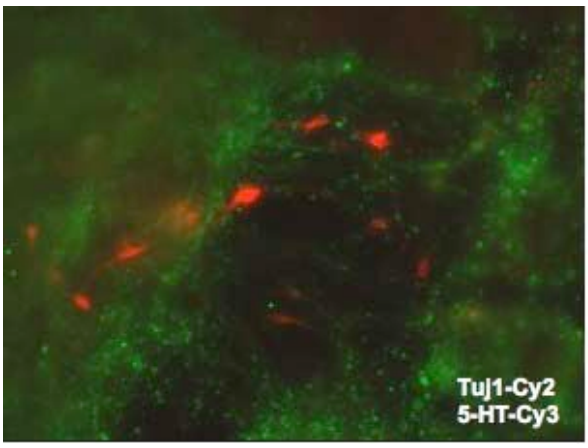


Figure 9.17 ePet transgenic constructs a) 3kb ePet drives the expression of EGFP b) 40kb ePet the expression of EYFP, both using β globin promoter. For details see materials and methods 6.1.15.2.

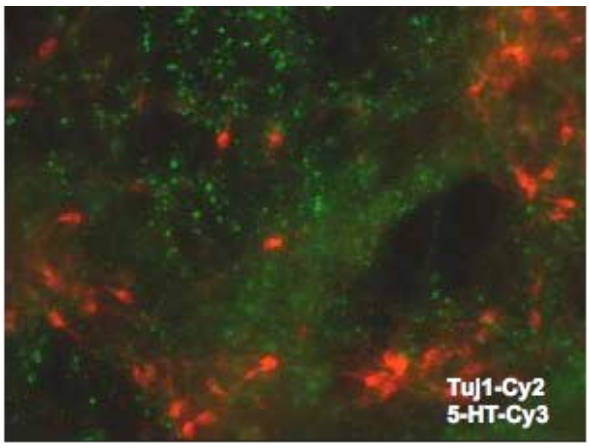
Despite the positive serotonergic outcome, neither 3kb ePet nor 40kb ePet were able to drive EGFP/EYFP expression during *in vitro* serotonergic differentiation (Figure 9.19). We still do not know the reason for the failure of those two transgenic constructs, as their functionality in serotonergic neurons is previously described *in vivo.* Thus, until the end of this work, we were not able to select 5-HT neurons during *in vitro* differentitation and to

perform the microarray analysis as all three selection cassettes failed to show the fluorescence during neuronal differentiation (Tph2 “selection-expression” cassette, 3kb ePet-EGFP and 40kb ePet-EYFP).

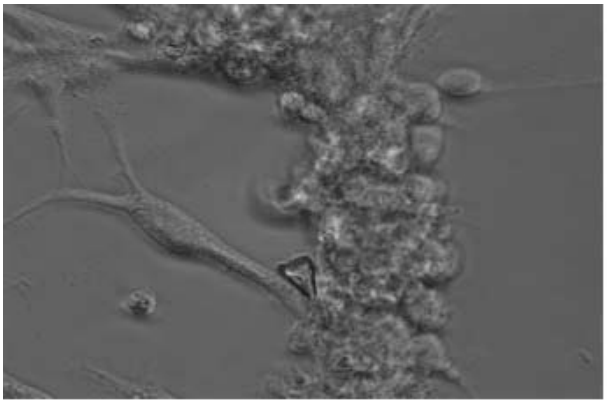
a)



b)

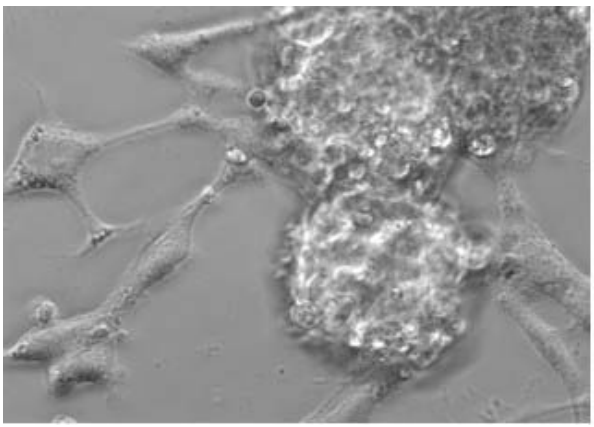


c)



d)

e)



f)

g) h)

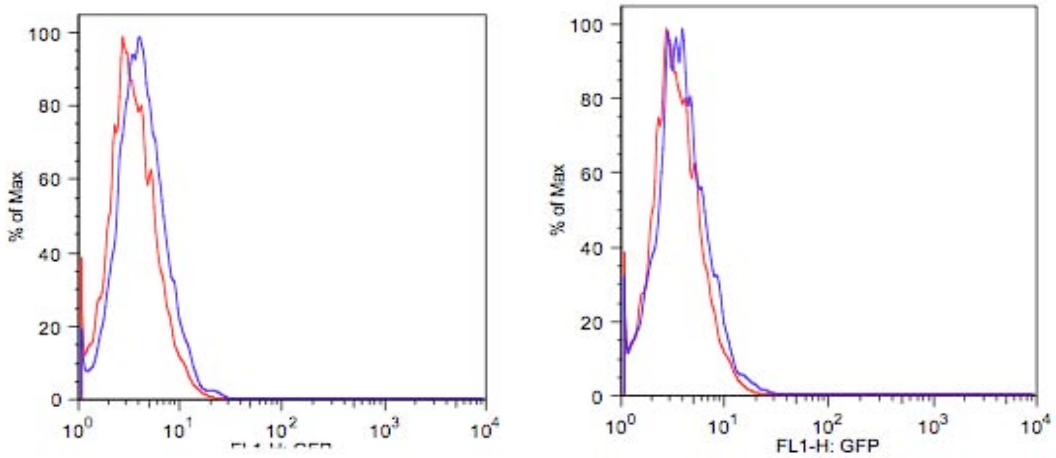


Figure 9.18. ePet$^+$ clones (2 of 30 are shown: 20 were 3kb ePet-EGFP, and 10 ePet 40kb – EYFP transgenic) a) b) 5-HT immunostaining, c) d) and e) f) *in vivo* GFP+ fluorescence and g) h) FACS analysis. We were not able to detect ePet driven EGFP/EYFP expression in cell culture.

IV DISCUSSION

10. TPH2 DEFICIENT MOUSE

Serotonin synthesis in mammals is initiated by two distinct tryptophan hydroxylases (TPH) (introduction 1.5.1.2.1), TPH1 and TPH2, encoded by two distinct genes (Walther and Bader 2003, Walther et al. 2003). *Tph1* is mainly expressed in the gut generating serotonin that is distributed into the whole body by thrombocytes, and in pineal gland, where the resulting 5-HT is metabolized to melatonin. The *Tph1* deficient mice (Walther et al. 2003, Cote et al. 2003, Savelieva et al. 2008) revealed that 95% of peripheral serotonin is produced by TPH1. TPH2, however, is responsible for the synthesis of serotonin in the raphe nuclei of the brain stem, from where all central serotonergic projection originate (Dahlström and Fuxe, 1964). Until recently, it was unclear if TPH1 and TPH2 are exclusively producing peripheral and central serotonin, respectively, as data were present showing *Tph1* expression in raphe nuclei of the rat (Patel et al. 2004, Malek et al. 2005), the mouse (Gundlah et al. 2005) and humans (Zill et al. 2007).

Generation of a Tph2 deficient mouse by us and others (Gutknecht et al. 2008, Savelieva et al. 2008) showed almost complete lack of serotonin in the whole brain as well as in the specific brain areas (results Table 8.1) and no detectable amounts of its metabolite 5-HIAA. TPH1 can be responsible for residual amounts of serotonin, since it is expressed in the brain at the levels of around 4% compared to TPH2 (Abumaria et al. 2008). However, TPH1 is not able to compensate for the loss of TPH2, confirming that TPH2 is the only relevant enzyme for central serotonergic synthesis. Furthermore, there was no difference in serotonin levels in any other organ or in the blood of *Tph2* deficient animals confirming that peripheral serotonin synthesis is not dependent on TPH2 (results Table 8.1). The bowel is an exception, as it expresses TPH1 in enterochromaffine cells (Walther et al. 2003) and TPH2 in enteric neuron (Gershon et al. 2009). Still, we were not able to detect the change in serotonin concentration, as TPH2 derived serotonin is only a minor portion of total intestinal serotonin.

Serotonergic neurons were still present in their normal location and pattern in the raphe nuclei of *Tph2*$^{-/-}$ mice as detected by *in situ* hybridization for serotonin transporter

(SERT) mRNA (results Fig 8.3). We could not detect any developmental changes in the brain of *Tph2*$^{-/-}$ animals, but a detailed analysis is still pending. It seems, however, that 5-HT itself is not required for serotonergic neuron development, maturation, outgrowth, maintainance and survival (our observation and Gutknecht et al. 2008). This distinguishes our model from the mice deficient in *Pet1* (Hendricks et al. 2003), *Lmx1b* (Ding et al. 2003, Zhao et al. 2006) and *Nkx2.2* (Briscoe et al. 1999), where a certain percentage of serotonergic neurons are arrested during development, while the remaining 5-HT neurons show only transient (*Lmx1b*$^{-/-}$ mice) or lower expression of serotonin specific genes, *Tph2* and *Sert* (*Pet1*$^{-/-}$ mice). It also contradicts the evidences present in literature that support 5-HT as an important morphogen during neuronal development (Gaspar et al. 2003). However, at least during embryonal development, the contribution of serotonin synthesized by TPH1 cannot be excluded, as it is not clear when is the brain blood barrier fully formed (Rubin 1999, Saunders et al. 1991). The presence of terminal synaptic connections in our model may cause the release of other substances present in serotonergic vesicles, like substance P and thyrotropin-realising hormone (TRH) that may meliorate the phenotypic changes (Pelletier et al. 1981, Richerson et al. 2004).

Tph2 deficient mice are viable until adulthood and show no gross morphological abnormalities. However, depletion of central serotonin leads to growth retardation and about 50% lethality (C57Bl/6 background) in the first 4 weeks of postnatal life.

Central serotonin is necessary for regulation of autonomic processes. Telemetric recordings revealed more extended daytime sleep, suppressed respiration, altered body temperature control and decreased blood pressure and heart rate during night time.

Tph2$^{-/-}$ *females exhibit impaired maternal care* that might be one of the reasons for the poor survival of the pups in the first weeks of life. The fertility and milk production of *Tph2* deficient females is not impaired, neither *Tph2* deficient males show any obvious reproductive abnormalities.

Thus, the hallmark of Tph2 ablation in mice is that central serotonin is involved in the regulation of behavior and autonomic pathways, but it is not essential for adult life.

10.1. Survival and growth

Tph2$^{-/-}$ *animals born at normal Mendelian ratios* and at the day of birth were undistinguishable from their WT littermates. However, in the next 3 weeks they were visibly smaller and more fragile then WT littermates and showed considerable lethality (27% mixed background, 21.4% FVB/N background and 47.4% C57Bl/6 background) in the first 2 weeks of life (results Figure 8.14). However, after the catch-up growth, we were not able to detect any visible morphological changes between *Tph2*$^{-/-}$ and *Tph2*$^{+/+}$ littermates, neither was increased mortality observed until 1.5 years of age on the mixed genetic background.

We do not know the reason for the lack of weight gain in early postnatal life. Newborn *Tph2*$^{-/-}$ mice drink milk and show normal digestion. All trials to quantify food intake or to force feed failed, since any handling of the pups worsened the phenotype. Pups raised in litters with mixed genotypes showed the same phenotype as pups raised in pure *Tph2*$^{-/-}$ litters excluding competition with the control littermates as cause for the effect. The growth retardation also did not depend on thegenotype of the mother since it appeared in pups born and raised by *Tph2*$^{-/-}$ and *Tph2*$^{+/-}$ dams. *Tph2*$^{-/-}$ vocalize equally as controls when separated from their mothers excluding a deficit in this behavior as cause of growth retardation (experiment done by Valentina Mosienko). Serum levels of IGF-I are markedely lower in Tph2-/- mice until two months of age but afterwards reached the levels of control animals, when the animals had attained normal size (results Figure 8.7). Albeit that these reduced IGF-I concentration are probably causing the postnatal growth retardation in *Tph2*$^{-/-}$ mice, they could easily be secondary to undernutrition (Thissen et al. 1994). Thus, further studies are required to clarify whether behavioral or sensory deficit causing malnutrition or a primary impairment of growth hormone/IGF-I axis by the lack of stimulatory serotonin actions (Papageorgiou et al. 2007) lead to the impaired thriving of these animals.

The direct cause of the increased mortality of Tph2$^{-/-}$ *mice is still unknown.* Some physiological and behavioral parameters observed in *Tph2* knock-out may be taken into account (low respiration rate and maternal neglect, see below), but other possibilities, such as under-nutrition, depression, lack of competitiveness or any other socio-physiological characteristics influenced by serotonin, could be the cause of observed lethality.

10.2. The role of central serotonin synthesis in the autonomous nervous system control

The autonomous nervous system (ANS) presents the part of peripheral nervous system working without conscious control. It includes the sympathetic and the parasympathetic nervous system. The function of two is complementary – they function in opposition to each other, and are usually referred to as "fight or flight" (sympathetic) and "rest and digest" (parasympathetic) systems. Anatomically, the reflex arcs with the sensory and motor arm comprise the autonomic nervous system. The sensory arms are located in the cranial sensory ganglia and consist of primary sensory neurons. They are sensors for oxygen levels, carbon dioxide and sugar levels in the blood and chemicals in the peritoneum and gut, but also for some consciously percepted stimuli, like taste. They project to secondary (visceral) sensory neurons in the medulla oblongata and form the nucleus tractus solitarius (NTS). NTS is processing informations received from other chemosensory centers and the cerebrospinal fluid and modulate the activity of motor neurons. Motor neurons of sympathetic and parasympathetic nervous system are located in two sympathetic ganglia chains next to the spinal cord or form ganglia in the close proximity to target organs, respectively. Together, they influence in opposite ways, vasodilatation, heart rate, breathing, peristalsis, and, generally, the processes involved in arousal and energy metabolism. However, except maybe the enteric ones, the ganglia of the autonomous nervous system are not really autonomous – they are prone to neuronal and hormonal control, enabling them to function as a part of a body, maintaining the homeostasis necessary for survival under the stress stimuli (Guyton 2007).

The neuronal innervation of the ANS is located in the brainstem that is interconnected with higher areas of the brain, all the way up to prefrontal cortex (Cerqueira et al. 2007). The hypothalamus, through the hypothalamo-pituitary axis (HPA) and hormonal secretion is involved in ANS regulation. Brainstem and the hypothalamus are, among other pathways, connected through extensive serotonergic projections, originating in the *raphe nuclei* and forming the synapses mostly in the *nucleus suprachiasmaticus* (NSC).

We investigated the role of central serotonin in the autonomous processes and hormonal regulation. To this purpose a telemetry method and different metabolic measurements were performed, as described in the materials and methods section.

10.2.1. Temperature regulation

Thermoregulation is the ability of the organism to keep its body temperature stable, independent of the environment. It is an aspect of homeostasis, a dynamic state of stability that keeps internal milieu stable, in a certain range of external changes. In mammals, it is regulated via the central nervous system through endocrine, autonomic and behavioral mechanisms.

First studies localized thermoregulatory region in the preoptic area (POA) of the hypothalamus that contains thermosensitive neurons and receives signals from peripheral receptors. Later it became apparent that other hypothalamic areas are involved, particularly the dorsomedial hypothalamus (DMH), most probably a common integrative center for the stress response (DiMicco et al. 2002). The integrative reaction is transmitted to the effectors via the sympathetic nervous system. As a response to decreased ambient temperature or "cold stress", shivering (skeletal muscle contraction) and non-shivering (metabolic activation of adipose tissue) thermogenesis occurs under the control of sympathetic outflow. The generated heat is transmitted systemically by inducing at the same time tachycardia and activating the hypothalamo-pituitary-adrenal axis, that stimulates glucocorticoid secretion as a response (DiMicco and Zaretsky 2007, Morrison 2004). DMH communicates bidirectionally with rostral *raphe pallidus* (rRP) that contains, among the others, serotonergic neurons, shown to project to the sympathetic regions of the spinal cord like the intermediolateral column, and to dorsal horns where the thermoreceptors terminate (Henry and Calaresu. 1974, Basbaum et al. 1978, Holstege and Kuypers et al. 1982, Bacon et al. 1990). Moreover, synaptic projections of rRP and *raphe magnus* (RM) were detected in brown adipose tissues (BAT) by retrograde tracing studies (Cano and Passerin 2003), suggesting that RM belongs to the sympathetic pathway.

Studies including the $GABA_A$ receptor antagonist, muscimol, imply tonic inhibition of DMH and rRP by the GABAergic neurons located in POA. Desinhibition of those regions leads to the activation of the sympathetic pathway involved in thermogenesis.

Involvement of serotonin in thermoregulation is acknowledged through several lines of evidence: serotonergic drugs cause pronounced hypothermia, and 8-OH-DPAT, an agonist of 5-HT_{1A} receptors abolishes sympathetic effects induced by hypothermia caused by anesthetic treatment. The experimental simulation of the fever response, by LPS or PGE_2 treatment that causes acute hyperthermia, showed that the same pathway, involving PAO – DMH – rRP, might be included. The signal was again attenuated after muscimol treatment and no body temperature change could be observed after LPS or PGE2 stimulation (DiMicco and Zaretsky 2007).

The net effect of the complete serotonergic depletion in the brain on thermoregulation was unknown, until recently. Pharmacological modulations and raphe nuclei lesions were giving contradictory results, most probably by involving only the manipulation of different subsets of serotonergic neurons. Hodges and collaborators (Hodges et al. 2008) showed that depletion of serotonin in the brain leads to impaired shivering and non shivering thermogenesis and lack of thermoregulation, without impaired temperature sensation or cutaneous vasoconstriction.

Impaired thermogenesis was observed in $Tph2^{-/-}$ mice. Basic body temperature was normal in *$Tph2^{-/-}$* mice, but after a challenge by a cold (4°C) environment, the observed drop in body temperature was more pronounced in serotonin-deficient mice than in control animals (results Figure 8.21). The data are in accordance with the study by Hodges et al. as mentioned above, but our mice show lower phenotypic penetration, most probably due to excretion of other substances present in serotonin vesicles (discussion 10). We confirmed that the lack of serotonin in the brain leads to the impaired temperature homeostasis under changed environmental conditions.

Thus, serotonergic neurons play a modulatory role in temperature regulation without influencing the temperature of the body under physiological conditions. Thermoregulation involves integration of several stimuli: serotonin acting on specific type of

receptors (like 5-HT1A), and, at the same time, synchronized activity of other neurotransmitters: excitatory glutamate (through glutamate ionotropic receptors) and inhibitory GABA (through $GABA_A$ receptors) in several areas of the brain (DiMicco and Zaretsky 2007). It seems that serotonin is fine-tuning the excitatory-inhibitory response of other neurotransmitters during the response to environmental challenges, and is not of primary importance for thermoregulation under normal environmental condition.

10.2.2. Breathing regulation

Breathing is a rhythmic process that enables the systemic consumption and transfer of oxygen to the organs and tissues, and transport of carbon dioxide in the opposite direction. The inhalation and expiration of the air-breathing mammals is facilitated by the contraction and relaxation of diaphragm, a muscle layer separating the thoraxic and abdominal cavities.

Respiratory rhythm is generated by brain stem neurons. A ventrolateral column contains respiratory premotor neurons projecting to spinal and cranial motor neurons that innervate diaphragm by forming the phrenic nerve. A small portion in the rostral area of the column, designated as preBötzinger Complex (preBötzC), is thought to be responsible for the rhythm generation, as lesions in and around this area lead to severe defects in breathing. It is also proposed that those neurons generate the rhythm through a "pacemaker" activity, as the blockade of synaptic transmission to this area does not influence the breathing (Rekling and Feldman 1998, Onimaru et al. 1989), but the identity of the real pacemaker neurons remains still unclear, mostly due to the lack of specific markers in this area (Feldman et al. 2003). It is also not known if there is only one generic center, or different groups that are coupled and rhythmically active. Early postnatal changes that may influence rhythm generation, like intracellular Cl^- concentration and Ca^{2+} channel expression, in this area are observed, but its physiological significance is still unclear. Those changes may contribute to the postnatal lethality of some genetically altered mice models, like NMDAR1 deficient mice (Funk et al. 1997, Sprengel and Single. 1999), MAOA deficient mice (Bou-Flores et al. 2000) or brain derived neurotrophic factor (BDNF) deficient mice (Balkowiec and Katz 1998, Erickson et al. 1996, Katz and Balkowiec 1997).

Plasticity in the context of respiration is the adaptivity of respiratory rhythm to different challenges, like hypoxia, hypercapnia, exercise, development and aging, neural injury or

sensory denervation. Most of these processes are serotonin dependent and present through the animal kingdom – *Aplysia* uses serotonin to facilitate the sensory motor synapse that mediates the gill withdrawal reflex (respiratory-defense reflex) (Kandel et al. 2001, Sutton et al. 2001). Respiratory long-term facilitation (LTF) is the long lasting increase in ventilation produced by hypoxia when induced in episodic pattern (Baker and Mitchell 2000) and can be abolished by serotonin receptors antagonists or serotonin depletion (Bach and Mitchell 1996). LTF is shown to be age and gender dependent (Mitchell et al. 2001), but it is mostly studied during early development, as it might be involved in sudden infant death syndrome (SIDS) (Kinney et al. 2001a, 2001b). It is postulated that chemosensitive neurons that sense hypoxia send signals to *raphe* serotonergic neurons - they project to phrenic and hypoglossal motoneurons and facilitate respiration. The mechanism is, however, not so clear, as blocking serotonergic receptors during hypoxic stimulation does not influence LTF (Fuller et al. 2001). Serotonergic neurons, thus, might be necessary for triggering LTF response, but other neurotransmitter systems must be involved in its maintenance. How the serotonin exerts its effect on motoneurons is still unknown, but an important role of BDNF is suggested (Mitchell et al. 2001), which again might be the reason for the postnatal lethality of the mice deficient in BDNF synthesis. Nitric oxide might also be involved, as LTF is not observed in the mutant mice deficient in NOS-1 (Kline et al. 2001), but this might be indirectly, due to inhibition of 5-HTR2 family (Nozik-Grayck et al. 2002) or decreased BDNF secretion in the hippocampus (Canossa et al. 2002) observed in this model.

Chemosensitivity is the ability of an organism to sense the relative concentrations of O_2/CO_2 and pH in the blood, and reflects the efficacy of breathing relative to the metabolism. O_2 chemoreceptors are outside of the brain in the carotid bodies, but CO_2/pH chemoreceptors are located in the carotid bodies and in the brain, the latter referred to as central chemoreceptors. Even small changes in pH/CO_2 can affect breathing. Several different experimental approaches located central chemoreceptors in the brain stem, including the regions of nucleus *tractus solitarius*, *locus coeruleus* and midline *medullary raphe* (Feldman et al. 2003), and it appears that this distinct regions can complement each other – disruption of any single site does not abolish CO_2 response, suggesting they were arousing in evolution, providing a more sophisticated control system with the progressive complexity of the nervous system.

Are serotonergic neurons chemosensitive? The evidences confirming this hypothesis are accumulating (Richerson 2004), but they are certainly not the only ones – the noradrenergic neurons of *locus ceruleus* are involved in the respiratory perturbation as the result of environmental changes, and pharmacological modulations of the glutamatergic and GABAergic neurons in the ventral medulla decrease CO_2 response. Recently, several groups reported that the depletion of serotonin activity in the brain leads to blunted hypercapnic ventilatory response (Hodges et al. 2008, Audero et al. 2008), but the effects of hypoxia or hyperoxia are attenuated, and most probably compensated by other pathways.

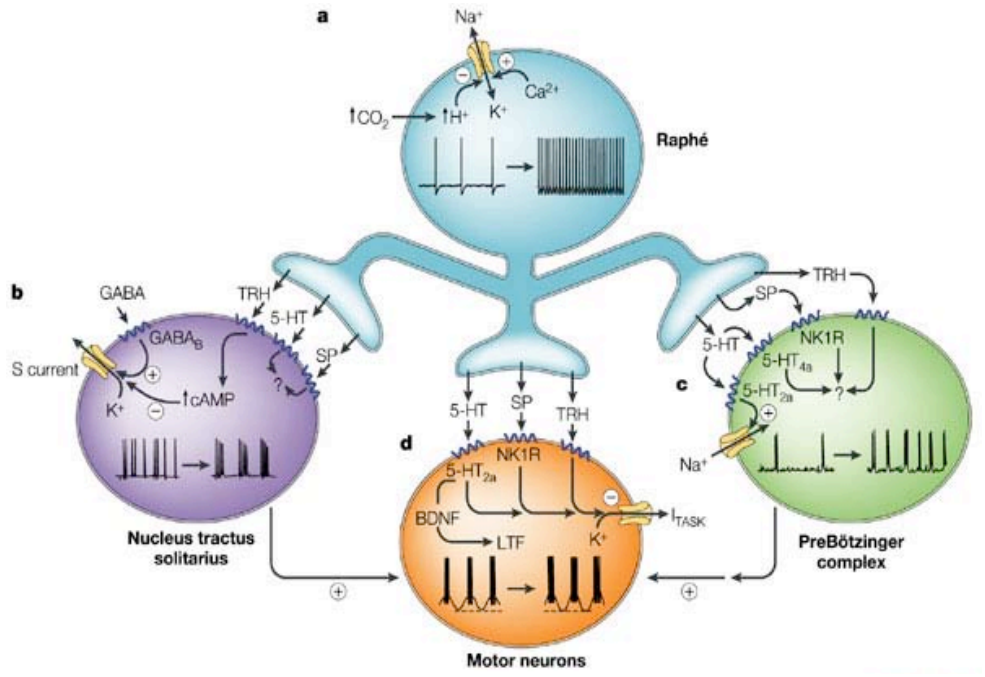


Figure 10.1. The regulation of respiration. *Raphé* neurons use many mechanisms to enhance respiratory output, modulating neurons at multiple sites within the respiratory network. The effects of 5-HT, tyreotropin releasing hormone (TRH) and substance P (all present in the vesicles of serotonergic neurons), on downstream targets might be complementary and supra-additive, and as the firing rate of *raphé* neurons increases, the relative proportion of each of these neurotransmitters that is released might change, with the neuropeptides becoming most important at high firing rates. Withdrawal of tone from serotonergic neurons, such as occurs during sleep or after *raphé* lesions, would be expected to lead to loss of tonic drive to the respiratory network. This would in turn cause a decrease in baseline ventilation and blunting of the carbon dioxide response, as has been observed. Therefore, output of the respiratory network might depend crucially on carbon dioxide- and state-dependent drive from raphé neurons (Richerson 2004).

Our model showed clearly reduced respiration rate. Such dampening of respiration has been described in other pharmacologic and genetic models of partial serotonin deficiency in the CNS (Hodges et al. 2008, Mueller et al. 1984, Erickson et al. 2007, Richerson 2004). However, there have also been contradictory studies postulating that serotonin inhibits breathing (Richerson 2004). Our data strongly support the concept of a tonic excitatory input of serotonergic fibers on respiratory neurons.

The early postnatal death of some of the Tph2$^{-/-}$ mice may be caused by the autonomic disturbances described above. Transgenic animals overexpressing autoinhibitory 5-HT1A receptors and thereby being functionally deficient in serotonergic activity (Audero et al. 2008) as well as mice lacking most of serotonergic neurons also showed this phenotype (Hodges et al. 2008). Together with recent data from patients (Paterson et al. 2006, Moon et al. 2007), these mouse models corroborate a role of the serotonin system in the sudden infant death syndrome, SIDS.

10.2.3. Sleep/awake pattern and circadian rhythm

Sleeping is an evolutionary advanced behavior present throughout the animal kingdom (reptiles, birds, mammals) and aimed to restore the vital functions of the body. It is suggested to be involved in development, protection, metabolism and memory consolidation. For a long time sleep was regarded as a passive unconscious state of the organism, but, after the development of the electroencephalogram (EEG) at the beginning of the 20th century, it became apparent that sleep includes active mechanisms of falling asleep and arousal, as well as several physiologically and behavioural different phases with marked different neuronal activity (REM and NREM sleep), that sometimes might be observed in what was believed to be the fully awaked state (Datta and MacLean 2007).

Sleep timing is a feature of mammalian sleep that appears during the predictable times in 24h cycle. It indicates that circadian processes are involved in regulation of sleep and in sleep timing. The timing of sleep is species specific, and most probably presents an evolutionary adaptation: animals are active when the possibility to gain food exceeds the risk of predators.

Nucleus suprachiasmaticus (NSC) in the hypothalamus is the central sensor for the circadian rhythm. Via the retinohypothalamic tract it receives the light signals from the retina, that is reset during the night phase by the excessive melatonin synthesis in the pineal gland or epiphysis. The pineal gland contains the highest concentration of serotonin compared to other organs, although it does not belong to the brain, but to the endocrine system of the organism. It is separated from the brain by the brain blood barrier, but extensive projections are leading to NSC. The epiphysis itself is regulated by the peripheral nervous system, notably noradrenergic fibers of the sympathetic nervous system. Massive projection to NSC are also coming from raphé nuclei and it is known that serotonergic neurons fire tonically in awake behaving animals, with lower activity during the NREM sleep and the lowest activity during the REM sleep (Fornal et al. 1985), which is found to be the pattern for all monoaminergic neurotransmitters (Aston-Jones et al. 1981, Steininger et al. 1999).

Waking up. The ascending arousal system promotes wakefulness and has two branches originating from the brain stem: thalamo-cortical, that is mostly cholinergic (ACh) and the branch that surpasses thalamus, and leads through the lateral hypothalamic area to the cerebral cortex. This branch involves: the noradrenergic locus ceruleus, the serotonergic dorsal and median raphé nuclei, the dopaminergic ventral periaqueductal grey matter and histaminergic tuberomammillary neurons. The lateral hypothalamus complements by adding the signal from peptidergic neurons (melanin concentrating hormone (MCH) and orexin/hypocretin) and basal forebrain adds cholinergic and GABAergic fibers before reaching the cortex (Saper et al. 2005).

Falling asleep. It was long time suspected that there must be an inhibiting region that shuts down the activity of arousal system to promote sleeping, and might be the cause of insomnia if destructed. Lesion experiments identified such a region in the basal ganglia and anterior lateral hypothalamus (McGinty 1968). Hypothalamical ventrolateral preoptic region (VLPO), where sleep active neurons containing inhibitory neurotransmitters, GABA and galanin, project to the monoaminergic system in the brain stem (McGinty and Szymusiak 2000). It also receives afferent projections from these neurons suggesting mutual inhibition (Sherin et al. 1996, Gaus et al. 2002, Sherin et al. 1998, Szymusiak et al. 1998).

Flip flop switch. Mutually inhibiting elements are self-reinforcing: the activity of one inhibits the activity of the other, reinforcing its own activity. As a paradigmatic model present in electrical engineering, the flip flop switch mechanism seems to occur in natural processes that are showing the sharp transition, such as waking up/falling asleep change. The model could explain the abrupt waking up, or sudden falling asleep, and it might have evolved as a defense mechanism of increased alertness. There are some safety mechanisms of the switch transition. For example, orexin neurons, active during the motor activity of the animal, are sending signals to the monoaminergic arousal system, keeping the animal safe not to fall unexpectedly asleep (Chemelli 1999, Peyron 1998, Marcus 2001).

What is pulling the switch towards falling asleep? There are two main regulators of sleep/awake state: energy exhaustion and circadian rhythm. Circulating substances, named somnogens, that detect the low energy level of the organism, are switching the VLPO to inhibit the arousal system. Adenosine accumulates in blood when the synthesis of ATP is exhausted, and is one of the proposed candidates (Strecker 1999, Radulovacki 1984, Benington and Heller 1995). Other signals are coming from NSC, that is always active during the light cycle, while VLPO is active during the dark phase. NSC, however, does not have any adaptive abilities - this feature is signaled from another hypothalamic region, DHM (Deurveilher 2005, Chou 2003). DHM was mentioned above as the integrating thermoregulatory center, and it seems it serves the same function by recognizing whether it is a good time to fall asleep or not: activity, feeding, body temperature or stress can postpone the signals from NSC and maintain the animal awake if necessary for the overall fitness.

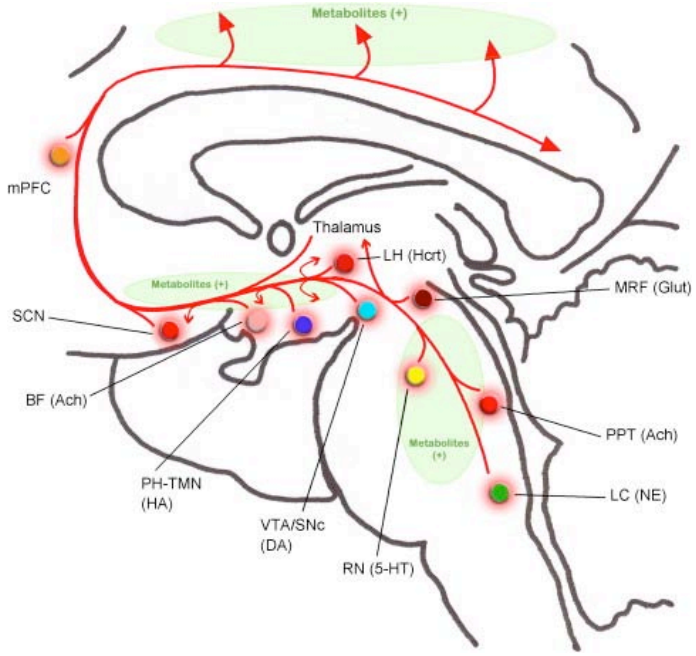


Figure 10.2. Monoaminergic arousal system. Ach - acetylcholine, Glut - glutamate, Hcrt - hypocretinergic/orexinergic cells, DA - dopamine, HA histamine, mPFC - medial prefrontal cortex, SCN - suprachiasmatic nucleus, BF - basal forebrain, PH-TMN - posterior hypothalamic tuberomammilary nucleus, VTA/SNc - ventral tegmental area substantia nigra compacta, RN - raphe nuclei, MRF - mesencephalic reticular formation, PPT - penduculopontine tegmentum, LC - locus coeruleus (Datta and MacLean 2007).

Serotonin and sleep. As a part of the fibers involved in the monoaminergic arousal system, it would be reasonable to suspect that the deprivation of serotonin leads to the impairment of arousal in the animals. However, pharmacological manipulations showed contradictory data. There is a long-lasting debate on the role of serotonin in the regulation of sleep and wakefulness. Both, sleep promotion and induction of arousal by 5-HT have been reported mostly depending on the origin of the serotonergic neurons analyzed (Saper et al. 2001, 2005).

Telemetric recordings in Tph2$^{-/-}$ animals revealed marked disturbances in sleep. During the day, these animals sleep more frequently and for longer periods of time than their control littermates. In the night, sleeping periods are more rare in both, *Tph2*$^{-/-}$ ands WT

animals, and do not significantly differ in length and frequency. Our animal model strongly supports sleep promotion and a suppression of arousal as the net effect of a lack of serotonin in the CNS.

10.2.4. Hypothalamo - Pituitary - Gonadal Axis: Growth and Ageing

Serotonergic fibers project massively to hypothalamus. The hypothalamus, except being involved in circadian rhythm regulation and other integrative processes, is a master regulator of neuroendocrine processes via the hypothalamo - hypophyseal - gonadal axes. The hormones, synthesized by the neuroendocrine cells of the hypothalamus, are released in the hypothalamo - hypophyseal portal system to regulate the activity of the hypophysis, the gonads and other organs. Hypothalamus is controlled by other brain regions, notably the brain stem and the hippocampus, but it also receives feedback impulses via the peripheral nervous system and the circulation. Hypothalamus activity is prone to ageing, and a decrease is observed in response to monoaminergic neurotransmitters and to a hormonal feedback (Rehman and Masson 2001). In humans, hindbrain serotonin levels are not decreased, but the number of 5-HT2 and 5-HT1D receptors (Arranz 1993), that can contribute to age related depression or disturbed sleep/awake pattern.

Dwarfism is a growth retardation that reduces either the size of the body parts or the total size of the body. There are many pathological conditions that can lead to dwarfism, one of which is “pituitary dwarfism” caused by growth hormone (GH) deficiency. Insufficiency in circulating growth hormone is impairing skeletal and muscle growth, leading to a proportionally smaller body.

Dwarfism, at least in mice, is connected with the life span, and several models of commercially available dwarf mouse strains have reported longer life expectancy then their wild type littermates (Ames dwarf mice or Snell dwarf mice, both having severe hypothyreoidism (Rusell and Kahn 2007)). Mutant mice deficient in growth hormone releasing hormone (GHRH) synthesis (Flurkey et al. 2001), GH receptors (Coschigano et al. 2000) or insulin-like growth factor I (IGF-I) receptors (Holzenberger et al. 2003) all show increased life span by up to 50%, that is also sex and diet restriction dependent, showing the involvement of other genetic and environmental influences.

Ageing is an ecological phenomenon that balances the reproductive phase and the time needed for the offspring to mature, with leaving enough space in the ecological niche for the next generation. It is a systemically regulated process, involving hormonal and neuroendocrine regulation. From the nineteenth century on, scientists noticed that the level of sex hormones decrease with ageing, some of them even trying to rejuvenate by injecting testosterone cocktails in the blood (Epelbaum 2008). The concentration of other hormones also changes during life - episodic GH secretion is markedly impaired (Finkelstein 1972). GH is pulsatively released from the hypophysis under the rhythmical stimulation of the hypothalamal GHRH. The frequency of GHRH release stays the same, but the amplitude is decreasing during ageing, causing reduced levels of GH. GH exerts its effect on tissues via IGF-I synthesized mainly by the liver, but also by other tissues on which it exerts its paracrine/autocrine function. Adiposity and ageing are associated with decreased plasma levels of GH and IGF-I (Copeland 1990) due to impaired protein anabolism. The negative feedback mechanism is also impaired – there is a significant reduction of IGF-1 receptors in the hippocampus and the hypothalamus associated with ageing (Kappeler 2008), as well as GH receptors and different parts of the brain (Lai et al. 1993).

Klotho. Recently, a novel gene involved in ageing was identified and named klotho, after one of the greek fates, involved in the spinning of the thread of life. It is a transmembrane protein with homology with β – glycosidase, whose extracellular part can be cleaved and circulates in the blood, functioning as a hormone. Klotho is involved via FGF23 signaling (Kurosu et al. 2006) in maintaining calcium and phosphate homeostasis (Yoshida et al. 2002), and preventing ectopic calcification occuring with ageing. Mostly synthesized by the brain and the kidney (Kuro-o et al. 1997, Tohyama et al. 2004), but expressed in other tissues like gonads, its overexpression in the brain or the testis, solely, can prolong life span (Kurosu et al. 2005), while the mice deficient in Klotho are developing severe growth retardation and the life span does not exceed more than 2 months (Kuro – o et al. 1997). Klotho deficient mice are IGF-I deficient (Mori et al. 2000, Utsugi et al. 2000) complicating the explanation of impaired insulin/IGF-I signaling and the prolonged life span. A possible synthesis of the contradictory data present in the literature might be the balance between mild and severe IGF-I reduction observed in *C. elegans*: moderately decreased signaling prolongs life span, while severe reduction in IGF-I signaling during development prevents maturation of larval stage (Rusell and Kahn 2007). The fine tuning between GH, insulin/IGF-I signaling and Klotho is

still to be elucidated. One is certain: the endocrine regulation of ageing employs the circulating proteins to maintain the cross talk between the tissues and secures simultaneous ageing of the whole organism.

Monoaminergic innervation of the hypothalamus is shown to regulate hormonal secretion (Sonntag et al. 1982) and recently, a mouse deficient in dopamine D2 receptor synthesis showed a growth retardation phenotype **(**García-Tornadú et al. 2006**).** The particular role of each neurotransmitter is still to be elucidated.

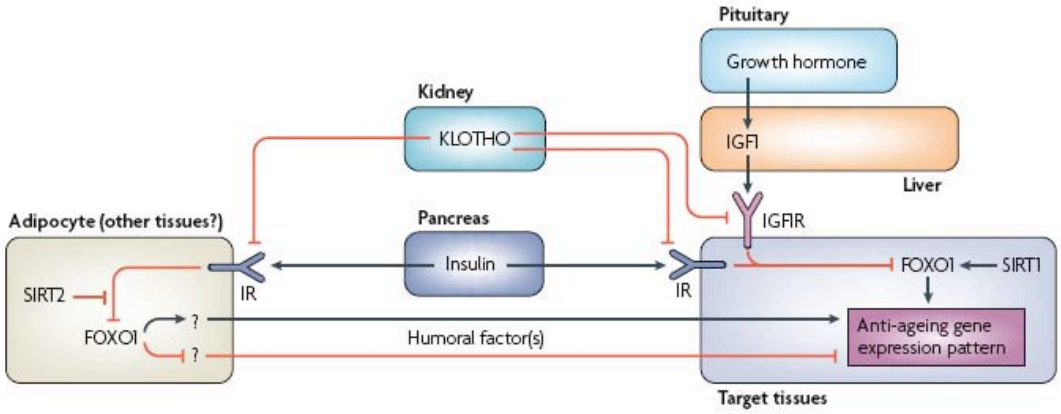


Figure 10.3. Cross - talk between tissues during ageing in mouse (Russel and Kahn 2007).

Does the lack of serotonin influence ageing? An interesting speculation appeared after the microarray analysis done on the brain samples of *Tph2*$^{-/-}$ animals. Klotho showed to be slightly downregulated in *Tph2*$^{-/-}$ animals (30%), even in the adult animals (for the microarray analysis, 4 months old females were used). Together with the observed impairment of IGF-I production (results Figure 8.7 and discussion 10.2.4), and the dwarf phenotype of *Tph2*$^{-/-}$ mice, it was reasonable to postulate an impairment in age related processes during the life span of serotonergic deficient animals. Therefore, we analysed the Ca^{2+}/PO^{4-} homeostasis and observed a slight elevation in Ca^{2+} blood concentration, as expected in Klotho deficient animals. Thus, all three parameters - downregulation of Klotho and IGF-I production, and impairment of ion homeostasis are showing a similarity to the Klotho deficient mouse model (Kur-o et al. 1997). Further investigations are required to prove a direct connection between the lack of serotonin, dwarfism and accelerated ageing. It will be very hard to dissect the age related phenomena and the spectrum of influences that serotonin deficiency causes.

Modulating the variety of stress stimuli, accelerated ageing in serotonin deficient mice may be caused by secondary effects - like depression, malnutrition or impaired neurogenesis and learning abilities. The requirement for extensive research in different areas of study, physiological and behavioural, as well as a high number of animals in different life stages will postpone the definite conclusion of the role of serotonin in ageing.

10.2.5. Cardiovascular parameters and serotonin: heart rate and blood pressure

In the CNS, serotonin is of major importance for cardiovascular control (reviewed in Ramage 2001, Jordan 2004). Considerable evidence supports a role of 5-HT1A receptors in the brain stem, in particular in centers involved in sympathetic regulation, which get strong serotonergic innervation from the dorsal group of *raphé nuclei*, B1 to B4. When these 5-HT1A receptors are activated, a fall in blood pressure is observed accompanied by a decrease in sympathetic nerve activity. Concurrently, centrally administered 5-HT1A agonists potentiate the vagal outflow to the heart (Jordan 2004). However, when these agonists are applied to forebrain nuclei, sympathoexcitation and hypertension is induced. These areas get their serotonergic input from the more rostral *raphé nuclei*, B5 to B9. To further complicate the issue, 5-HT2, 5-HT3, and 5-HT7 receptors have been shown to participate in central serotonergic regulation of the sympathetic and parasympathetic tone.

Our data clearly show that a ubiquitous lack of serotonin decreases blood pressure and heart rate at least at night-time probably by inhibition of the sympathetic and stimulation of the parasympathetic nervous system. The functional role of particular receptors and the cause for circadian variations in control of blood pressure and heart rate are still to be elucidated.

10.3. Fertility and maternal neglect

Fertility in the context of serotonergic deficiency may be discussed on genetic or behavioral level. Developmental role of serotonin is reviewed elsewhere (Gaspar et al. 2003) and is mostly focused on neuronal and behavioral alterations, but its involvement in sperm and oocyte maturation is not yet clear. 5-HT7 recoptors are involved in oocyte arrest (G2 phase) before maturation (Sheng et al. 2004). Depletion of 5-HT by pCPA treatment in rats (Aragõn et al. 2005) was accompanied by loss of germ cells in the testis followed by a decrease in sperm production. In addition, 5-HT stimulates sperm motility (Parisi et al. 1984). Since TPH2 is expressed in testis (Tenner 2008), the ability to produce offspring, and the lack of difference in the number of pups per litter even when both parents were lacking serotonin came as a surprise. However, the obvious aggressivity of *Tph2*$^{-/-}$ animals has been noticed to slow down the frequency of mating in *Tph2*$^{-/-}$ deficient breeding couples, and is influencing maternal behavior after the delivery.

Maternal care comprises a series of physiologicaly determined behavioral patterns such as nest building, pup retrieval, cleaning and nursing (Gammie 2005). Involvement of monoaminergic neurotransmitters in maternal behavior was acknowledged previously on *Dbh*$^{-/-}$ (Thomas and Palmiter 1997) and *Pet1*$^{-/-}$ (Lerch-Haner et al. 2008) mice models, lacking adrenergic neurotransmitters and most of serotonergic neurons, respectively. It has been shown that maternal neglect can go along with aggressiveness in mice (Takayanagi et al. 2005) and, indeed, we observed a more pronounced aggressive behaviour of female and male *Tph2* deficient mice compared to controls. Even females housed with *Tph2*$^{-/-}$ females were often wounded by fighting that never happens in control animals of the same genetic background. These observations are consistent with the hypothesis that increased aggression is associated with states of low serotonergic system activity (Popova 2006). We did not observe changes in milk production, or suckling behavior of the pups, excluding the possibilty that malnutrition in the first days after the delivery can be the cause for increased mortality of the pups born by *Tph2*$^{-/-}$ mothers. Maternal behavior in mouse depends on olfaction – removal of the olfactory bulbs in mice results in cannibalism or abandonment of newborns (Parks and Bruce 1961, Bowers and Alexander 1967). *Tph2*$^{-/-}$ mice did not show any differences in the time it took to find a hidden cookie (results by Valentina Mosienko). Therefore, the maternal neglect in *Tph2*$^{-/-}$ mothers might be influenced by changes

in behavior due to serotonergic deficiency, and not by any other physiological malfunction. Interestingly, the frequent cannibalism they show towards their own offspring is not noticed in *Dbh*$^{-/-}$ mothers or *Pet1*$^{-/-}$ mothers, suggesting that increased aggression may mostly influence maternal behavior in the case of *Tph2*$^{-/-}$ mice. It is noteworthy that the medial preoptic area of the hypothalamus (MPOA) has been shown to be a crucial center for the control of nurturing behavior (Numan et al. 1988). Thus, the impaired hypothalamo-hypophyseal axis in serotonergic deficient animals (disscussion 10.2.4) may be the physiological explanation behind this behavioral phenomena.

11. ESC AND IN VITRO DIFFERENTIATION

11.1. The failure of Tph2 "knock-in cassette"

Tph2 - neoR - IVS - IRES - dsRed2 - pA expression cassette, created to select serotonergic neurons during *in vitro* differentiation and after switching on the *Tph2* promoter, failed to show either neoR or dsRed positive selection in culture. We thought of several explanations of this failure.

dsRed is not visible under suboptimal excitation or expression conditions. We suspected that dsRed could not be visualized under the FACS conditions we used to detect dsRed positive 5-HT neurons in culture, as it uses suboptimal wavelength (488nm) for the fluorophore activation (www.clontech.com). We created a CMV-dsRed transgenic cell line (C3) as a positive control for detecting dsRed fluorescence (data not shown and materials 5.5). It appeared we had no problems in detecting dsRed when inserted as a transgenic construct. This, however, did not exclude the possibility that the "knock-in" construct expressed from one allele only provided insufficient amounts of dsRed protein in the cells for its efficient detection, or that weak dsRed signal is due to the weakness of *Tph2* promoter used in the construct.

IRES failed to drive polycistronic mRNA transcription or/and translation. The internal ribosomal entry site (IRES) found in viruses, but also some eukaryotic mRNA (Martinez-Salas E, 1999) enables the ribosomal machinery to drive the translation from the secondary translation site of the same transcript. It consists of repetitive AUG triplets, which are a signal for ribosome to begin translation. The secondary structure, however, defines which of the AUGs will be used, if any at all. It is shown that physicochemical properties of the cell like hypoxia, but also physiological, like cell cycle or growth factors signaling may all influence IRES efficiency (Martinez – Salas 1999). It is used in biotechnology to enable double selection (e.g antibiotic resistance and fluorescence selection) from the same transcript with a single promoter. Clontech, however, was providing the attenuated IRES sequence driving the second translation with lesser efficiency, and designed for FACS detection only (www.clontech.com).

Is there an internal promoter or regulatory element in the intronic sequence of Tph2 gene? When starting the work, there were no available studies of *Tph2* promoter activity in neuronal tissue. As a putative promoter sequence of *Tph2* gene we considered the region immediately upstream of the exon1 and the first ATG of the published *Tph2* sequence (GenBank). It was not excluded, however, that, by deleting the first and second intron of *Tph2* gene (methods 6.1.14.1), we interfered with an internal promoter or enhancer in one of those introns. Alternative splicing of the *Tph1* gene uncovered an alternative promoter sequence in Exon 2b and the intronic binding motifs in rats (Walther 2003) that might be conserved in the *Tph2* gene. A recent study, however, showed that all tested sequences longer then 1kb upstream of the *Tph2* gene were sufficient to drive 5-HT specific expression but the signal was undetectable without immunostaining (Bezekhroufa K et al. 2009). The authors then used a transcriptional activation strategy to drive 5-HT specific transgene expression and overcome the weakness of the cell specific promoter. Our targeting construct used the 10kb long sequence directly upstream of *Tph2* start codon, and, thus, it is unlikely that we interfered with this region. Most probably, as in the case of Bezekhroufa et al. the weakness of *Tph2* promoter was the reason for failure of our Tph2 "selection-expression" cassette.

11.2. Transgenic ePet line – an unknown in vitro attenuation event?

The capacity of an enhancer of the Pet1 gene (ePet) to drive expression of any downstream inserted gene was discovered recently and shown to be exclusive for serotonergic neurons (Scott et al. 2005, Jensen et al. 2008). We decided to use the sequence to drive EYFP expression in the serotonergic neurons during *in vitro* differentiation. We were not able to detect any positive signal at different time points of 5-HT differentiation, neither with 3kb ePet region, nor with 40kb original BAC transgene obtained from E. Deneris lab. The expression of *Pet1* gene can be seen as early as in D8 of embryoid bodies differentiation (EB8), and thus, cannot be the reason for ePet transgene failure. The results are in contradiction with the observed lacZ expression driven by 40kb ePet independent of position of integration in ePet transgenic mice created in Evan S. Deneris lab.

A mosaicism of the transgene expression triggered by epigenetic downregulation as described in Kaufman et al. 2008 may be the cause for the transgene expression failure. The authors additionally show that omitting of the antibiotic during the isolation of stably

transfected cell line may be the cause for loss of transgene expression (Kaufman et al. 2008). We selected both ePet transgenic cell lines with an efficient antibiotic concentration (see methods 6.1.14.2). However, when applying the antibiotics in culture after replating on PDL/Lam matrix to select for neuronal precursors, the cells were dying within 2 days, during the mitotic phase of the differentiation. At the same antibiotic concentration EBs and ES cells of the same clone were surviving indefinitely in culture. Thus, after the neuronal precursors were selected, the differentiation was performed without antibiotics. It might be that three factors, epigenetic downregulation, lack of antibiotic selection and low percentage of serotonergic neurons obtained during the differentiation were the cause for the failure of ePet transgenic construct. Until now, we were not able to solve the selection-expression problem during *in vitro* neuronal differentiation.

11.3. FunGenES cooperation – Sanofi Aventis "Tph2 cluster"

In parallel to our attempt to select serotonergic neurons in culture, we analyzed microarray data of other labs in FunGenES consortium. Following the course of differentiation, Laurent Pradier and his associates in Paris did a detailed microarray analysis of RA treated and untreated (control) EB differentiation at several time points. In this data set, we were able to reveal a cluster of genes containing several already known genes of the serotonergic phenotype: *Tph2*, FEV (*Pet1*), VMAT2 and SERT. We defined the cluster as "Tph2 cluster", expecting it to contain other genes involved in defining serotonergic neurons. We chose several additional genes and confirmed their co-expression with the serotonergic markers (*Tph2* and *Pet1*) during the differentiation in our culture. Thus, we confirmed the reproducibility of the system in different labs, and the scientific rationale behind the FunGenES cooperation. We, at the same time, discovered five genes (GCH1, ASB4, CACNA2D1, FoxA1 and Pldxc2) among the selected sharing the same pattern of expression as *Tph2* and *Pet1* (see below). The functional analysis of those genes was not possible anymore in the frame of this thesis.

11.3.1. ASB4

ASB4 (Ankyrin repeat and SOCS box-containing 4) belongs to the family of four similar proteins containing ankyrin repeats and SOCS box repeats (ASB1, ASB2, ASB3, ASB4). Based on its expression pattern, tissue distribution and colocalisation, it is most probably involved in energy homeostasis during differentiation processes of different tissues (www.bioportfolio.com). It is reported to be involved in a stage-specific manner in murine spermatogenesis (Kim et al. 2008), as well as in endothelial differentiation (Ferguson et al, 2007). High levels of ASB4 expression coincide with drastic increases in oxygen tension, followed by dramatic downregulation when oxygen levels stabilize (Ferguson et al. 2007). ASB4 itself is hydroxylated in a process of FIH (factor inhibiting HIF1 alpha) mediated hydroxylation via oxygen-dependent mechanism. Thus, ASB4 might serve as a regulator of oxygen and pH homeostasis during the differentiation *in vitro,* and its expression might not be restricted to 5-HT neurons, only. This possibility, however, is not excluded – ASB4 *in situ* hybridization (Allen Brain Atlas (www.brain-map.org)) was shown to collocate with *Tph2* expression in the raphe nuclei of an adult mouse brain. A more detailed analysis of oxygen state during 5-HT differentiation *in vitro* is needed to confirm the hypothesis. Despite the lack of evidence for exclusivity of ASB4 in defining the serotonergic phenotype, the gene remained the favorite candidate to this role among the genes selected from "Tph2 cluster".

11.3.2. CACNA2D1

Cacna2d1 (calcium channel, voltage dependent, alpha 2/delta subunit 1), is a voltage gated calcium channel found in brain and muscle tissue (Taylor and Garrido 2008). A2d1 stands for $\alpha 2 - \delta 1$ subunits that form this particular calcium channel (CACN). There are three additional members of the family $\alpha 2 - \delta 2\text{-}4$, that share the similar structure and glycosylation pattern (Taylor and Garrido 2008). Even though we could trace similar expression pattern of CACNA2D1 with serotonergic marker genes *Tph2* and *Pet1*, I would speculate that the role of calcium channel upregulation during neuronal differentiation *in vitro* is of more general nature. Most probably, the density and number of $\alpha 2 - \delta 1$ calcium channel subunits amplifies with the formation of neuronal network and new neuronal synapses and circuits. Moreover, the immunostaining of the rat brain showed dense staining of the subunit in regions others then raphe nuclei (Taylor and Garrido 2007), implying its role in the development of other cell types then 5-HT neurons. Its presence in *substantia nigra* and *stria*

terminalis (Taylor and Garrido, 2007) and a high percentage of TH positive neurons present during 5-HT differentiation (Kawasaki et al. 2000, Lee et al. 2000) might, actually, point to probable involvement of CACNA2D1 in dopaminergic development and signaling. The ability of α2 – δ1 and δ2 subunit to bind the drug pregabalin and gabapentin (Dooley et al. 2007) in contrast to δ3 and δ4 variants, could be used to investigate its eventual role in efficiency of 5 HT neuron generation in culture.

11.3.3. GCH1

GCH1 (GTP cyclohydrolase 1) is involved in biochemical synthesis of tetrahydrobiopterin (BH4). It catalyzes the first and rate-limiting step in *de novo* BH4 synthesis, using GTP as a precursor to produce dihydroneopterin triphosphate (Gross and Levi 1992). Subsequent metabolism of dihydroneopterin triphosphate leads to the production of BH4, most probably in two additional steps, although it is not certain if all the intermediates of the reaction are identified (Gross and Levi 1992). BH4 is a cofactor, involved in the activity of several enzymes including phenylalanine hydroxylase (PAH), tyrosine hydroxylase (TH) and tryptophan hydroxilase (TPH). Therefore, the upregulation of GCH1 during 5 HT differentiation *in vitro*, is expected, and follows the increasing expression pattern of *Tph2*.

11.3.4. FoxA1

FoxA1 (forkhead box A1 (hepatocyte nuclear factor 3α)) is a member of the forkhead family of transcription factors known to be involved in development and differentiation of several organs including liver, kidney, pancreas, lung, prostate and mammary glands (Lupien et al. 2008). It obviously presents a part of the transcriptional network responsible for activating cell type specific transcriptional programs. Recently, it has been discovered that FoxA1 interacts with histone H3, dependent of the dimethylation status at lysine 4, acting, thus, as a translator of epigenetic signature through the changes in chromatin structure to activate and establish lineage specific transcriptional programs (Lupien et al. 2008). In that sense, FoxA1 may be involved in defining serotonergic lineage in neuronal tissue. Considering the heterogeneity of the *in vitro* differentiation from ES cells, and the heterogeneity of the EBs containing all three germ layer until D8 of differentiation, I

would suspect preferable involvement of FoxA1 in other cell lineages than neuronal, as previously reported (Friedman and Kaestner 2006, Kouros-Mehr et al. 2006, Spear et al. 2006). Its significantly higher expression in several non-neuronal types of cancers (breast cancer Badve et al 2007, Wolf et al 2007, prostatic and ductal carcinomas (MeSH)) supports this hypothesis.

11.3.5. Plxdc2

Plxdc2 (plexin domain containing 2 (tumor endothelial marker 7-related TEM7R)) is a still uncharacterized transmembrane protein, expressed from mid-embryonic stages (E9.5 – E11.5) of mouse development (Miller et al. 2006). Its expression in the brain, particularly midbrain – hindbrain boundary and midbrain floorplate could point to its involvement in definition of the serotonergic lineage, at least in the early stages. However, in E15.5, *Plxdc2* is observed throughout the brain and in the neuroepithelium. Present in all areas of the brain, from *choroid plexus*, through *dentate gyrus* of hippocampus and the floorplate in the hindbrain, *Plxdc2* most probably presents a common transmembrane protein involved in the support of neuronal cells networking, signaling and organization. Its abundance in Purkinje neurons of cerebellum is pointing to an unknown additional function, whereas its presence in glial wedge and fimbria (Miller et al. 2006) is showing their, at least partly, glial nature. *Plxdc2* expression is not restricted to the brain only – *in situ* hybridization showed its presence in the limbs, lung buds and developing heart (Miller et al, 2006), excluding the specific 5-HT or even neuronal phenotype characterization. *Plxdc2* (TEM7R) was characterized as an overexpressed transcript in the blood vessels of human solid tumors (Nanda et al. 2004). Thus, its putative role, and specificity of its function might be regarded as a part of multiple signaling cascade in which this transmembrane protein might be involved.

11.3.6. 3100002J23RIK

Several protein-coding genes with unknown function including 3100002J23RIK, were putative choices for the analysis of similarity of pattern with *Tph2* and *Pet1* gene. Most of them, however, did not share the similarity of expression with *Tph2*, as compared in the Allen Brain Atlas (www.brain-map.org). Therefore, we chose the only RIKEN sequence with unknown pattern (*in situ* hybridyzation of sagittal sections was inconclusive), to test the

possible serotonergic expression pattern. It appeared it was one of the rare genes we selected that did not have anything in common with the observed expression of the investigated genes, and was obviously belonging to another cluster of neuronal or glial phenotype present in culture. Together with very restricted expression and functional information present in the scientific literature and, including the failure of producing a knock out model for this gene (The Jackson Laboratories), we were not able to conclude any putative role for the 3100002J23RIK from "Tph2 cluster".

11.3.7. Egr2

Egr2 (early growth response 2) is a transcription factor involved in the rostral patterning of the hindbrain (Wassef et al. 2008), expressed exclusively in rhombomeres r3 and r5. Its regulation and function is rather complex, besides hindbrain patterning, involving the development of rhythmical respiratory network in the hindbrain (Chatonnet et al. 2007), active gene repression during myelination (Mager et al. 2008) or induction in the forebrain during attention-set-shifting tasks (DeSteno and Schmauss 2008). Recently, Patricia Jensen in collaboration with Evan S. Deneris showed the possibility of using the *Egr2* gene in tracing the serotonergic neurons derived from r3 and r5, by using special genetic tools combined with the methodology of intersectional and subtractive genetic fate (for details see Jensen P and al., 2008). Serotonergic neurons are very heterogeneous. They are anatomically clustered in different *raphe nuclei*, but also show different morphology and neurotoxin sensitivity and are implicated in variety of clinical disorders. Genetic tracing showed that 5-HT neurons located in different *raphe nuclei* belong to different genetic lineages during development. These lineages are not in concordance with their anatomical distribution, as, for example 5-HT neurons in B9, B8 and B5 are derived from different rhombomeres during development (Jensen et al. 2008). We showed that the level of expression and the pattern of *Egr2* followed the peaks of *Tph2* and *Pet1* expression during 5-HT differentiation, thus showing the presence of 5-HT neurons in culture that follow the genetic lineage of r3/r5 origin. The idea to trace 5-HT neurons originating from r2, using *Rse2* as a genetic marker, failed due to technical difficulties. Previously, we showed the expression of *En1*, as one of genetic markers for r1 derived neurons by RT-PCR (results Figure 9.11). Thus, the heterogeneity of the 5-HT neurons forming during 15 days differentiation from ES cells in culture is astonishingly recapitulating the heterogeneity of 5-HT neurons clustered in raphe nuclei *in vivo*.

V CONCLUSION AND PERSPECTIVES

The role of serotonin in autonomous processes, such as breathing, heart rate or thermoregulation, was postulated earlier and recently confirmed in other experimental models (Hodges et al. 2008, Erickson 2007). We confirmed these findings in *Tph2* deficient animals.

A crucial role of serotonin in sleeping dynamics was discovered. A role of peripheral serotonin in sleep, synthesized by *Tph1* as an intermediate of melatonin synthesis, was observed previously (Benloucif et al. 2005) and already clinically used to treat sleep disorders (Lewy et al. 1992, Smits et al. 2003). The mechanism underlying the observation of prolonged sleeping time in *Tph2* deficient animals is still to be elucidated.

The importance the central serotonin exerts on the HPA was also highlighted during the course of this work. The full importance and the spectrum of physiological changes caused by dysfunctional HPA are under investigation. The secondary effects of the ion and hormonal homeostasis disbalance will require long time observations and detailed investigation.

Serotonin has only a modulatory role in the brain since its depletion caused only mild phenotypes. Neither gross morphological changes nor developmental changes were, until now, observed. Most probably, the deficiency of brain serotonin will be shown to be important under stress conditions and in disease. The higher lethality of *Tph2* deficient animals may be caused by the slower respiratory rhythm in the early life, but the penetrance of the phenotype is never exceeding 50% of the animals born. Any other putatively lethal physiological changes caused by the serotonin depletion in the brain are still to be elucidated.

Future of Tph2 $^{-/-}$ animals. Differences in immune response and immunomodulation might be present in *Tph2* deficient animals in accordance with the observed autonomous physiological changes and impaired HPA. The role of brain serotonin levels in disease, such as Huntington chorea, may be concluded using *Tph2*$^{-/-}$ animals crossbred with mice models for Huntington´s disease (Kish et al. 1987, Reynolds et al. 1999). Other neurodegenerative and neuropsychiatric processes, such as anxiety, depression and compulsive disorders could be studied on this mouse model. New treatment options and applications of newly discovered

and commercially available drugs may be studied. *Tph2* deficient mice may be of importance in discovering the pathways of drugs of abuse, known to be involving the serotonergic brain system. The future crossbreeding of *Tph2*$^{-/-}$ and *Tph1*$^{-/-}$ animals created in our lab may give an insight in the phenotypic effects resulting from simultaneous central and peripheral serotonin deficiency. Possible complementation mechanisms, most probably involving the serotonin synthesis by other hydroxylases, such as PAH, will be investigated. Most probably, as there is no observed high lethality of the serotonin deficient animals in the case of double KO (our observation and Savelieva et al. 2008), the complementation involves not only the synthesis, but also the signaling cascade. It is known, for example, that dopamine is weakly binding the serotonergic receptor 5-HT3A involved in fast synaptic transmission (Werkman et al. 2006). The possibility that downstream molecules of signaling cascade, such as DARPP32 (Svenningsson et al. 2005), are taking over the signal transmission is not to be neglected. That, and other questions, opened during the phenotyping of *Tph2*$^{-/-}$ animals, like the physiological mechanism behind the maternal neglect behavior and the role of serotonin in early and late development, is still to be addressed. Creating the animal model with almost complete deficiency of serotonin in the brain, we hope to contribute to the investigation of the wide spectrum of effects this molecule exerts in mammals.

An efficient protocol of serotonergic differentiation developed partly during the course of this study, as well as efficient 5-HT neurons selection *in vitro* by using additional strategies such as one suggested recently by Benzekhroufa and Kasparov (Benzekhroufa et al. 2009) may open an exciting possibility of cell replacement therapy of serotonergic neurons and follow up studies in animals deficient in central serotonin synthesis. Eventually, we may be able to select a pure population of 5-HT neurons, and re-introduce them in the brain of *Tph2* deficient mice, thus, proving also their functionality *in vivo*. An additional goal, the discovery of unknown genes involved in the serotonergic development and profiling of serotonergic phenotype, might also be achieved.

Is there life without serotonin? Unfortunately, we still do not know. Apparently, there are complementary mechanisms responsible for serotonin synthesis even in the case of both tryptophan hydroxylase deficiency (Savalieva et al. 2008) suggesting a protective evolutionary mechanism involving the monoaminergic system as a whole, and, thus, pointing to the importance of monoamines in survival. Further cross-breeding of mouse lineages to

obtain the offspring deficient in multiple hydroxylases may give the answer to the question we posed before starting this work.

VI SUMMARY

Tryptophan hydroxylase (TPH) catalyzes the rate-limiting step of the synthesis of serotonin. Recently, it was discovered that in mammals, serotonin synthesis is initiated by two distinct tryptophan hydroxylases, TPH1 and TPH2. It was postulated that TPH1 is responsible for serotonin synthesis in peripheral tissues, whereas TPH2 synthesized serotonin within the central nervous system.

By genetically ablating TPH2, we created mice (*Tph2*$^{-/-}$ mice) which lack serotonin in the central nervous system. Thus, we confirmed that the majority of central serotonin is generated by TPH2.

Tph2-/- mice can be born and survive until adulthood. Depletion of serotonin signaling in the brain leads to growth retardation and 50% of lethality in the first four weeks of postnatal life.

The role of central serotonin in autonomous processes is revealed. Telemetric monitoring revealed more extended daytime sleep, suppressed respiration, altered body temperature control and decreased blood pressure and heart rate during nighttime in *Tph2*$^{-/-}$ animals.

The importance the central serotonin exerts on hypothalamo-pituitary axis (HPA) was also highlighted during the course of the study. The full spectrum of physiological changes caused by dysfunctional HPA requires further investigation and will be done on our model.

Tph2$^{-/-}$ *females, despite being fertile and producing milk, exhibit impaired maternal care* leading to poor survival of their pups.

TPH2 derived serotonin has a modulatory role in the brain, and is not essential for adult life. It is, however, involved in the regulation of behavioral and autonomic pathways.

We developed and characterized an efficient protocol of serotonergic differentiation from embryonic stem cells (ESC) during the course of the study. We proved the reproducibility of the system in different laboratories and confirmed the possibility of standardization of ESC differentiation. The protocol will be used to eventually select a pure population of 5-HT neurons differentiated *in vitro*. These neurons may be re-introduced in the brain of *Tph2*$^{-/-}$ mice, opening an exciting possibility of cell replacement therapy for central serotonergic system. An additional goal, the discovery of unknown genes involved in the serotonergic development and profiling of serotonergic phenotype, might also be achieved.

ZUSAMMENFASSUNG

Tryptophan-Hydroxylase (TPH) katalysiert den geschwindigkeitsbestimmenden Schritt bei der Synthese von Serotonin. Vor kurzem wurde entdeckt, dass die Serotoninsynthese bei Säugetiere von 2 bestimmten Tryptophan-Hydroxylasen, TPH1 und TPH2, ausgelöst wird. Es wurde angenommen, dass TPH1 für die Serotoninsynthese in peripheren Geweben zuständig ist, wohin gegen TPH2 Serotonin im zentralen Nervensystem synthesiert.

Durch die genetische Abtragung von TPH2 gelang es uns Mäuse (Tph2$^{-/-}$ Mäuse), denen Serotonin im zentralen Nervensystem fehlte, zu schaffen und hierdurch zu bestätigten, dass der Großteil des zentralen Serotonins von TPH2 erzeugt wird.

Tph2$^{-/-}$ *Mäuse können geboren werden* und bis zum Erwachsenenalter überleben. Der Schwund von Serotoninsignalen im Gehirn führt allerdings zu Wachstumsrückstand und 50% Letalität in den ersten 4 Wochen nach der Geburt.

Während der Dauer unserer Arbeit ist es uns gelungen, die Funktion des zentralen Serotonins in eigenständigen Prozessen zu enthüllen. Durch telemetrisches Monitoring konnten wir ausgedehnte Tagesschlaf, unterdrückte Atmung, veränderte Körpertemperaturkontrolle und verminderten Blutdruck und Herzschlag, währen der Nachzeit, bei $Tph2^{-/-}$ -Tieren beobachten.

Zusätzlich gelang es uns die Wichtigkeit des zentralen Serotonins für die Hypothalamo-hypophysäre Schilddrüsen-Achse (HPA), im Laufe unserer Untersuchung hervorzuheben.

Um das volle Spektrum der physiologischen Veränderungen, verursacht durch eine dysfunktionale HPA, zu erfassen, werden weitere Untersuchung benötigt, welche auf der Basis unseres Modells stattfinden sollen.

Von TPH2 stammendes Serotonin hat eine regulierende Rolle im Gehirn und ist nicht essentielle fürs Erwachsenenleben. Allerdings ist es involviert in die Regulierung von Verhalten und autonomen Nervenbahnen. $Tph2^{-/-}$ Weibchen weisen, trotz Ihrer Fruchtbarkeit und er Fähigkeit Milch zu produzieren, geminderte Mutterinstinkte auf, welche zu einer niedrigen Überlebensrate Ihrer Jungen führt.

Im Laufe meiner Arbeit entwickelten und charakterisierten wir eine effiziente Methode, zur serotoninergen Differenzierungen embryonaler Stammzellen (ES-Zellen). Wir belegten die Reproduzierbarkeit des Systems und bestätigten die Möglichkeit der Standardisierung von ES-Zellen-Differenzierung.

Unsere Methode wird schlussendlich genutzt werden um eine reine Population von 5-HT Neuronen im Reagenzglas zu selektieren. Diese Neuronen können dann in das Gehirn von $Tph2^{-/-}$ Mäusen evtl. wiedereingesetzt werden und so eine vielversprechende Möglichkeit zur Therapie des zentralen serotonergen Systems eröffnen. Ein zusätzliches Ziel, die Entdeckung unbekannter Gene, involviert in die serotonerge Entwicklung und die Profilierung serotonerger Phänotypen, könnte so ebenso erreicht werden.

VII BIBLIOGRAPHY

Abate,C., Smith,J.A., & Joh,T.H. Characterization of the catalytic domain of bovine adrenal tyrosine hydroxylase. *Biochem. Biophys. Res. Commun.* **151**, 1446-1453 (1988).

Abbott, N. J., Ronnback, L. and Hansson E. Astrocyte-endothelial interactions at the blood-brain barrierr, Nat. Rev. Neurosci. (Band 7), Nr. 1, 41-53 (2006)

Abumaria,N., Ribic,A., Anacker,C., Fuchs,E., & Flugge,G. Stress upregulates TPH1 but not TPH2 mRNA in the rat dorsal raphe nucleus: identification of two TPH2 mRNA splice variants. *Cell Mol. Neurobiol.* **28**, 331-342 (2008).

Alenina,N., Bashammakh,S., & Bader,M. Specification and differentiation of serotonergic neurons. *Stem Cell Rev.* **2**, 5-10 (2006).

Alvarez,C. *et al.* Effects of genetic depletion of monoamines on somatosensory cortical development. *Neuroscience* **115**, 753-764 (2002).

Aragon,M.A. *et al.* Serotoninergic system blockage in the prepubertal rat inhibits spermatogenesis development. *Reproduction.* **129**, 717-727 (2005).

Arranz,B., Eriksson,A., Mellerup,E., Plenge,P., & Marcusson,J. Effect of aging in human cortical pre- and postsynaptic serotonin binding sites. *Brain Res.* **620**, 163-166 (1993).

Aston-Jones,G. & Bloom,F.E. Activity of norepinephrine-containing locus coeruleus neurons in behaving rats anticipates fluctuations in the sleep-waking cycle. *J. Neurosci.* **1**, 876-886 (1981).

Audero,E. *et al.* Sporadic autonomic dysregulation and death associated with excessive serotonin autoinhibition. *Science* **321**, 130-133 (2008).

Austin,M.C. & O'Donnell,S.M. Regional distribution and cellular expression of tryptophan hydroxylase messenger RNA in postmortem human brainstem and pineal gland. *J. Neurochem.* **72**, 2065-2073 (1999).

Azmitia,E.C. & Whitaker-Azmitia,P.M. Awakening the sleeping giant: anatomy and plasticity of the brain serotonergic system. *J. Clin. Psychiatry* **52 Suppl**, 4-16 (1991).

Azmitia,E.C. Modern views on an ancient chemical: serotonin effects on cell proliferation, maturation, and apoptosis. *Brain Res. Bull.* **56**, 413-424 (2001).

Azmitia,E.C. Serotonin and brain: evolution, neuroplasticity, and homeostasis. *Int. Rev. Neurobiol.* **77**, 31-56 (2007).

Bach,K.B. & Mitchell,G.S. Hypoxia-induced long-term facilitation of respiratory activity is serotonin dependent. *Respir. Physiol* **104**, 251-260 (1996).

Bacon,S.J., Zagon,A., & Smith,A.D. Electron microscopic evidence of a monosynaptic pathway between cells in the caudal raphe nuclei and sympathetic preganglionic neurons in the rat spinal cord. *Exp. Brain Res.* **79**, 589-602 (1990).

Badve,S. *et al.* FOXA1 expression in breast cancer--correlation with luminal subtype A and survival. *Clin. Cancer Res.* **13**, 4415-4421 (2007).

Bain,G., Kitchens,D., Yao,M., Huettner,J.E., & Gottlieb,D.I. Embryonic stem cells express neuronal properties in vitro. *Dev. Biol.* **168**, 342-357 (1995).

Baker,T.L. & Mitchell,G.S. Episodic but not continuous hypoxia elicits long-term facilitation of phrenic motor output in rats. *J. Physiol* **529 Pt 1**, 215-219 (2000).

Balkowiec,A. & Katz,D.M. Brain-derived neurotrophic factor is required for normal development of the central respiratory rhythm in mice. *J. Physiol* **510 (Pt 2)**, 527-533 (1998).

Baluda,V.P., Dorofeev,V.M., & Khnychev,S.S. [Effect of endogenous and exogenous serotonin on hemostasis]. *Biull. Eksp. Biol. Med.* **68**, 17-19 (1969).

Barbosa,R.M. *et al.* Control of pulsatile 5-HT/insulin secretion from single mouse pancreatic islets by intracellular calcium dynamics. *J. Physiol* **510 (Pt 1)**, 135-143 (1998).

Barnes,N.M. & Sharp,T. A review of central 5-HT receptors and their function. *Neuropharmacology* **38**, 1083-1152 (1999).

Basbaum,A.I., Clanton,C.H., & Fields,H.L. Three bulbospinal pathways from the rostral medulla of the cat: an autoradiographic study of pain modulating systems. *J. Comp Neurol.* **178**, 209-224 (1978).

Bellivier,F. *et al.* Serotonin transporter gene polymorphisms in patients with unipolar or bipolar depression. *Neurosci. Lett.* **255**, 143-146 (1998).

Bengel,D. *et al.* Altered brain serotonin homeostasis and locomotor insensitivity to 3, 4-methylenedioxymethamphetamine ("Ecstasy") in serotonin transporter-deficient mice. *Mol. Pharmacol.* **53** , 649-655 (1998).

Benington,J.H. & Heller,H.C. Restoration of brain energy metabolism as the function of sleep. *Prog. Neurobiol.* **45**, 347-360 (1995).

Benloucif,S. *et al.* Responsiveness of the aging circadian clock to light. *Neurobiol. Aging* **27**, 1870-1879 (2006).

Benzekhroufa,K., Liu,B.H., Teschemacher,A.G., & Kasparov,S. Targeting central serotonergic neurons with lentiviral vectors based on a transcriptional amplification strategy. *Gene Ther.* **16**, 681-688 (2009).

Birnboim,H.C. & Doly,J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7**, 1513-1523 (1979).

Blaugrund,E. *et al.* Distinct subpopulations of enteric neuronal progenitors defined by time of development, sympathoadrenal lineage markers and Mash-1-dependence. *Development* **122**, 309-320 (1996).

Boadle-Biber,M.C. Regulation of serotonin synthesis. *Prog. Biophys. Mol. Biol.* **60**, 1-15 (1993).

Bosin,T.R., Campaigne,E., Dinner,A., Rogers,R.B., & Maickel,R.P. Comparative toxicological studies of indole, benzo[b] thiophene, and 1-methylindole derivatives. *J. Toxicol. Environ. Health* **1**, 515-520 (1976).

Bou-Flores,C. & Hilaire,G. 5-Hydroxytryptamine(2A) and 5-hydroxytryptamine(1B) receptors are differently affected by the monoamine oxidase A-deficiency in the Tg8 transgenic mouse. *Neurosci. Lett.* **296**, 141-144 (2000).

Bou-Flores,C. *et al.* Abnormal phrenic motoneuron activity and morphology in neonatal monoamine oxidase A-deficient transgenic mice: possible role of a serotonin excess. *J. Neurosci.* **20**, 4646-4656 (2000).

Boularand,S., Darmon,M.C., Ganem,Y., Launay,J.M., & Mallet,J. Complete coding sequence of human tryptophan hydroxylase. *Nucleic Acids Res.* **18**, 4257 (1990).

Boularand,S., Darmon,M.C., & Mallet,J. The human tryptophan hydroxylase gene. An unusual splicing complexity in the 5'-untranslated region. *J. Biol. Chem.* **270**, 3748-3756 (1995).

Boularand,S. *et al.* Tyrosine hydroxylase in the european eel (Anguilla anguilla): cDNA cloning, brain distribution, and phylogenetic analysis. *J. Neurochem.* **71**, 460-470 (1998).

Boutrel,B., Monaca,C., Hen,R., Hamon,M., & Adrien,J. Involvement of 5-HT1A receptors in homeostatic and stress-induced adaptive regulations of paradoxical sleep: studies in 5-HT1A knock-out mice. *J. Neurosci.* **22**, 4686-4692 (2002).

Bowers,J.M. & Alexander,B.K. Mice: individual recognition by olfactory cues. *Science* **158**, 1208-1210 (1967).

Bradford,M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254 (1976).

Briscoe,J. *et al.* Homeobox gene Nkx2.2 and specification of neuronal identity by graded Sonic hedgehog signalling. *Nature* **398**, 622-627 (1999).

BRODIE,B.B. & COSTA,E. Some current views on brain monoamines. *Psychopharmacol. Serv. Cent. Bull.* **2**, 1-25 (1962).

Brodski,C. *et al.* Location and size of dopaminergic and serotonergic cell populations are controlled by the position of the midbrain-hindbrain organizer. *J. Neurosci.* **23**, 4199-4207 (2003).

Brunet,J.F. & Pattyn,A. Phox2 genes - from patterning to connectivity. *Curr. Opin. Genet. Dev.* **12**, 435-440 (2002).

Brunner,D., Buhot,M.C., Hen,R., & Hofer,M. Anxiety, motor activation, and maternal-infant interactions in 5HT1B knockout mice. *Behav. Neurosci.* **113**, 587-601 (1999).

Burnet,H. *et al.* Altered respiratory activity and respiratory regulations in adult monoamine oxidase A-deficient mice. *J. Neurosci.* **21**, 5212-5221 (2001).

Callahan,T., Young,H.M., Anderson,R.B., Enomoto,H., & Anderson,C.R. Development of satellite glia in mouse sympathetic ganglia: GDNF and GFR alpha 1 are not essential. *Glia* **56**, 1428-1437 (2008).

Cano,G. *et al.* Anatomical substrates for the central control of sympathetic outflow to interscapular adipose tissue during cold exposure. *J. Comp Neurol.* **460**, 303-326 (2003).

Canossa,M., Giordano,E., Cappello,S., Guarnieri,C., & Ferri,S. Nitric oxide down-regulates brain-derived neurotrophic factor secretion in cultured hippocampal neurons. *Proc. Natl. Acad. Sci. U. S. A* **99**, 3282-3287 (2002).

Cases,O. *et al.* Aggressive behavior and altered amounts of brain serotonin and norepinephrine in mice lacking MAOA. *Science* **268**, 1763-1766 (1995).

Cases,O. *et al.* Lack of barrels in the somatosensory cortex of monoamine oxidase A-deficient mice: role of a serotonin excess during the critical period. *Neuron* **16**, 297-307 (1996).

Cases,O. *et al.* Plasma membrane transporters of serotonin, dopamine, and norepinephrine mediate serotonin accumulation in atypical locations in the developing brain of monoamine oxidase A knock-outs. *J. Neurosci.* **18**, 6914-6927 (1998).

Cash,C.D. Why tryptophan hydroxylase is difficult to purify: a reactive oxygen-derived species-mediated phenomenon that may be implicated in human pathology. *Gen. Pharmacol.* **30**, 569-574 (1998).

Cassone,V.M. Effects of melatonin on vertebrate circadian systems. *Trends Neurosci.* **13**, 457-464 (1990).

Cerqueira,J.J., Mailliet,F., Almeida,O.F., Jay,T.M., & Sousa,N. The prefrontal cortex as a key target of the maladaptive response to stress. *J. Neurosci.* **27**, 2781-2787 (2007).

Chambers,I. The molecular basis of pluripotency in mouse embryonic stem cells. *Cloning Stem Cells* **6**, 386-391 (2004).

Champier,J. *et al.* Evidence for tryptophan hydroxylase and hydroxy-indol-O-methyl-transferase mRNAs in human blood platelets. *Life Sci.* **60**, 2191-2197 (1997).

Chatonnet,F. *et al.* Distinct roles of Hoxa2 and Krox20 in the development of rhythmic neural networks controlling inspiratory depth, respiratory frequency, and jaw opening. *Neural Dev.* **2**, 19 (2007).

Chemelli,R.M. *et al.* Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. *Cell* **98**, 437-451 (1999).

Cheng,L. *et al.* Lmx1b, Pet-1, and Nkx2.2 coordinately specify serotonergic neurotransmitter phenotype. *J. Neurosci.* **23**, 9961-9967 (2003).

Chou,T.C. *et al.* Critical role of dorsomedial hypothalamic nucleus in a wide range of behavioral circadian rhythms. *J. Neurosci.* **23**, 10691-10702 (2003).

Chung,Y.I., Park,D.H., Kim,M., Baker,H., & Joh,T.H. Immunochemical characterization of brain and pineal tryptophan hydroxylase. *J. Korean Med. Sci.* **16**, 489-497 (2001).

Clarke,A.R. Manipulating the germline: its impact on the study of carcinogenesis. *Carcinogenesis* **21**, 435-441 (2000).

Coleman,C.M. & Neckameyer,W.S. Serotonin synthesis by two distinct enzymes in Drosophila melanogaster. *Arch. Insect Biochem. Physiol* **59**, 12-31 (2005).

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

Compan,V., Scearce-Levie,K., Crosson,C., Daszuta,A., & Hen,R. Enkephalin contributes to the locomotor stimulating effects of 3,4-methylenedioxy-N-methylamphetamine. *Eur. J. Neurosci.* **18**, 383-390 (2003).

Consolazione, A. and Cuello, A.C. CNS serotonin pathways, The biology of serotonergic transmission 29-61, John Wiley & Sons, New York.

Conti,L. *et al.* Niche-independent symmetrical self-renewal of a mammalian tissue stem cell. *PLoS. Biol.* **3**, e283 (2005).

Coon,S.L. *et al.* The human serotonin N-acetyltransferase (EC 2.3.1.87) gene (AANAT): structure, chromosomal localization, and tissue expression. *Genomics* **34**, 76-84 (1996).

Copeland,K.C., Colletti,R.B., Devlin,J.T., & McAuliffe,T.L. The relationship between insulin-like growth factor-I, adiposity, and aging. *Metabolism* **39**, 584-587 (1990).

Cordes,S.P. Molecular genetics of the early development of hindbrain serotonergic neurons. *Clin. Genet.* **68**, 487-494 (2005).

Coschigano,K.T., Clemmons,D., Bellush,L.L., & Kopchick,J.J. Assessment of growth parameters and life span of GHR/BP gene-disrupted mice. *Endocrinology* **141**, 2608-2613 (2000).

Cote,F. *et al.* Disruption of the nonneuronal tph1 gene demonstrates the importance of peripheral serotonin in cardiac function. *Proc. Natl. Acad. Sci. U. S. A* **100**, 13525-13530 (2003).

Craven,S.E. *et al.* Gata2 specifies serotonergic neurons downstream of sonic hedgehog. *Development* **131**, 1165-1173 (2004).

Dahlstrom,A. & Fuxe,K. Localization of monoamines in the lower brain stem. *Experientia* **20**, 398-399 (1964).

Datta,S. & Maclean,R.R. Neurobiological mechanisms for the regulation of mammalian sleep-wake behavior: reinterpretation of historical evidence and inclusion of contemporary cellular and molecular evidence. *Neurosci. Biobehav. Rev.* **31**, 775-824 (2007).

Daubner,S.C., Lohse,D.L., & Fitzpatrick,P.F. Expression and characterization of catalytic and regulatory domains of rat tyrosine hydroxylase. *Protein Sci.* **2**, 1452-1460 (1993).

Delarue,C., Becquet,D., Idres,S., Hery,F., & Vaudry,H. Serotonin synthesis in adrenochromaffin cells. *Neuroscience* **46**, 495-500 (1992).

DeSteno,D.A. & Schmauss,C. Induction of early growth response gene 2 expression in the forebrain of mice performing an attention-set-shifting task. *Neuroscience* **152**, 417-428 (2008).

Deurveilher,S. & Semba,K. Indirect projections from the suprachiasmatic nucleus to major arousal-promoting cell groups in rat: implications for the circadian control of behavioural state. *Neuroscience* **130**, 165-183 (2005).

DiMicco,J.A., Samuels,B.C., Zaretskaia,M.V., & Zaretsky,D.V. The dorsomedial hypothalamus and the response to stress: part renaissance, part revolution. *Pharmacol. Biochem. Behav.* **71**, 469-480 (2002).

DiMicco,J.A. & Zaretsky,D.V. The dorsomedial hypothalamus: a new player in thermoregulation. *Am. J. Physiol Regul. Integr. Comp Physiol* **292**, R47-R63 (2007).

Ding,Y.Q. *et al.* Lmx1b is essential for the development of serotonergic neurons. *Nat. Neurosci.* **6**, 933-938 (2003).

Doetschman,T. *et al.* Targetted correction of a mutant HPRT gene in mouse embryonic stem cells. *Nature* **330**, 576-578 (1987).

Doetschman,T.C., Eistetter,H., Katz,M., Schmidt,W., & Kemler,R. The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *J. Embryol. Exp. Morphol.* **87**, 27-45 (1985).

Dolberg,O.T., Iancu,I., Sasson,Y., & Zohar,J. The pathogenesis and treatment of obsessive-compulsive disorder. *Clin. Neuropharmacol.* **19**, 129-147 (1996).

Dooley,D.J., Taylor,C.P., Donevan,S., & Feltner,D. Ca2+ channel alpha2delta ligands: novel modulators of neurotransmission. *Trends Pharmacol. Sci.* **28**, 75-82 (2007).

Dumas,S., Darmon,M.C., Delort,J., & Mallet,J. Differential control of tryptophan hydroxylase expression in raphe and in pineal gland: evidence for a role of translation efficiency. *J. Neurosci. Res.* **24**, 537-547 (1989).

Dyck,L.E., Kazakoff,C.W., & Dourish,C.T. The role of catecholamines, 5-hydroxytryptamine and m-tyramine in the behavioural effects of m-tyrosine in the rat. *Eur. J. Pharmacol.* **84**, 139-149 (1982).

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

Eaton,M.J. *et al.* Distribution of aromatic L-amino acid decarboxylase mRNA in mouse brain by in situ hybridization histology. *J. Comp Neurol.* **337**, 640-654 (1993).

Edagawa,Y., Saito,H., & Abe,K. Endogenous serotonin contributes to a developmental decrease in long-term potentiation in the rat visual cortex. *J. Neurosci.* **21**, 1532-1537 (2001).

Epelbaum,J. Neuroendocrinology and aging. *J. Neuroendocrinol.* **20**, 808-811 (2008).

Erickson,J.T. *et al.* Mice lacking brain-derived neurotrophic factor exhibit visceral sensory neuron losses distinct from mice lacking NT4 and display a severe developmental deficit in control of breathing. *J. Neurosci.* **16**, 5361-5371 (1996).

Erickson,J.T., Shafer,G., Rossetti,M.D., Wilson,C.G., & Deneris,E.S. Arrest of 5HT neuron differentiation delays respiratory maturation and impairs neonatal homeostatic responses to environmental challenges. *Respir. Physiol Neurobiol.* **159**, 85-101 (2007).

ERSPAMER,V. & ASERO,B. Identification of enteramine, the specific hormone of the enterochromaffin cell system, as 5-hydroxytryptamine. *Nature* **169**, 800-801 (1952).

Evans,M.J. & Kaufman,M.H. Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292**, 154-156 (1981).

Fekkes,D., Timmerman,L., & Pepplinkhuizen,L. Effects of clomipramine on plasma amino acids and serotonergic parameters in panic disorder and depression. *Eur. Neuropsychopharmacol.* **7**, 235-239 (1997).

Feldman,J.L., Mitchell,G.S., & Nattie,E.E. Breathing: rhythmicity, plasticity, chemosensitivity. *Annu. Rev. Neurosci.* **26**, 239-266 (2003).

Ferguson,J.E., III *et al.* ASB4 is a hydroxylation substrate of FIH and promotes vascular differentiation via an oxygen-dependent mechanism. *Mol. Cell Biol.* **27**, 6407-6419 (2007).

Finkelstein,J.W., Roffwarg,H.P., Boyar,R.M., Kream,J., & Hellman,L. Age-related change in the twenty-four-hour spontaneous secretion of growth hormone. *J. Clin. Endocrinol. Metab* **35**, 665-670 (1972).

Finocchiaro,L.M. *et al.* Serotonin and melatonin synthesis in peripheral blood mononuclear cells: stimulation by interferon-gamma as part of an immunomodulatory pathway. *J. Interferon Res.* **8**, 705-716 (1988).

Fiorica-Howells,E., Hen,R., Gingrich,J., Li,Z., & Gershon,M.D. 5-HT(2A) receptors: location and functional analysis in intestines of wild-type and 5-HT(2A) knockout mice. *Am. J. Physiol Gastrointest. Liver Physiol* **282**, G877-G893 (2002).

Flurkey,K., Papaconstantinou,J., Miller,R.A., & Harrison,D.E. Lifespan extension and delayed immune and collagen aging in mutant mice with defects in growth hormone production. *Proc. Natl. Acad. Sci. U. S. A* **98**, 6736-6741 (2001).

Fon,E.A. *et al.* Vesicular transport regulates monoamine storage and release but is not essential for amphetamine action. *Neuron* **19**, 1271-1283 (1997).

Fornal,C., Auerbach,S., & Jacobs,B.L. Activity of serotonin-containing neurons in nucleus raphe magnus in freely moving cats. *Exp. Neurol.* **88**, 590-608 (1985).

Frengen,E. *et al.* A modular, positive selection bacterial artificial chromosome vector with multiple cloning sites. *Genomics* **58**, 250-253 (1999).

Friedman,J.R. & Kaestner,K.H. The Foxa family of transcription factors in development and metabolism. *Cell Mol. Life Sci.* **63**, 2317-2328 (2006).

Frungieri,M.B. *et al.* Serotonin in golden hamster testes: testicular levels, immunolocalization and role during sexual development and photoperiodic regression-recrudescence transition. *Neuroendocrinology* **69**, 299-308 (1999).

Fujimoto,S., Ueda,H., & Kagawa,H. Immunocytochemistry on the localization of 5-hydroxytryptamine in monkey and rabbit taste buds. *Acta Anat. (Basel)* **128**, 80-83 (1987).

Fuller,D.D., Zabka,A.G., Baker,T.L., & Mitchell,G.S. Phrenic long-term facilitation requires 5-HT receptor activation during but not following episodic hypoxia. *J. Appl. Physiol* **90**, 2001-2006 (2001).

Funk,G.D. *et al.* Functional respiratory rhythm generating networks in neonatal mice lacking NMDAR1 gene. *J. Neurophysiol.* **78**, 1414-1420 (1997).

Furuya,S., Sawada,M., Nagatsu,T., Suzuki,O., & Higashida,H. Localization of [3H]serotonin in neuroblastoma x glioma hybrid cells. *Brain Res.* **361**, 77-90 (1985).

Fuxe,K. EVIDENCE FOR THE EXISTENCE OF MONOAMINE NEURONS IN THE CENTRAL NERVOUS SYSTEM. IV. DISTRIBUTION OF MONOAMINE NERVE TERMINALS IN THE CENTRAL NERVOUS SYSTEM. *Acta Physiol Scand. Suppl*SUPPL (1965).

Gal,E.M. & Sherman,A.D. L-kynurenine: its synthesis and possible regulatory function in brain. *Neurochem. Res.* **5**, 223-239 (1980).

Gammie,S.C. Current models and future directions for understanding the neural circuitries of maternal behaviors in rodents. *Behav. Cogn Neurosci. Rev.* **4**, 119-135 (2005).

Garcia-Tornadu,I. *et al.* GH in the dwarf dopaminergic D2 receptor knockout mouse: somatotrope population, GH release, and responsiveness to GH-releasing factors and somatostatin. *J. Endocrinol.* **190**, 611-619 (2006).

Gaspar,P., Cases,O., & Maroteaux,L. The developmental role of serotonin: news from mouse molecular genetics. *Nat. Rev. Neurosci.* **4**, 1002-1012 (2003).

Gaus,S.E., Strecker,R.E., Tate,B.A., Parker,R.A., & Saper,C.B. Ventrolateral preoptic nucleus contains sleep-active, galaninergic neurons in multiple mammalian species. *Neuroscience* **115**, 285-294 (2002).

George J. Siegel M.D (editor). 1998. Basic Neurochemistry. Molecular, Cellular and Medical Aspects. Sixth Edition

Gershon,M.D., Drakontides,A.B., & Ross,L.L. serotonin: synthesis and release from the myenteric plexus of the mouse intestine. *Science* **149**, 197-199 (1965).

Gershon,M.D. Enteric serotonergic neurones ... finally! *J. Physiol* **587**, 507 (2009).

Giammanco,S., Ernandes,M., & La,G.M. Effects of environmental lighting and tryptophan devoid diet on the rat vaginal cycle. *Arch. Physiol Biochem.* **105**, 445-449 (1997).

Gondo,Y. Trends in large-scale mouse mutagenesis: from genetics to functional genomics. *Nat. Rev. Genet.* **9**, 803-810 (2008).

Goodwill,K.E. *et al.* Crystal structure of tyrosine hydroxylase at 2.3 A and its implications for inherited neurodegenerative diseases. *Nat. Struct. Biol.* **4**, 578-585 (1997).

Green,C.B. & Besharse,J.C. Tryptophan hydroxylase expression is regulated by a circadian clock in Xenopus laevis retina. *J. Neurochem.* **62**, 2420-2428 (1994).

Grenett,H.E., Ledley,F.D., Reed,L.L., & Woo,S.L. Full-length cDNA for rabbit tryptophan hydroxylase: functional domains and evolution of aromatic amino acid hydroxylases. *Proc. Natl. Acad. Sci. U. S. A* **84**, 5530-5534 (1987).

Griebel,G. Variability in the effects of 5-HT-related compounds in experimental models of anxiety: evidence for multiple mechanisms of 5-HT in anxiety or never ending story? *Pol. J. Pharmacol.* **48**, 129-136 (1996).

Grimsby,J. *et al.* Increased stress response and beta-phenylethylamine in MAOB-deficient mice. *Nat. Genet.* **17**, 206-210 (1997).

Gross,C., Santarelli,L., Brunner,D., Zhuang,X., & Hen,R. Altered fear circuits in 5-HT(1A) receptor KO mice. *Biol. Psychiatry* **48**, 1157-1163 (2000).

Gross,C. *et al.* Serotonin1A receptor acts during development to establish normal anxiety-like behaviour in the adult. *Nature* **416**, 396-400 (2002).

Gross,S.S. & Levi,R. Tetrahydrobiopterin synthesis. An absolute requirement for cytokine-induced nitric oxide generation by vascular smooth muscle. *J. Biol. Chem.* **267**, 25722-25729 (1992).

Gundlah,C. *et al.* Estrogen receptor-beta regulates tryptophan hydroxylase-1 expression in the murine midbrain raphe. *Biol. Psychiatry* **57**, 938-942 (2005).

Gutknecht,L. *et al.* Deficiency of brain 5-HT synthesis but serotonergic neuron formation in Tph2 knockout mice. *J. Neural Transm.* **115**, 1127-1132 (2008).

Guyton Arthur C., Hall John E. Textbook of medical physiology. W B Saunders Co, 11th Ed. (2008)

Hart, R.P. Yang, R, Riley, L.A., Green, T.L. Post-transcriptional control of tryptophan hydroxylase gene expression in rat brain stem and pineal gland, Mol. Cell. Neurosci, 2, 71-77.

Hasegawa,H., Yanagisawa,M., Inoue,F., Yanaihara,N., & Ichiyama,A. Demonstration of non-neural tryptophan 5-mono-oxygenase in mouse intestinal mucosa. *Biochem. J.* **248**, 501-509 (1987).

Haycock,J.W., Kumer,S.C., Lewis,D.A., Vrana,K.E., & Stockmeier,C.A. A monoclonal antibody to tryptophan hydroxylase: applications and identification of the epitope. *J. Neurosci. Methods* **114**, 205-212 (2002).

Hedlund,P.B. *et al.* No hypothermic response to serotonin in 5-HT7 receptor knockout mice. *Proc. Natl. Acad. Sci. U. S. A* **100**, 1375-1380 (2003).

Heisler,L.K., Chu,H.M., & Tecott,L.H. Epilepsy and obesity in serotonin 5-HT2C receptor mutant mice. *Ann. N. Y. Acad. Sci.* **861**, 74-78 (1998).

Heisler,L.K. *et al.* Central serotonin and melanocortin pathways regulating energy homeostasis. *Ann. N. Y. Acad. Sci.* **994**, 169-174 (2003).

Hellon,R.F. Monoamines, pyrogens and cations: their actions on central control of bodytemperature. *Pharmacol. Rev.* **26**, 289-321 (1974).

Hendricks,T., Francis,N., Fyodorov,D., & Deneris,E.S. The ETS domain factor Pet-1 is an early and precise marker of central serotonin neurons and interacts with a conserved element in serotonergic genes. *J. Neurosci.* **19**, 10348-10356 (1999).

Hendricks,T.J. *et al.* Pet-1 ETS gene plays a critical role in 5-HT neuron development and is required for normal anxiety-like and aggressive behavior. *Neuron* **37**, 233-247 (2003).

Heninger,G.R., Delgado,P.L., & Charney,D.S. The revised monoamine theory of depression: a modulatory role for monoamines, based on new findings from monoamine depletion experiments in humans. *Pharmacopsychiatry* **29**, 2-11 (1996).

Henry,D.P. & Bowsher,R.R. An improved radioenzymatic assay for plasma norepinephrine using purified phenylethanolamine N-methyltransferase. *Life Sci.* **38**, 1473-1483 (1986).

Henry,J.L. & Calaresu,F.R. Origin and course of crossed medullary pathways to spinal sympathetic neurons in the cat. *Exp. Brain Res.* **20** , 515-526 (1974).

Herman,J.K., O'Halloran,K.D., Janssen,P.L., & Bisgard,G.E. Dopaminergic excitation of the goat carotid body is mediated by the serotonin type 3 receptor subtype. *Respir. Physiol Neurobiol.* **136**, 1-12 (2003).

Hixson,E.J., Lehrmann,G.V., & Maickel,R.P. Contractile responses to tryptamine analogues in isolated smooth muscle. *Arch. Int. Pharmacodyn. Ther.* **229**, 4-14 (1977).

Hodges,M.R. & Richerson,G.B. Interaction between defects in ventilatory and thermoregulatory control in mice lacking 5-HT neurons. *Respir. Physiol Neurobiol.* **164**, 350-357 (2008).

Hogan, B. Constantini, F. Lacy, E. Manipulating the mouse embryo – a laboratory manual, 1-332 (1986).

Holstege,G. & Kuypers,H.G. The anatomy of brain stem pathways to the spinal cord in cat. A labeled amino acid tracing study. *Prog. Brain Res.* **57**, 145-175 (1982).

Holzenberger,M. *et al.* IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. *Nature* **421**, 182-187 (2003).

Hoyer,D., Hannon,J.P., & Martin,G.R. Molecular, pharmacological and functional diversity of 5-HT receptors. *Pharmacol. Biochem. Behav.* **71**, 533-554 (2002).

Hufton,S.E., Jennings,I.G., & Cotton,R.G. Structure and function of the aromatic amino acid hydroxylases. *Biochem. J.* **311 (Pt 2)**, 353-366 (1995).

Huh,S.O., Park,D.H., Cho,J.Y., Joh,T.H., & Son,J.H. A 6.1 kb 5' upstream region of the mouse tryptophan hydroxylase gene directs expression of E. coli lacZ to major serotonergic brain regions and pineal gland in transgenic mice. *Brain Res. Mol. Brain Res.* **24**, 145-152 (1994).

Ichinose,H. *et al.* Increased heterogeneity of tyrosine hydroxylase in humans. *Biochem. Biophys. Res. Commun.* **195**, 158-165 (1993).

Isobe,T. *et al.* Distinct forms of the protein kinase-dependent activator of tyrosine and tryptophan hydroxylases. *J. Mol. Biol.* **217**, 125-132 (1991).

Iwaki,M., Phillips,R.S., & Kaufman,S. Proteolytic modification of the amino-terminal and carboxyl-terminal regions of rat hepatic phenylalanine hydroxylase. *J. Biol. Chem.* **261**, 2051-2056 (1986).

Jacobs,B.L. & Azmitia,E.C. Structure and function of the brain serotonin system. *Physiol Rev.* **72**, 165-229 (1992).

Jacobs,B.L., Martin-Cora,F.J., & Fornal,C.A. Activity of medullary serotonergic neurons in freely moving animals. *Brain Res. Brain Res. Rev.* **40**, 45-52 (2002).

Jaeger,C.B. *et al.* Aromatic L-amino acid decarboxylase in the rat brain: immunocytochemical localization in neurons of the brain stem. *Neuroscience* **11**, 691-713 (1984).

Jansen, R.C. and J.P. Nap Genetical genomics: the added value from segregation. Trends in Genetics 17: 388-391 (2001).

Jensen,P. *et al.* Redefining the serotonergic system by genetic lineage. *Nat. Neurosci.* **11**, 417-419 (2008).

Johnston,J.P. Some observations upon a new inhibitor of monoamine oxidase in brain tissue. *Biochem. Pharmacol.* **17**, 1285-1297 (1968).

Jordan,D. Vagal control of the heart: central serotonergic (5-HT) mechanisms. *Exp. Physiol* **90**, 175-181 (2005).

Jung, M. J. Substrates and inhibitors of aromatic amino acid decarboxylase, Bioorg. Chem., 14, 429 – 443 (1986).

Juorio,A.V. & Boulton,A.A. The effect of some precursor amino acids and enzyme inhibitors on the mouse striatal concentration of tyramines and homovanillic acid. *J. Neurochem.* **39**, 859-863 (1982).

Kandel,E.R. The molecular biology of memory storage: a dialog between genes and synapses. *Biosci. Rep.* **21**, 565-611 (2001).

Kaneda,N. *et al.* Isolation of a novel cDNA clone for human tyrosine hydroxylase: alternative RNA splicing produces four kinds of mRNA from a single gene. *Biochem. Biophys. Res. Commun.* **146**, 971-975 (1987).

Kappeler,L. *et al.* Brain IGF-1 receptors control mammalian growth and lifespan through a neuroendocrine mechanism. *PLoS. Biol.* **6**, e254 (2008).

Katz,D.M. & Balkowiec,A. New insights into the ontogeny of breathing from genetically engineered mice. *Curr. Opin. Pulm. Med.* **3**, 433-439 (1997).

Kaufman,S. & Fisher,D.B. Purification and some physical properties of phenylalanine hydroxylase from rat liver. *J. Biol. Chem.* **245**, 4745-4750 (1970).

Kaufman,W.L. *et al.* Homogeneity and persistence of transgene expression by omitting antibiotic selection in cell line isolation. *Nucleic Acids Res.* **36**, e111 (2008).

Kawasaki,H. *et al.* Induction of midbrain dopaminergic neurons from ES cells by stromal cell-derived inducing activity. *Neuron* **28**, 31-40 (2000).

Keller,G. Embryonic stem cell differentiation: emergence of a new era in biology and medicine. *Genes Dev.* **19**, 1129-1155 (2005).

Kim,J.J. *et al.* Selective enhancement of emotional, but not motor, learning in monoamine oxidase A-deficient mice. *Proc. Natl. Acad. Sci. U. S. A* **94**, 5929-5933 (1997).

Kim,K.S. *et al.* Molecular cloning and characterization of cDNA encoding tryptophan hydroxylase from rat central serotonergic neurons. *Brain Res. Mol. Brain Res.* **9**, 277-283 (1991).

Kim,S.K. *et al.* Stage-specific expression of ankyrin and SOCS box protein-4 (Asb-4) during spermatogenesis. *Mol. Cells* **25**, 317-321 (2008).

Kinney,H.C., Burger,P.C., Harrell,F.E., Jr., & Hudson,R.P., Jr. 'Reactive gliosis' in the medulla oblongata of victims of the sudden infant death syndrome. *Pediatrics* **72**, 181-187 (1983).

Kinney,H.C., Filiano,J.J., & White,W.F. Medullary serotonergic network deficiency in the sudden infant death syndrome: review of a 15-year study of a single dataset. *J. Neuropathol. Exp. Neurol.* **60**, 228-247 (2001).

Kish SJ, Shannak K, Hornykiewicz O. Elevated serotonin and reduced dopamine in subregionally divided Huntington's disease striatum. Ann Neurol. 1987 Sep;22(3):386-9.

Kitahama,K. *et al.* Immunohistochemistry of aromatic L-amino acid decarboxylase in the cat forebrain. *J. Comp Neurol.* **270**, 337-353 (1988).

Kline,D.D. & Prabhakar,N.R. Role of nitric oxide in short-term potentiation and long-term facilitation: involvement of NO in breathing stability. *Adv. Exp. Med. Biol.* **499**, 215-219 (2001).

Kobayashi,K. *et al.* Targeted disruption of the tyrosine hydroxylase locus results in severe catecholamine depletion and perinatal lethality in mice. *J. Biol. Chem.* **270**, 27235-27243 (1995).

Kodama,H. *et al.* MC3T3-G2/PA6 preadipocytes support in vitro proliferation of hemopoietic stem cells through a mechanism different from that of interleukin 3. *J. Cell Physiol* **129**, 20-26 (1986).

Kouros-Mehr,H., Slorach,E.M., Sternlicht,M.D., & Werb,Z. GATA-3 maintains the differentiation of the luminal cell fate in the mammary gland. *Cell* **127**, 1041-1055 (2006).

Kuhn,D.M., Meyer,M.A., & Lovenberg,W. Comparisons of tryptophan hydroxylase from a malignant murine mast cell tumor and rat mesencephalic tegmentum. *Arch. Biochem. Biophys.* **199**, 355-361 (1980).

Kumer,S.C., Mockus,S.M., Rucker,P.J., & Vrana,K.E. Amino-terminal analysis of tryptophan hydroxylase: protein kinase phosphorylation occurs at serine-58. *J. Neurochem.* **69**, 1738-1745 (1997).

Kuro-o M *et al.* Mutation of the mouse klotho gene leads to a syndrome resembling ageing. *Nature* **390**, 45-51 (1997).

Kurosu,H. *et al.* Suppression of aging in mice by the hormone Klotho. *Science* **309**, 1829-1833 (2005).

Kurosu,H. *et al.* Regulation of fibroblast growth factor-23 signaling by klotho. *J. Biol. Chem.* **281**, 6120-6123 (2006).

Lai,Z. *et al.* Age-related reduction of human growth hormone-binding sites in the human brain. *Brain Res.* **621**, 260-266 (1993).

Lauder,J.M. Neurotransmitters as growth regulatory signals: role of receptors and second messengers. *Trends Neurosci.* **16**, 233-240 (1993).

Leathwood,P.D. & Fernstrom,J.D. Effect of an oral tryptophan/carbohydrate load on tryptophan, large neutral amino acid, and serotonin and 5-hydroxyindoleacetic acid levels in monkey brain. *J. Neural Transm. Gen. Sect.* **79**, 25-34 (1990).

Lee,S.H., Lumelsky,N., Studer,L., Auerbach,J.M., & McKay,R.D. Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. *Nat. Biotechnol.* **18**, 675-679 (2000).

Lenders,J.W. *et al.* Specific genetic deficiencies of the A and B isoenzymes of monoamine oxidase are characterized by distinct neurochemical and clinical phenotypes. *J. Clin. Invest* **97**, 1010-1019 (1996).

Leonard,S. *et al.* Nicotinic receptor function in schizophrenia. *Schizophr. Bull.* **22**, 431-445 (1996).

Lerch-Haner,J.K., Frierson,D., Crawford,L.K., Beck,S.G., & Deneris,E.S. Serotonergic transcriptional programming determines maternal behavior and offspring survival. *Nat. Neurosci.*(2008).

Lesurtel,M. *et al.* Platelet-derived serotonin mediates liver regeneration. *Science* **312**, 104-107 (2006).

Lewy,A.J., Ahmed,S., Jackson,J.M., & Sack,R.L. Melatonin shifts human circadian rhythms according to a phase-response curve. *Chronobiol. Int.* **9**, 380-392 (1992).

Li,W., Cogswell,C.A., & LoTurco,J.J. Neuronal differentiation of precursors in the neocortical ventricular zone is triggered by BMP. *J. Neurosci.* **18**, 8853-8862 (1998).

Lidov,H.G. & Molliver,M.E. Immunohistochemical study of the development of serotonergic neurons in the rat CNS. *Brain Res. Bull.* **9**, 559-604 (1982).

Lindvall,O., Kokaia,Z., & Martinez-Serrano,A. Stem cell therapy for human neurodegenerative disorders-how to make it work. *Nat. Med.* **10 Suppl**, S42-S50 (2004).

Lovenberg,W., Jequier,E., & Sjoerdsma,A. Tryptophan hydroxylation: measurement in pineal gland, brainstem, and carcinoid tumor. *Science* **155**, 217-219 (1967).

Lucas,J.J. & Hen,R. New players in the 5-HT receptor field: genes and knockouts. *Trends Pharmacol. Sci.* **16**, 246-252 (1995).

Ludecke,B. *et al.* Recessively inherited L-DOPA-responsive parkinsonism in infancy caused by a point mutation (L205P) in the tyrosine hydroxylase gene. *Hum. Mol. Genet.* **5**, 1023-1028 (1996).

Lupien,M. *et al.* FoxA1 translates epigenetic signatures into enhancer-driven lineage-specific transcription. *Cell* **132**, 958-970 (2008).

Lydic,R. Reticular modulation of breathing during sleep and anesthesia. *Curr. Opin. Pulm. Med.* **2**, 474-481 (1996).

Mager,G.M. *et al.* Active gene repression by the Egr2.NAB complex during peripheral nerve myelination. *J. Biol. Chem.* **283**, 18187-18197 (2008).

Malek,Z.S., Pevet,P., & Raison,S. Circadian change in tryptophan hydroxylase protein levels within the rat intergeniculate leaflets and raphe nuclei. *Neuroscience* **125**, 749-758 (2004).

Marcus,J.N. *et al.* Differential expression of orexin receptors 1 and 2 in the rat brain. *J. Comp Neurol.* **435**, 6-25 (2001).

Markey,K.A., Kondo,H., Shenkman,L., & Goldstein,M. Purification and characterization of tyrosine hydroxylase from a clonal pheochromocytoma cell line. *Mol. Pharmacol.* **17**, 79-85 (1980).

Martin,G.R. & Evans,M.J. Differentiation of clonal lines of teratocarcinoma cells: formation of embryoid bodies in vitro. *Proc. Natl. Acad. Sci. U. S. A* **72**, 1441-1445 (1975).

Martin,G.R. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. U. S. A* **78**, 7634-7638 (1981).

Martinez-Salas,E. Internal ribosome entry site biology and its use in expression vectors. *Curr. Opin. Biotechnol.* **10**, 458-464 (1999).

Matsuda,M. *et al.* Serotonin regulates mammary gland development via an autocrine-paracrine loop. *Dev. Cell* **6**, 193-203 (2004).

Mauri,M.C. *et al.* Neurobiological and psychopharmacological basis in the therapy of bulimia and anorexia. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **20**, 207-240 (1996).

McGinty,D. & Szymusiak,R. The sleep-wake switch: A neuronal alarm clock. *Nat. Med.* **6**, 510-511 (2000).

McGinty,D.J. & Sterman,M.B. Sleep suppression after basal forebrain lesions in the cat. *Science* **160**, 1253-1255 (1968).

McKinney,J., Knappskog,P.M., & Haavik,J. Different properties of the central and peripheral forms of human tryptophan hydroxylase. *J. Neurochem.* **92**, 311-320 (2005).

Miller,S.F. *et al.* Expression of Plxdc2/TEM7R in the developing nervous system of the mouse. *Gene Expr. Patterns.* **7**, 635-644 (2007).

Mitchell,G.S. *et al.* Invited review: Intermittent hypoxia and respiratory plasticity. *J. Appl. Physiol* **90**, 2466-2475 (2001).

Mockus,S.M., Kumer,S.C., & Vrana,K.E. Carboxyl terminal deletion analysis of tryptophan hydroxylase. *Biochim. Biophys. Acta* **1342**, 132-140 (1997).

Mockus,S.M., Kumer,S.C., & Vrana,K.E. A chimeric tyrosine/tryptophan hydroxylase. The tyrosine hydroxylase regulatory domain serves to stabilize enzyme activity. *J. Mol. Neurosci.* **9**, 35-48 (1997).

Mockus,S.M., Yohrling,G.J., & Vrana,K.E. Tyrosine hydroxylase and tryptophan hydroxylase do not form heterotetramers. *J. Mol. Neurosci.* **10**, 45-51 (1998).

Mockus,S.M. & Vrana,K.E. Advances in the molecular characterization of tryptophan hydroxylase. *J. Mol. Neurosci.* **10**, 163-179 (1998).

Moon R. Y., Horne R. S., Hauck F. R., *Lancet* 370, 1578-1587 (2007).

Mori,K. *et al.* Disruption of klotho gene causes an abnormal energy homeostasis in mice. *Biochem. Biophys. Res. Commun.* **278**, 665-670 (2000).

Morrison,S.F. Central pathways controlling brown adipose tissue thermogenesis. *News Physiol Sci.* **19**, 67-74 (2004).

Mossner,R. & Lesch,K.P. Role of serotonin in the immune system and in neuroimmune interactions. *Brain Behav. Immun.* **12**, 249-271 (1998).

Mueller,R.A., Towle,A.C., & Breese,G.R. Supersensitivity to the respiratory stimulatory effect of TRH in 5,7-dihydroxytryptamine-treated rats. *Brain Res.* **298**, 370-373 (1984).

Nakano,T., Kodama,H., & Honjo,T. Generation of lymphohematopoietic cells from embryonic stem cells in culture. *Science* **265**, 1098-1101 (1994).

Nakata,H. & Fujisawa,H. Tryptophan 5-monooxygenase from mouse mastocytoma P815. A simple purification and general properties. *Eur. J. Biochem.* **124**, 595-601 (1982).

Nakata,H. & Fujisawa,H. Purification and properties of tryptophan 5-monooxygenase from rat brain-stem. *Eur. J. Biochem.* **122**, 41-47 (1982).

Nakshatri,H. & Badve,S. FOXA1 as a therapeutic target for breast cancer. *Expert. Opin. Ther. Targets.* **11**, 507-514 (2007).

Nanda,A. *et al.* Identification of a binding partner for the endothelial cell surface proteins TEM7 and TEM7R. *Cancer Res.* **64**, 8507-8511 (2004).

Nebigil,C.G., Launay,J.M., Hickel,P., Tournois,C., & Maroteaux,L. 5-hydroxytryptamine 2B receptor regulates cell-cycle progression: cross-talk with tyrosine kinase pathways. *Proc. Natl. Acad. Sci. U. S. A* **97**, 2591-2596 (2000).

Nebigil,C.G. *et al.* Ablation of serotonin 5-HT(2B) receptors in mice leads to abnormal cardiac structure and function. *Circulation* **103**, 2973-2979 (2001).

Neckameyer,W.S. & White,K. A single locus encodes both phenylalanine hydroxylase and tryptophan hydroxylase activities in Drosophila. *J. Biol. Chem.* **267**, 4199-4206 (1992).

Newman,C., Wang,D., & Cutz,E. Serotonin (5-hydroxytryptamine) expression in pulmonary neuroendocrine cells (NE) and a netumor cell line. *Adv. Exp. Med. Biol.* **337**, 73-78 (1993).

Nishikawa,S.I. *et al.* In vitro generation of lymphohematopoietic cells from endothelial cells purified from murine embryos. *Immunity.* **8**, 761-769 (1998).

Nozik-Grayck,E. *et al.* Pulmonary vasoconstriction by serotonin is inhibited by S-nitrosoglutathione. *Am. J. Physiol Lung Cell Mol. Physiol* **282**, L1057-L1065 (2002).

Numan,M. Neural basis of maternal behavior in the rat. *Psychoneuroendocrinology* **13**, 47-62 (1988).

Onimaru,H., Arata,A., & Homma,I. Firing properties of respiratory rhythm generating neurons in the absence of synaptic transmission in rat medulla in vitro. *Exp. Brain Res.* **76**, 530-536 (1989).

Onishi,A., Liotta,L.J., & Benkovic,S.J. Cloning and expression of Chromobacterium violaceum phenylalanine hydroxylase in Escherichia coli and comparison of amino acid sequence with mammalian aromatic amino acid hydroxylases. *J. Biol. Chem.* **266**, 18454-18459 (1991).

Padbury,J.F. *et al.* Placental biogenic amine transporters: cloning and expression. *Brain Res. Mol. Brain Res.* **45**, 163-168 (1997).

Papageorgiou,A. & Denef,C. Stimulation of growth hormone release by 5-hydroxytryptamine (5-HT) in cultured rat anterior pituitary cell aggregates: evidence for mediation by 5-HT2B, 5-HT7, 5-HT1B, and ketanserin-sensitive receptors. *Endocrinology* **148**, 4509-4522 (2007).

Parisi,E., De,P.P., Capasso,A., & del,P.M. Serotonin and sperm motility. *Cell Biol. Int. Rep.* **8**, 95 (1984).

PARKES,A.S. & BRUCE,H.M. Olfactory stimuli in mammalian reproduction. *Science* **134**, 1049-1054 (1961).

Patel,P.D., Pontrello,C., & Burke,S. Robust and tissue-specific expression of TPH2 versus TPH1 in rat raphe and pineal gland. *Biol. Psychiatry* **55**, 428-433 (2004).

Patient,R.K. & McGhee,J.D. The GATA family (vertebrates and invertebrates). *Curr. Opin. Genet. Dev.* **12**, 416-422 (2002).

Pattyn,A., Vallstedt,A., Dias,J.M., Sander,M., & Ericson,J. Complementary roles for Nkx6 and Nkx2 class proteins in the establishment of motoneuron identity in the hindbrain. *Development* **130**, 4149-4159 (2003).

Pattyn,A. *et al.* Coordinated temporal and spatial control of motor neuron and serotonergic neuron generation from a common pool of CNS progenitors. *Genes Dev.* **17**, 729-737 (2003).

Pattyn,A. *et al.* Ascl1/Mash1 is required for the development of central serotonergic neurons. *Nat. Neurosci.* **7**, 589-595 (2004).

Pelletier,G., Steinbusch,H.W., & Verhofstad,A.A. Immunoreactive substance P and serotonin present in the same dense-core vesicles. *Nature* **293**, 71-72 (1981).

Penev,P.D., Zee,P.C., & Turek,F.W. Monoamine depletion blocks triazolam-induced phase advances of the circadian clock in hamsters. *Brain Res.* **637**, 255-261 (1994).

Perkins,M.N. & Stone,T.W. An iontophoretic investigation of the actions of convulsant kynurenines and their interaction with the endogenous excitant quinolinic acid. *Brain Res.* **247**, 184-187 (1982).

Perkins,M.N. & Stone,T.W. An iontophoretic investigation of the actions of convulsant kynurenines and their interaction with the endogenous excitant quinolinic acid. *Brain Res.* **247**, 184-187 (1982).

Persico,A.M. *et al.* Barrel pattern formation requires serotonin uptake by thalamocortical afferents, and not vesicular monoamine release. *J. Neurosci.* **21**, 6862-6873 (2001).

Persico,A.M. *et al.* Reduced programmed cell death in brains of serotonin transporter knockout mice. *Neuroreport* **14**, 341-344 (2003).

Peterson DS, Trachtenberg FL, Thompson ED, et al. Multiple serotonergic brainstem abnormalities in sudden infant death syndrome. JAMA 2006

Peyron,C. *et al.* Neurons containing hypocretin (orexin) project to multiple neuronal systems. *J. Neurosci.* **18** , 9996-10015 (1998).

Popova,N.K. & Amstislavskaya,T.G. 5-HT2A and 5-HT2C serotonin receptors differentially modulate mouse sexual arousal and the hypothalamo-pituitary-testicular response to the presence of a female. *Neuroendocrinology* **76**, 28-34 (2002).

Popova,N.K. & Amstislavskaya,T.G. Involvement of the 5-HT(1A) and 5-HT(1B) serotonergic receptor subtypes in sexual arousal in male mice. *Psychoneuroendocrinology* **27**, 609-618 (2002).

Popova,N.K. From genes to aggressive behavior: the role of serotonergic system. *Bioessays* **28**, 495-503 (2006).

Powels, P. J., 5-HAT receptors and their ligands, Tocris Reviews No.25, Septembar 2003.

Purves Dale, Augustine J. George, Fitzpatrick David, Hall William C., LaMantia Anthony-Samuel, McNamara James O., and White Leonard E. Neuroscience, Fourth Edition. 2008

Principles of Neuropsychopharmacology: Robert S. Feldman, Jerrold S. Meyer, Linda F. Quenzer. Sinauer Associates; 1 edition (January 1997)

Radulovacki,M., Virus,R.M., Djuricic-Nedelson,M., & Green,R.D. Adenosine analogs and sleep in rats. *J. Pharmacol. Exp. Ther.* **228**, 268-274 (1984).

Ramage,A.G. Central cardiovascular regulation and 5-hydroxytryptamine receptors. *Brain Res. Bull.* **56**, 425-439 (2001).

Rang HP, Dale MM,. Ritter JM and Moore PK. Pharmacology. Fifth Edition Churchill. Livingstone, Edinburgh, 2003

Rapport MM, Green AA, Page IH. (1948) Partial purification of the vasoconstrictor in beef serum. J Biol Chem 174:735-741

Rapport MM. (1949) Serum vasoconstrictor (serotonin) the presence of creatinine in the complex; a proposed structure of the vasoconstrictor principle. J Biol Chem 180:961-969

Raymond,J.R. *et al.* Multiplicity of mechanisms of serotonin receptor signal transduction. *Pharmacol. Ther.* **92** , 179-212 (2001).

Rebsam,A., Seif,I., & Gaspar,P. Refinement of thalamocortical arbors and emergence of barrel domains in the primary somatosensory cortex: a study of normal and monoamine oxidase a knock-out mice. *J. Neurosci.* **22**, 8541-8552 (2002).

Rehman,H.U. & Masson,E.A. Neuroendocrinology of ageing. *Age Ageing* **30**, 279-287 (2001).

Rekling,J.C. & Feldman,J.L. PreBotzinger complex and pacemaker neurons: hypothesized site and kernel for respiratory rhythm generation. *Annu. Rev. Physiol* **60**, 385-405 (1998).

RENSON,J., WEISSBACH,H., & UDENFRIEND,S. Hydroxylation of tryptophan by phenylalanine hydroxylase. *J. Biol. Chem.* **237**, 2261-2264 (1962).

Reynolds GP, Pearson SJ. Decreased glutamic acid and increased 5-hydroxytryptamine in Huntington's disease brain. Neurosci Lett. 1987 Jul 22;78(2):233-8.

Richerson,G.B. Serotonergic neurons as carbon dioxide sensors that maintain pH homeostasis. *Nat. Rev. Neurosci.* **5**, 449-461 (2004).

Rocha,B.A. *et al.* Increased vulnerability to cocaine in mice lacking the serotonin-1B receptor. *Nature* **393**, 175-178 (1998).

Rubin,L.L. & Staddon,J.M. The cell biology of the blood-brain barrier. *Annu. Rev. Neurosci.* **22**, 11-28 (1999).

Russell,S.J. & Kahn,C.R. Endocrine regulation of ageing. *Nat. Rev. Mol. Cell Biol.* **8**, 681-691 (2007).

Salichon,N. *et al.* Excessive activation of serotonin (5-HT) 1B receptors disrupts the formation of sensory maps in monoamine oxidase a and 5-ht transporter knock-out mice. *J. Neurosci.* **21**, 884-896 (2001).

Sambrook, J., Fritsch, E.F., Maniatis, T. Molecular cloning: a laboratory manual. 2nd edition, 771 (1989).

Saper,C.B., Chou,T.C., & Scammell,T.E. The sleep switch: hypothalamic control of sleep and wakefulness. *Trends Neurosci.* **24**, 726-731 (2001).

Saper,C.B., Scammell,T.E., & Lu,J. Hypothalamic regulation of sleep and circadian rhythms. *Nature* **437**, 1257-1263 (2005).

Saper,C.B., Cano,G., & Scammell,T.E. Homeostatic, circadian, and emotional regulation of sleep. *J. Comp Neurol.* **493**, 92-98 (2005).

Saudou,F. *et al.* Enhanced aggressive behavior in mice lacking 5-HT1B receptor. *Science* **265**, 1875-1878 (1994).

Saunders,N.R., Dziegielewska,K.M., & Mollgard,K. The importance of the blood-brain barrier in fetuses and embryos. *Trends Neurosci.* **14**, 14-15 (1991).

Savelieva,K.V. *et al.* Genetic disruption of both tryptophan hydroxylase genes dramatically reduces serotonin and affects behavior in models sensitive to antidepressants. *PLoS. ONE.* **3**, e3301 (2008).

Scott,M.M. *et al.* A genetic approach to access serotonin neurons for in vivo and in vitro studies. *Proc. Natl. Acad. Sci. U. S. A* **102**, 16472-16477 (2005).

Scott,M.M., Krueger,K.C., & Deneris,E.S. A differentially autoregulated Pet-1 enhancer region is a critical target of the transcriptional cascade that governs serotonin neuron development. *J. Neurosci.* **25**, 2628-2636 (2005).

Sheng,Y. *et al.* A serotonin receptor antagonist induces oocyte maturation in both frogs and mice: evidence that the same G protein-coupled receptor is responsible for maintaining meiosis arrest in both species. *J. Cell Physiol* **202**, 777-786 (2005).

Sherin,J.E., Shiromani,P.J., McCarley,R.W., & Saper,C.B. Activation of ventrolateral preoptic neurons during sleep. *Science* **271**, 216-219 (1996).

Sherin,J.E., Elmquist,J.K., Torrealba,F., & Saper,C.B. Innervation of histaminergic tuberomammillary neurons by GABAergic and galaninergic neurons in the ventrolateral preoptic nucleus of the rat. *J. Neurosci.* **18**, 4705-4721 (1998).

Shuey,D.L., Sadler,T.W., Tamir,H., & Lauder,J.M. Serotonin and morphogenesis. Transient expression of serotonin uptake and binding protein during craniofacial morphogenesis in the mouse. *Anat. Embryol. (Berl)* **187**, 75-85 (1993).

Sibille,E., Pavlides,C., Benke,D., & Toth,M. Genetic inactivation of the Serotonin(1A) receptor in mice results in downregulation of major GABA(A) receptor alpha subunits, reduction of GABA(A) receptor binding, and benzodiazepine-resistant anxiety. *J. Neurosci.* **20**, 2758-2765 (2000).

Silva,J., Chambers,I., Pollard,S., & Smith,A. Nanog promotes transfer of pluripotency after cell fusion. *Nature* **441**, 997-1001 (2006).

Silva,J. & Smith,A. Capturing pluripotency. *Cell* **132**, 532-536 (2008).

Smidt,M.P. *et al.* A second independent pathway for development of mesencephalic dopaminergic neurons requires Lmx1b. *Nat. Neurosci.* **3**, 337-341 (2000).

Smith,A. The battlefield of pluripotency. *Cell* **123**, 757-760 (2005).

Smithies,O., Gregg,R.G., Boggs,S.S., Koralewski,M.A., & Kucherlapati,R.S. Insertion of DNA sequences into the human chromosomal beta-globin locus by homologous recombination. *Nature* **317**, 230-234 (1985).

Smits,M.G. *et al.* Melatonin improves health status and sleep in children with idiopathic chronic sleep-onset insomnia: a randomized placebo-controlled trial. *J. Am. Acad. Child Adolesc. Psychiatry* **42**, 1286-1293 (2003).

Sole,M.J., Madapallimattam,A., & Baines,A.D. An active pathway for serotonin synthesis by renal proximal tubules. *Kidney Int.* **29**, 689-694 (1986).

Son,J.H. *et al.* Immortalization of neuroendocrine pinealocytes from transgenic mice by targeted tumorigenesis using the tryptophan hydroxylase promoter. *Brain Res. Mol. Brain Res.* **37**, 32-40 (1996).

Sonntag,W.E. *et al.* L-dopa restores amplitude of growth hormone pulses in old male rats to that observed in young male rats. *Neuroendocrinology* **34**, 163-168 (1982).

Soprano,D.R. & Soprano,K.J. Retinoids as teratogens. *Annu. Rev. Nutr.* **15**, 111-132 (1995).

Sourkes,T.L. Kinetics of tryptophan transport into the brain. *J. Neural Transm. Suppl*107-114 (1979).

Sourkes, T.L. DOPA decarboxylase (aromatic amino acid decarboxylase)., Horn, A.S., Korf, J. and Westerinkm B.H.C., The Neurobiology of Dopamine, 123-132, Academic Press

Spear,B.T., Jin,L., Ramasamy,S., & Dobierzewska,A. Transcriptional control in the mammalian liver: liver development, perinatal repression, and zonal gene regulation. *Cell Mol. Life Sci.* **63**, 2922-2938 (2006).

Sprengel,R. & Single,F.N. Mice with genetically modified NMDA and AMPA receptors. *Ann. N. Y. Acad. Sci.* **868**, 494-501 (1999).

Steininger,T.L., Alam,M.N., Gong,H., Szymusiak,R., & McGinty,D. Sleep-waking discharge of neurons in the posterior lateral hypothalamus of the albino rat. *Brain Res.* **840**, 138-147 (1999).

Stoll,J., Kozak,C.A., & Goldman,D. Characterization and chromosomal mapping of a cDNA encoding tryptophan hydroxylase from a mouse mastocytoma cell line. *Genomics* **7**, 88-96 (1990).

Strauer,B.E. & Kornowski,R. Stem cell therapy in perspective. *Circulation* **107**, 929-934 (2003).

Strecker,R.E. *et al.* Behavioral state-related changes of extracellular serotonin concentration in the pedunculopontine tegmental nucleus: a microdialysis study in freely moving animals. *Sleep Res. Online.* **2**, 21-27 (1999).

Sucov,H.M. & Evans,R.M. Retinoic acid and retinoic acid receptors in development. *Mol. Neurobiol.* **10**, 169-184 (1995).

Sutton,M.A., Masters,S.E., Bagnall,M.W., & Carew,T.J. Molecular mechanisms underlying a unique intermediate phase of memory in aplysia. *Neuron* **31**, 143-154 (2001).

Svenningsson,P., Nairn,A.C., & Greengard,P. DARPP-32 mediates the actions of multiple drugs of abuse. *AAPS. J.* **7**, E353-E360 (2005).

Szymusiak,R., Alam,N., Steininger,T.L., & McGinty,D. Sleep-waking discharge patterns of ventrolateral preoptic/anterior hypothalamic neurons in rats. *Brain Res.* **803**, 178-188 (1998).

Takahashi,N. *et al.* VMAT2 knockout mice: heterozygotes display reduced amphetamine-conditioned reward, enhanced amphetamine locomotion, and enhanced MPTP toxicity. *Proc. Natl. Acad. Sci. U. S. A* **94**, 9938-9943 (1997).

Takayanagi,Y. *et al.* Pervasive social deficits, but normal parturition, in oxytocin receptor-deficient mice. *Proc. Natl. Acad. Sci. U. S. A* **102**, 16096-16101 (2005).

Taylor,C.P. & Garrido,R. Immunostaining of rat brain, spinal cord, sensory neurons and skeletal muscle for calcium channel alpha2-delta (alpha2-delta) type 1 protein. *Neuroscience* **155**, 510-521 (2008).

Tenner, K. Funktionalle Charakterisierung der humanen Tryptophanehydroxylase 2, Doctoral thesis, Freie university Berlin (2008).

Thissen,J.P., Ketelslegers,J.M., & Underwood,L.E. Nutritional regulation of the insulin-like growth factors. *Endocr. Rev.* **15**, 80-101 (1994).

Thomas,K.R., Folger,K.R., & Capecchi,M.R. High frequency targeting of genes to specific sites in the mammalian genome. *Cell* **44**, 419-428 (1986).

Thomas,K.R. & Capecchi,M.R. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* **51**, 503-512 (1987).

Thomas,S.A., Matsumoto,A.M., & Palmiter,R.D. Noradrenaline is essential for mouse fetal development. *Nature* **374**, 643-646 (1995).

Thomas,S.A. & Palmiter,R.D. Impaired maternal behavior in mice lacking norepinephrine and epinephrine. *Cell* **91**, 583-592 (1997).

Thomson,J.A. *et al.* Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145-1147 (1998).

Tohyama,O. *et al.* Klotho is a novel beta-glucuronidase capable of hydrolyzing steroid beta-glucuronides. *J. Biol. Chem.* **279**, 9777-9784 (2004).

Tong,J.H. & Kaufman,S. Tryptophan hydroxylase. Purification and some properties of the enzyme from rabbit hindbrain. *J. Biol. Chem.* **250** , 4152-4158 (1975).

TWAROG,B.M. & PAGE,I.H. Serotonin content of some mammalian tissues and urine and a method for its determination. *Am. J. Physiol* **175**, 157-161 (1953).

TWAROG,B.M. Responses of a molluscan smooth muscle to acetylcholine and 5-hydroxytryptamine. *J. Cell Physiol* **44**, 141-163 (1954).

Uchida,K., Usami,M., Bandow,H., & Harada,I. Characteristics of substrates and inhibitors in binding to rat liver L-tryptophan 2,3-dioxygenase: a Fourier transform infrared and kinetic study. *Biochim. Biophys. Acta* **1121**, 153-159 (1992).

Upton,A.L. *et al.* Excess of serotonin (5-HT) alters the segregation of ispilateral and contralateral retinal projections in monoamine oxidase A knock-out mice: possible role of 5-HT uptake in retinal ganglion cells during development. *J. Neurosci.* **19**, 7007-7024 (1999).

Upton,A.L. *et al.* Lack of 5-HT(1B) receptor and of serotonin transporter have different effects on the segregation of retinal axons in the lateral geniculate nucleus compared to the superior colliculus. *Neuroscience* **111**, 597-610 (2002).

Utsugi,T. *et al.* Decreased insulin production and increased insulin sensitivity in the klotho mutant mouse, a novel animal model for human aging. *Metabolism* **49**, 1118-1123 (2000).

Vacher,C.M. *et al.* Monoaminergic control of vasopressin and VIP expression in the mouse suprachiasmatic nucleus. *J. Neurosci. Res.* **71**, 791-801 (2003).

van Doorninck,J.H. *et al.* GATA-3 is involved in the development of serotonergic neurons in the caudal raphe nuclei. *J. Neurosci.* **19**, RC12 (1999).

Vialli M, Erspamer V. (1937) Ricerche sul secrete delle cellule enterocromaffini. IX. Intorno alla natura chimica della sostanza specifica. Boll Soc med -chir Pavia 51:1111-1118

Voltattorni,C.B., Giartosio,A., & Turano,C. Aromatic-L-amino acid decarboxylase from pig kidney. *Methods Enzymol.* **142**, 179-187 (1987).

Walther,D.J. & Bader,M. Serotonin synthesis in murine embryonic stem cells. *Brain Res. Mol. Brain Res.* **68**, 55-63 (1999).

Walther,D.J. & Bader,M. A unique central tryptophan hydroxylase isoform. *Biochem. Pharmacol.* **66**, 1673-1680 (2003).

Walther,D.J. *et al.* Synthesis of serotonin by a second tryptophan hydroxylase isoform. *Science* **299**, 76 (2003).

Walther, D. J. Die dualität des serotonergen Systems, Doctoral thesis, Freie University Berlin (2003).

Wang,L., Erlandsen,H., Haavik,J., Knappskog,P.M., & Stevens,R.C. Three-dimensional structure of human tryptophan hydroxylase and its implications for the biosynthesis of the neurotransmitters serotonin and melatonin. *Biochemistry* **41**, 12569-12574 (2002).

Wang,Y.M. *et al.* Knockout of the vesicular monoamine transporter 2 gene results in neonatal death and supersensitivity to cocaine and amphetamine. *Neuron* **19**, 1285-1296 (1997).

Wassef,M.A. *et al.* Rostral hindbrain patterning involves the direct activation of a Krox20 transcriptional enhancer by Hox/Pbx and Meis factors. *Development* **135**, 3369-3378 (2008).

Weber,L.J. & Horita,A. A study of 5-hydroxytryptamine formation from L-tryptophan in the brain and other tissues. *Biochem. Pharmacol.* **14**, 1141-1149 (1965).

Weihe,E. & Eiden,L.E. Chemical neuroanatomy of the vesicular amine transporters. *FASEB J.* **14**, 2435-2449 (2000).

Werkman,T.R., Glennon,J.C., Wadman,W.J., & McCreary,A.C. Dopamine receptor pharmacology: interactions with serotonin receptors and significance for the aetiology and treatment of schizophrenia. *CNS. Neurol. Disord. Drug Targets.* **5**, 3-23 (2006).

Whitaker-Azmitia,P.M. Role of serotonin and other neurotransmitter receptors in brain development: basis for developmental pharmacology. *Pharmacol. Rev.* **43**, 553-561 (1991).

Whitaker-Azmitia,P.M. The discovery of serotonin and its role in neuroscience. *Neuropsychopharmacology* **21**, 2S-8S (1999).

Wilson PG. and Stice S. 2006. Development and differentiation of neural rosettes derived from human embryonic stem cells 2006. Stem Cell Reviews. 2:67-78

Wolf,I. *et al.* FOXA1: Growth inhibitor and a favorable prognostic factor in human breast cancer. *Int. J. Cancer* **120**, 1013-1022 (2007).

Wong,E.A. & Capecchi,M.R. Analysis of homologous recombination in cultured mammalian cells in transient expression and stable transformation assays. *Somat. Cell Mol. Genet.* **12**, 63-72 (1986).

WOOLLEY,D.W. & SHAW,E. Some neurophysiological aspects of serotonin. *Br. Med. J.* **2**, 122-126 (1954).

Xu,P., Santos,R.A., Bader,M., & Alenina,N. Alterations in gene expression in the testis of angiotensin-(1-7)-receptor Mas-deficient mice. *Regul. Pept.* **138**, 51-55 (2007).

Xu,P. *et al.* Endothelial dysfunction and elevated blood pressure in MAS gene-deleted mice. *Hypertension* **51**, 574-580 (2008).

Yang,X.J. & Kaufman,S. High-level expression and deletion mutagenesis of human tryptophan hydroxylase. *Proc. Natl. Acad. Sci. U. S. A* **91**, 6659-6663 (1994).

Ye,W. *et al.* Distinct regulators control the expression of the mid-hindbrain organizer signal FGF8. *Nat. Neurosci.* **4**, 1175-1181 (2001).

Yee,S.P. & Rigby,P.W. The regulation of myogenin gene expression during the embryonic development of the mouse. *Genes Dev.* **7**, 1277-1289 (1993).

Ying,Q.L. *et al.* The ground state of embryonic stem cell self-renewal. *Nature* **453**, 519-523 (2008).

Ying,Y., Qi,X., & Zhao,G.Q. Induction of primordial germ cells from murine epiblasts by synergistic action of BMP4 and BMP8B signaling pathways. *Proc. Natl. Acad. Sci. U. S. A* **98**, 7858-7862 (2001).

Yoshida,T., Fujimori,T., & Nabeshima,Y. Mediation of unusually high concentrations of 1,25-dihydroxyvitamin D in homozygous klotho mutant mice by increased expression of renal 1alpha-hydroxylase gene. *Endocrinology* **143**, 683-689 (2002).

Young,M.R. *et al.* Stimulation of splenic T-lymphocyte function by endogenous serotonin and by low-dose exogenous serotonin. *Immunology* **80**, 395-400 (1993).

Zambrowicz,B.P., Turner,C.A., & Sands,A.T. Predicting drug efficacy: knockouts model pipeline drugs of the pharmaceutical industry. *Curr. Opin. Pharmacol.* **3**, 563-570 (2003).

Zeitz,K.P. *et al.* The 5-HT3 subtype of serotonin receptor contributes to nociceptive processing via a novel subset of myelinated and unmyelinated nociceptors. *J. Neurosci.* **22**, 1010-1019 (2002).

Zhao,G., Xia,T., Song,J., & Jensen,R.A. Pseudomonas aeruginosa possesses homologues of mammalian phenylalanine hydroxylase and 4 alpha-carbinolamine dehydratase/DCoH as part of a three-component gene cluster. *Proc. Natl. Acad. Sci. U. S. A* **91**, 1366-1370 (1994).

Zhao,Z.Q. *et al.* Lmx1b is required for maintenance of central serotonergic neurons and mice lacking central serotonergic system exhibit normal locomotor activity. *J. Neurosci.* **26**, 12781-12788 (2006).

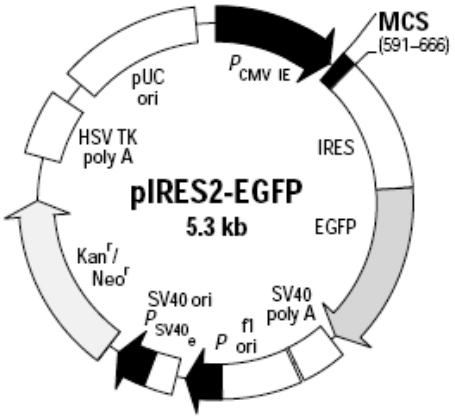
Zhou,Q.Y., Quaife,C.J., & Palmiter,R.D. Targeted disruption of the tyrosine hydroxylase gene reveals that catecholamines are required for mouse fetal development. *Nature* **374**, 640-643 (1995).

Zill,P. *et al.* Analysis of tryptophan hydroxylase I and II mRNA expression in the human brain: a post-mortem study. *J. Psychiatr. Res.* **41**, 168-173 (2007).

APPENDIX I

pIRES2-EGFP Vector Information PT3267-5

Catalog #6029-1



591 601 611 621 631 641 651 661

GCTAGCGCTACCGGACTCAGATCTCGAGCTCAAGCTTCGAATTCTGCAGTCGACGGTACCGCGGGCCCGGGATCC...IRES

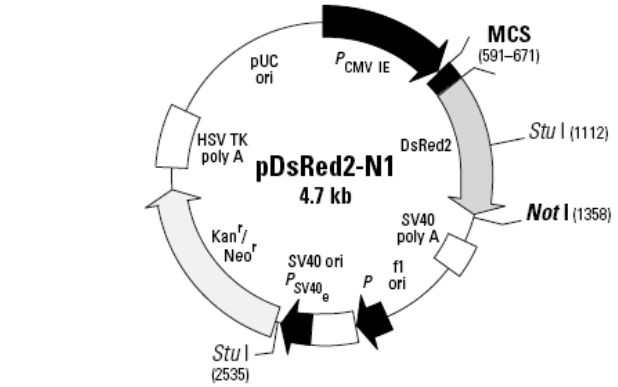
***Nhe* I** ***Eco*47 III** ***Bgl* II** ***Xho* I** *Hin*d III ***Sac* I** ***Ecl*136 II** ***Eco*R I** ***Pst* I** ***Sal* I** ***Acc* I** *Kpn* I *Asp*718 I *Ape* I *Bsp*120 I ***Sac* II** ***Xma* I** ***Sma* I** ***Bam*H I**

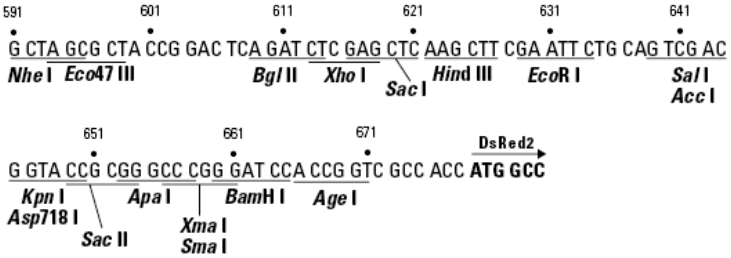
Restriction Map and Multiple Cloning Site (MCS) of pIRES2-EGFP Vector. Unique restriction sites are in bold. Note that the *Eco*47 III site has not been confirmed in the final construct.

APPENDIX II

pDsRed2-N1 Vector Information PT3604-5

Cat. No. 632406





Restriction Map and Multiple Cloning Site (MCS) of pDsRed2-N1 Vector. Unique restriction sites are in bold. The *Not* I site follows the DsRed2 stop codon.

APPENDIX III

1439635_at	1.432	**0.00320**	Rgs9	**regulator of G-protein signaling 9**	chr11 E1|11 70.0 cM
1455806_x_at	1.546	**0.00387**	LOC669626; Ndufa12	**NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 12; similar to 13kDa differentiation-associat...**	chr10 F
1427515_at	1.368	**0.00424**	A530088I07Rik	**RIKEN cDNA A530088I07 gene**	chr5 A3
1432452_at	1.476	**0.00696**	1700054O19Rik	**RIKEN cDNA 1700054O19 gene**	chr14 A3
1423400_at	-1.439	**0.01141**	**Kl**	**klotho**	chr5 G3
1446392_at	1.339	**0.01310**	**Slit2**	Slit homolog 2 (Drosophila)	chr5 B3
1455981_at	1.512	**0.01432**	EG214738; EG626259; EG62957...	**ribosomal protein S6; predicted gene, EG214738; similar to 40S ribosomal protein S6; predicted ge...**	chr14 D3; chr15 D3; chr17 E...
1446598_at	1.379	**0.01479**	Prkca	**Protein kinase C, alpha**	chr11 E1|11 68.0 cM
1435415_x_at	1.410	**0.01531**	**Marcksl1**	MARCKS-like 1	chr4 D2.2|4 59.0 cM
1450174_at	1.604	**0.01956**	**Ptprt**	protein tyrosine phosphatase, receptor type, T	chr2 H2|2 93.0 cM
1459894_at	1.301	**0.01996**	**Iqgap2**	IQ motif containing GTPase activating protein 2	chr13 D1
1458114_at	1.492	**0.02020**	**Samd12**	sterile alpha motif domain containing 12	chr15 C-D1
1421329_a_at	-1.315	**0.03190**	**Smyd1**	**SET and MYND domain containing 1**	chr6 C1|6 30.5 cM
1426127_x_at	-1.376	**0.03221**	**Klra18**	**killer cell lectin-like receptor, subfamily A, member 18**	chr6 F3
1439437_x_at	1.546	**0.03528**	**Cpe; LOC677374**	carboxypeptidase E; similar to carboxypeptidase E	chr8 B3.1|8 32.6 cM
1436981_a_at	1.612	**0.03566**	**Ywhaz**	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	chr15 B3.1
1456112_at	1.419	**0.03915**	**Tpr**	translocated promoter region	chr1 G1
1458830_at	-1.528	**0.03942**	**Fgf14**	Fibroblast growth factor 14	chr14 E5|14 59.0 cM
1426951_at	1.364	**0.04038**	**Crim1**	cysteine rich transmembrane BMP regulator 1 (chordin like)	chr17 E2
1430561_at	1.391	**0.04083**	**Dnajb14**	DnaJ (Hsp40) homolog, subfamily B, member 14	chr3 G3
1458307_at	1.575	**0.04232**	B230334C09Rik	**RIKEN cDNA B230334C09 gene**	---
1437001_at	1.362	**0.04236**	**Gsk3b**	glycogen synthase kinase 3 beta	chr16 B4
1446144_at	1.508	**0.04373**	**Pex2**	peroxin 2	chr3 B
1445957_at	1.481	**0.04376**	**Evl**	Ena-vasodilator stimulated phosphoprotein	chr12 F2
1456119_at	1.298	**0.04481**	**Grm5**	glutamate receptor, metabotropic 5	chr7 D3
1459788_at	1.450	**0.04490**	**Gpr107**	G protein-coupled receptor 107	chr2 B
1422849_a_at	1.294	**0.04518**	**Pabpn1**	poly(A) binding protein, nuclear 1	chr14 C2|14 19.5 cM
1443235_at	1.310	**0.04521**	**Eif2ak4**	Eukaryotic translation initiation factor 2 alpha kinase 4	---
1439005_x_at	1.306	**0.04525**	**Ywhaz**	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	chr15 B3.1

IX PUBLICATIONS

Alenina, N., ***Kikic, D***., Todiras, M., Mosienko, V., Qadri, F., Plehm, R., Boye, P., Vilianovitch, L., Sohr, R., Tenner, K., Hörtnagl, H., and Bader, M. (2009) Growth retardation and altered autonomic control in mice lacking brain serotonin, PNAS (in print).