Brain-specific conditional and time-specific inducible Tph2 knockout mice possess normal serotonergic gene expression in the absence of serotonin during adult life

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1. Introduction

Serotonin (5-HT) synthesis in the brain is exclusively catalysed by the rate-limiting enzyme tryptophan hydroxylase (TPH) 2 (Gutknecht et al., 2009). Thus, the genetic set-point of brain 5-HT synthesis, which might result in behavioural abnormalities, seems to be due to variation at the TPH2 rather than the TPH1 locus. Mouse strains which exhibit a prolinc-to-arginine (Pro447Arg) mutation of Tph2, which is accompanied by a two-fold lower activity of Tph2 enzyme and substantially lower levels of 5-HT in the brain (Zhang et al., 2004) consequently showed changed activity of Tph2 enzyme and substantially lower levels of 5-HT in the brain-specific Tph2 KO and the 5-HT-reduction models. These findings confirm the specificity of Tph2 in brain 5-HT synthesis across the lifespan, yet also suggest that neither developmental nor adult 5-HT synthesis is required for the expression of genes specific for serotonergic signalling. The formation of the serotonergic system thus seems to be a preserved expression pattern due to intrinsic cellular programs which occurs also in the absence of its key molecule, namely 5-HT.
conditional Lmx1b knockout (KO) mice (Ding et al., 2003; Zhao et al., 2006) are viable without apparent developmental abnormalities despite the fact that these animals completely lack 5-HT in the brain. Contrary to TPH2, which can be found in brain and periphery (Gutknecht et al., 2009), detection of TPH1 expression was limited to peripheral organs including the pineal gland suggesting the possibility of physiological adaptation to different needs for regulation of 5-HT production in the brain and in peripheral organs. This might also play a role in 5-HT-associated pathological changes in neuropsychiatric disorders like depression. Therefore, animal models distinguishing between both systems (peripheral and central serotonergic system) might be an adequate model for neuropsychiatric research. Furthermore, targeted inactivation of one gene ab initio in every cell as accomplished in the classical constitutive KO models might affect many developmental processes throughout ontogeny and compensatory mechanisms may be activated in both central and peripheral nervous systems. The development of Tph2 conditional knockout (cKO) mice, in which Tph2 is inactivated in a tissue- or time-specific manner, can circumvent these disadvantages and thus provide clues regarding the differential impact of peripheral and central 5-HT synthesis, e.g. in the development of brain and behaviour. Hence, different brain-specific Tph2 cKO models were generated and the function of several genes of the serotonergic system was examined. These models include one model specific for Tph2 deletion in all neuronal cells (Tph2-cKO<sup>nes</sup>) and two models specific for Tph2 deletion only in 5-HT cells (Tph2-cKO<sup>Pet1</sup> and Tph2-iKO), the latter featuring the advantage of inducible knockout (iKO) providing a time-specific deletion of Tph2. In contrast to the non-inducible cKO models (cKO<sup>Pet1</sup> and cKO<sup>nes</sup>), both of which produce gene deletion from embryonic stages E10–E11 on, the inducible model was tamoxifen-injected at the stage of adulthood avoiding putative compensatory effects from other systems during brain development which probably might balance reduced 5-HT availability. Comparison of all three Tph2 cKO models, considering the induction of gene deletion at different time points, provides a means to further study the brain-specific modulation of the serotonergic system.

2. Methods

2.1. Mouse strains

Wildtype and different Tph2 cKO and Tph2 iKO mice resulting from crosses of transgenic Nestin-Cre, Pet1-Cre and Pet1-CreER (for iKO) lines with a Tph2-floxed (Tph2<sup>fl/fl</sup>) line were used for this study. Wildtype mice as well as mice expressing either Cre-recombinase (transgenic Cre-lines) or carrying the floxed Tph2 gene (in no case, both together) were used as controls. Most of the mice had a cleared C57/BL6 strain background, while the remaining mice were crossbreeds of C57/BL6, 129/SvEMS and FVB/N strains. All mice were kept and generated under normal light-dark-cycles. Tph2 iKOs and their respective controls (in order to estimate the stress-induced changes) received tamoxifen injections to induce Cre<i>LoxP</i> mediated gene deletion. Tamoxifen injections were given four to five times (6–8 mg per 40 g of body weight) with a 1-week interval between the first and the second injection, and a 1-day interval between the following injections. Tamoxifen-induced mice were killed 3 weeks after the last injection.

All mice were instantaneously euthanized by cervical dislocation after CO2 anesthesia. Immediately after euthanasia brains and/or peripheral tissues including pineal gland were dissected and stored at −80°C. Afterward, Preparation of brain regions for quantitative real-time (qRT)-PCR was done on a cooling plate (−20°C). All investigated animals were in the stage of adulthood (2–3 months).

2.2. Cre-<i>LoxP</i> mediated Tph2 knockout

Tph2 KO mice were generated by tissue-specific Cre<i>LoxP</i> mediated gene deletion. Elimination of exon 5 of Tph2 resulting in a truncated non-functional Tph2 protein by creating a shift in the reading frame was used to generate Tph2<sup>5/6</sup> KO mice (Ding et al., 2003). Exon 5 is coding for an amino acid sequence at the start of the catalytic domain and ends with a partial codon. Therefore, a floxed Tph2 gene construct leading to a Tph2 allele with exons 5 flanked by two <i>LoxP</i> sites (floxed Tph2 allele) was generated by cloning and introduced into mouse ES cells genome by homologous recombination (Gutknecht et al., 2008). The Tph2-floxed line (i<i>LoxP</i>-flanked Tph2 gene) was crossed with three different transgenic lines expressing the cyclization recomb.
In addition, investigation of Tph1 expression in the different Tph2 expression reduction model, whereas Tph2-cKO Pet1 can be considered an absolute and 5-HT neuron-specific Tph2 KO model. Complete loss of Tph2 protein was only shown in Tph2-cKO Pet1. In contrast to Tph2-cKO Pet1, where no Tph2-positive cells were detected neither in the rostral nor in the caudal raphe nuclei (Fig. 2f and l), Tph2-cKO Pet1 exhibited some remaining Tph2-positive cells mainly in the dorsal part of rostral raphe region (Fig. 2e and k). Similar pattern of remaining Tph2-positive cells were detected in Tph2-iKO. Comparison of a 5-month-old Tph2-iKO animal injected four times with a 3-month-old animal injected five times with tamoxifen showed significant differences of Tph2 expression in the rostral as well as the caudal raphe region (Fig. 2c, i, d and j). The brain of the five times injected mouse exhibited less Tph2-positive cells as compared to the four times injected animal in both the rostral and the caudal part. Detection of remaining Tph2 expression in Tph2-cKO Pet1 and Tph2-iKO indicates that these strains rather represent a Tph2 knockout mosaic and thus a Tph2 expression reduction model, whereas Tph2-cKO Pet1 can be considered an absolute and 5-HT neuron-specific Tph2 KO model.

In addition, investigation of Tph1 expression in the rostral and the caudal (not shown) raphe region (Fig. 2, lower panel). Specific and effective binding of Tph1 antibodies was proven by their strong staining in the pineal gland (Fig. 2, inserts of a and b).

3.3. Quantitative real-time PCR

qRT-PCR studies were performed to detect expression of seven different genes specific for the serotonergic system: 5-HT1a, 5-HT1b, 5-HT2a, 5-HT2c receptors, serotonin transporter (Sert), Tph2 and Tph1 in eight different brain regions of interest: frontal and prefrontal cortex (marked as cortex frontal), striatum, hippocampus, hypothalamus, amygdala, rostral and caudal raphe region, and entorhinal cortex. Since there were no significant differences between males and females, statistical evaluation concerning genotype differences was accomplished using the means of females and males to increase the amount of animals per group. Strong expression differences ($p < 0.001$) of Tph2-mRNA were detected in the rostral and caudal raphe regions of the different genotypes confirming previous IHC results (Fig. 3F). Confirming our IHC data, Tph1 expression in the brain was almost undetectable in comparison to Tph1 control tissues like intestine and pineal gland (Fig. 3G and H). In addition, Tph1 expression values in the different brain regions of all genotypes are negligible as compared to Tph2, suggesting no physiologically relevant Tph1 expression in Tph2 cKOs. In all other investigated genes, significant changes of expression among the different genotypes could only be detected for 5-HT1a receptor expression in the hippocampus (Fig. 3A). However, further statistical comparison between Tph2 cKO and their corresponding controls using accurate Fisher test yielded only statistical trends or borderline significance (cKO Pet1).
Fig. 2. Tph2 and Tph1 immunohistochemistry (IHC) in different Tph2 conditional knockout models vs. specific controls. Upper panel illustrates IHC with specific Tph2 antibodies in rostral (a–f) and caudal (g–l) raphe region. Highest amount of Tph2-positive cells was detected in wildtype (WT) and iKO-control (pictures a, g and h). A Tph2 iKO of 5 months age which was treated four times with tamoxifen also possesses a relative high amount of Tph2-positive cells, mainly in the dorsal part of the rostral raphe region (c) and likewise in the caudal raphe region (l). Whereas a 3 months old Tph2-iKO which was injected five times showed less Tph2-positive cells in both raphe regions (d and j). In comparison to Tph2-cKO Pet1, which exhibited no Tph2-positive cells neither in the rostral nor in the caudal raphe nuclei, Tph2-cKO Nes possesses Tph2-positive cells mainly in the dorsal part of rostral raphe region. Lower panel shows IHC with specific Tph1 antibodies in rostral raphe region using WT (a and e) and injected WT (iKO-control, c) for comparison of Tph2-cKO Nes (b) and Tph2-cKO Pet1 (f) or a 3 months old Tph2 iKO which was injected five times (d). In none of the investigated groups Tph1-positive cells could be detected, indicating no compensatory effect of Tph1 expression in brains of Tph2 cKO models. Specific and effective binding of Tph1 antibodies was proven by their strong staining in the pineal gland (inserts of a and b).

221 $p = 0.0374; \text{cKO}^{Nes} p = 0.0730; \text{iKO} p = 0.0585$). Finally, expression of the 5-HT1a receptor in the hippocampus was not importantly altered in the different cKO models.

222 4. Discussion

223 4.1. Efficiency of Tph2 knockout

224 Efficiency of Tph2 KO was tested in all Tph2 cKO models with IHC and validated quantitatively by qRT-PCR. As expected, all cKO models possess a significantly reduced amount of cells and fibres staining positive for Tph2 in the raphe region, including a complete loss of Tph2 enzyme in the brain of Tph2-cKO Pet1. For as yet unknown reasons Tph2-cKO Nes exhibit some residual Tph2-positive cells mainly in the dorsal part of the rostral raphe, which could also be observed in Tph2 iKO mice. In the latter, this results presumably from less effective, however time-specific KO. Double labelling of β-galactosidase and Tph2 antibodies in the brain-specific Tph2 iKOs furthermore controls the efficiency of both Cre recombination as well as Tph2 gene deletion, which should be linked to each other. Additional experiments demonstrate as well that a variation of first-time tamoxifen injection and the repetition of injections cause serious alterations in the amount of Tph2-positive cells, in that earlier and repetitive injection resulted in a higher efficiency of the knockout (see Fig. 2 upper panel c, i and d, j). Accordingly, we used mice for qRT-PCR at the stage of early adulthood (2–4 months) and injected them five times which led to a higher reduction of Tph2 expression. The Tph2-mRNA levels obtained by qRT-PCR are in complete accordance to IHC and demonstrate a deletion of Tph2 in both the rostral and the caudal part of raphe region. Based on the values of the respective controls, an 80% reduction of overall Tph2-mRNA expression was obtained in rostral and caudal raphe region of Tph2 iKO mice as well as in the rostral part of Tph2-cKO Nes. In the caudal raphe of Tph2-cKO Nes, this was even more pronounced featuring approximately 90% of reduction. The assumption of an absolute and brain-specific knockout in Tph2-cKO Pet1 could be verified by a reduction of Tph2-mRNA over 90% in caudal and more than 95% in rostral raphe.

225 Not only the reduction of detectable Tph2 expression, but also the lack of putative compensatory Tph1 expression which was shown by IHC and high sensitive qRT-PCR, gives rise to the notion of loss of 5-HT in the brain of brain-specific Tph2 cKO models and further confirms the exclusive specificity of Tph2 in brain 5-HT synthesis across the lifespan. Furthermore, high reduction of 5-HT was demonstrated by HPLC analysis and no 5-HT-positive cells were detected with IHC in constitutive Tph2 KO mice resulting from a spontaneous germline transmission of Tph2-cKO Nes (Gutknecht et al., 2008). Even under this extreme condition, Tph1 remained undetectable in the raphe nuclei rendering any spontaneous brain Tph1 expression unlikely. Finally, a 5-HT-specific antibody failed to detect 5-HT-positive cells in the Tph2-cKO Pet1, whereas at least a reduction of 5-HT-positive cells could be observed in the Tph2 iKO (data not shown).

226 In contrast to non-inducible conditional knockout models (Tph2-cKO Pet1 and Tph2-cKO Nes) which both produce gene deletion from embryonic stages E10–E11 on, the inducible model was tamoxifen-injected at the stage of adulthood avoiding putative compensatory effects from other systems during brain development which probably might balance reduced 5-HT availability. To our best knowledge, this is the first report on brain-specific inducible gene deletion with a user-controlled first onset in the stage of early adulthood.

227 4.2. Expression of serotonergic genes in 5-HT deletion mouse models

228 By studying the expression of several genes of the serotonergic system using qRT-PCR we tested the proper formation of the serotonergic system in Tph2 cKO mice. Different 5-HT receptor
subtypes and the Sert were investigated in eight different brain regions of interest. Interestingly, there was no significant change of expression of any of the genes in any investigated brain region between the different genotypes and their respective controls. Unchanged expression of genes of the serotonergic system was unexpected in the brain-specific Tph2 cKO models. One possible explanation might be that Tph2 deletion does not start at E0, but at the time of expression of the serotonergic transcription factor Pet1. Pet1 operates rather at the end of a transcription factor cascade necessary for proper formation of serotonergic phenotypes and brain development (Ding et al., 2003). However, Pet1 seems to orchestrate a variety of molecules relevant for serotonergic signalning, and thus its disruption results in undifferentiated 5-HT neurons lacking proteins for 5-HT production (i.e., Tph2), uptake, and storage (Hendricks et al., 2003). Therefore, if a cell once is programmed to express the serotonergic phenotype (a process which is however already starting before Pet1 is switched on), it might not stop this program until its end. Thus, 5-HT-associated proteins are produced in a normal manner and localized to their specific sites, although they are presumably not needed for the development of serotonergic neurons and have a questionable function in the absence of 5-HT. However, a growing body of literature supports the idea of promiscuity among neurotransmitter-associated proteins. For example, the different monoamine transporters provide uptake of multiple amines in addition to their “native” transmitter. The dopamine and noradrenaline transporter (DAT, NET) are able to transport 5-HT under conditions in which SERT activity is eliminated by pharmacological inhibition. SERT also can accomplish low dopamine and noradrenaline transport (for a review see Daws, 2009). Thus the absence of 5-HT in Tph2 cKO models might not lead to disruption of 5-HT-associated proteins like Sert and 5-HT receptors in the brain, as they still might be required for functions not related to 5-HT.

Although no significant changes between the different genotypes could be measured for the different investigated genes of the serotonergic sector, each investigated gene displays a specific expression pattern among the different brain regions. Thereby, previous findings on the distribution of the different 5-HT receptors could be confirmed and further specified. Each receptor subtype has a particular region where its expression shows the highest level: the hippocampus in the case of the 5-HT1a, striatum for the 5-HT1b, frontal and prefrontal cortex for the 5-HT2a, and the raphe region for the 5-HT2c receptor. For Sert-mRNA, as expected, expression was concentrated in raphe nuclei since those contain the somata of all 5-HT neurons in the brain.

Fig. 3. Quantitative real-time PCR on distinct brain regions of different Tph2 conditional knockout models. Illustration of the relative mRNA expression of 5-HT1a (A), 5-HT1b (B), 5-HT2a (C), 5-HT2c receptor (D), serotonin transporter (Sert) (E), Tph2 (F), and Tph1 (G and H) in different murine brain regions. Calculated quantitative values were normalized by GeNorm (Vandesompele et al., 2002) with the two most stable housekeeping genes of four. Statistical evaluation was performed using the non-parametric Kruskal–Wallis test and the parametric ANOVA test (Statview program, SAS institute Inc., version 5.0). Groups were further statistically analysed with the accurate Fisher test if parametric and non-parametric tests showed p-values < 0.05. Data are means ± SEM of 6–10 mice. *Shows significant differences (**p < 0.0001) of the particular genotype from their respective controls.
4.3. Animal models for human affective disorders?

For many years 5-HT has been implicated in the etiopathogenesis of affective disorders. In addition, most of the currently used antidepressants interfere with the serotonergic system. Further data supportive of this notion are around since the early seventies, where it was observed that depressive symptoms could be ameliorated by administration of 5-HT precursors like 5-hydroxytryptophan (5-HTP) and tryptophan (Mendels et al., 1975). On the other hand, aggravation of symptoms could be evoked in depressed patients by administering a tryptophan depleting diet (Delgado et al., 1990; Van der Does, 2001).

Likewise, tryptophan depletion causes gloomy mood in healthy subjects with a family history for depression and remitted patients previously suffering from depression (Ruhé et al., 2007). In contrast to monoamines like 5-HT, amino acids like tryptophan or the 5-HT precursor 5-HTP are able to cross the blood brain barrier. Thus, modulation of availability of precursors for neuronal 5-HT synthesis might have an important influence in developing depression-like behavioural changes during adulthood. Our inducible Tph2 KO model might be a model for both reduced availability of 5-HT in the brain (i.e., mirroring tryptophan depletion) and modulation of the 5-HT system during adulthood.

Taken together, our findings on the one hand confirm the exclusive specificity of Tph2 in brain 5-HT synthesis across the lifespan proven by all three Tph2 cKO models. On the other hand, our data indicate that neither developmental nor adult Tph2-dependent 5-HT synthesis is required for normal serotonergic system formation, although Tph1 does not compensate the lack of 5-HT amount in the brain of Tph2 cKO models. Considering the induction of gene deletion at the stage of adulthood – provides a means to further study brain-specific modulation of 5-HT system, independently of the developmental mechanisms which can then be kept normal, and its impact on behaviour and the etiopathogenesis of affective disorders.

Conflict of interest

There is no conflict of interest to disclose.

Uncited reference

Mendels and Frazer (1975).

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