



## Research article

## Increased adult neurogenesis in mice with a permanent overexpression of the postsynaptic 5-HT<sub>1A</sub> receptor



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## HIGHLIGHTS

- Depression and hippocampal adult neurogenesis (HAN) are influenced by 5-HT<sub>1A</sub>Rs.
- Here, we studied HAN in mice with postsynaptic overexpression of 5-HT<sub>1A</sub>R (OE).
- Proliferation, survival and differentiation of newborn cells were increased in OE.
- Alterations in survival were only detected in the female subgroup of OE mice.
- We propose a leading role of postsynaptic 5-HT<sub>1A</sub>Rs in HAN.

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## ABSTRACT

Depression is among the leading causes of disability and disease burden. Recent studies point to an involvement of altered serotonin<sub>1A</sub> receptor (5-HT<sub>1A</sub>R) –mediated adult neurogenesis in depression. However, the exact underlying mechanisms remain unclear, mainly due to the complexity of the serotonergic system with its various receptors and their locations. Mice with permanent overexpression of postsynaptic 5-HT<sub>1A</sub>Rs (OE mice) represent a unique tool for investigating the involvement of postsynaptic 5-HT<sub>1A</sub>Rs in this context. Correct 5-HT<sub>1A</sub>R coupling and functioning has been demonstrated earlier, indicating that more postsynaptic 5-HT<sub>1A</sub>Rs can be activated in these mice. Initially we examined morphometric parameters of the dentate gyrus (DG) and the prefrontal cortex as they are involved in adult hippocampal neurogenesis and/or depression. The volume of the DG in OE mice was increased in comparison to wildtype controls. We therefore investigated parameters of adult neurogenesis by the bromodeoxyuridine method. Proliferation and survival of newborn cells in the DG of OE mice were significantly increased. Significant increases in survived neurons were only detected in the female but not in the male subgroup. Additional staining for early precursor cells (Sox2) and progenitor cells of the neuronal lineage (doublecortin) showed an increase in type-1/2a as well as in type-2b/3 cells in OE mice. Our study suggests a leading role of the postsynaptic 5-HT<sub>1A</sub>R in adult hippocampal neurogenesis and might open an important link to depression.

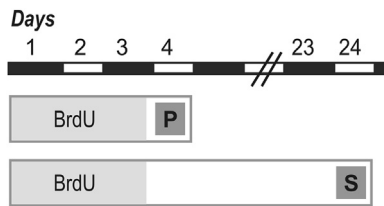
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## 1. Introduction

With a life-time prevalence of about 15% in high-income countries, depression represents a common mental disorder with an enormous social and economic impact [10]. To date, depression is

among the leading causes of disability and disease burden [34]. Depressive disorder is characterized by low mood and/or anhedonia, combined with several cognitive and vegetative symptoms. In many cases, these symptoms can be reduced by selective serotonin (5-HT) reuptake inhibitors (SSRIs), which are generally recommended for first line-treatment in depressive disorders [4]. The efficacy of these drugs as well as studies in depressive patients led to the monoamine hypothesis of depression, which postulates a prominent pathophysiological role of decreased monoamine levels, especially 5-HT, in depression [1].

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**Fig. 1.** Treatment regime for investigation of proliferation and survival of newborn cells. Adult 5-HT<sub>1A</sub>R overexpressing mice and controls were treated on three consecutive days with an i.p. injection of 50 mg/kg BrdU and killed either one day (proliferation (P)) or 21 days (survival (S)) after the last injection for immunohistochemical studies.

Among an impressive variety of identified 5-HT receptors, the 5-HT<sub>1A</sub> receptor (5-HT<sub>1A</sub>R) represents one of the most abundant subtypes expressed in the mammalian brain [25]. 5-HT<sub>1A</sub>Rs are located presynaptically as somatodendritic autoreceptors on 5-HT neurons, where their activation inhibits the firing rate of the serotonergic neurons, and are postsynaptically expressed in corticolimbic regions that are implicated in mood and emotion [2]. Over the last decades, they have been investigated intensely in the context of pathophysiology and treatment of depression. Indeed, animal studies as well as studies in depressive patients, including pharmacologic and genetic studies, propose that 5-HT<sub>1A</sub>Rs are important players in the mediation of antidepressant effects and antidepressant-like behavior [16].

More recent studies shed light on a possible involvement of altered adult neurogenesis in the pathophysiology of depression. For several drugs it has been demonstrated that adult hippocampal neurogenesis is required for the mediation of antidepressant-like behavioral effects in laboratory animals [26]. The proneurogenic effect seems to be, at least partly, mediated by 5-HT and some of its receptor subtypes with a proposed pivotal role for the 5-HT<sub>1A</sub>R [19,28]. However, the exact underlying mechanisms remain unclear, mainly due to the diverse external and internal influences on neurogenesis and the complexity of the serotonergic system with its various receptors and locations [13].

In 2004, Kusserow et al. generated a transgenic mouse line with a permanent 5-HT<sub>1A</sub>R overexpression (OE mice) in cortical, hippocampal and further limbic areas, while unaltered receptor densities were detected in the raphe nuclei, where serotonergic autoreceptors are located [14,20]. In the present study, we used this animal model to examine the role of postsynaptic 5-HT<sub>1A</sub>Rs on hippocampal adult neurogenesis in mice. Initially, we investigated morphometric parameters of the dentate gyrus (DG) and the prefrontal cortex (PFC), because of their relation to the serotoner-

gic system and/or involvement in adult hippocampal neurogenesis and depression [7,19,31]. Afterwards, proliferation, differentiation and survival of new-born neurons of OE mice were analyzed.

## 2. Materials and methods

All experiments were done in accordance with the European Communities Council Directive (86/609/EEC) and in compliance with the German Animal Welfare Act (T0270/13; G0013/14). Maximum care was taken for optimizing welfare and to minimize the number of animals used.

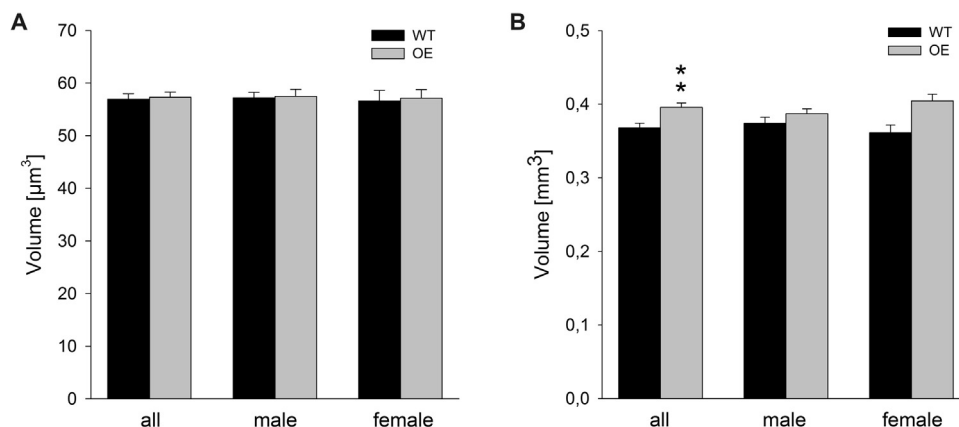
### 2.1. Animals

The present experiments were carried out in a total of 72 transgenic mice (background: NMRI mice by Harlan-Winkelmann, Borcheln, Germany) and NMRI WT mice of both genders, aged 10–13 weeks. In the OE animals, stable overexpression of postsynaptic 5-HT<sub>1A</sub>Rs, especially in the outer cortical layers and the CA3 region was demonstrated previously [14]. Furthermore, recent [<sup>35</sup>S]GTPγS binding studies revealed correct 5-HT<sub>1A</sub>R coupling and functionality [23]. All mice were born and kept under controlled environmental conditions (23–25 °C, 50–60% humidity, 12 h light/dark cycle) and had free access to standard diet and water. To analyze proliferation and survival of newborn neurons, animals received intraperitoneal injections of bromodeoxyuridine (BrdU; 50 mg/kg body weight dissolved in 0.9% NaCl; Abcam) on three consecutive days (see Fig. 1). Animals were killed either one day (proliferation) or 21 days (survival) after the last injection aged 10 or 13 weeks, respectively.

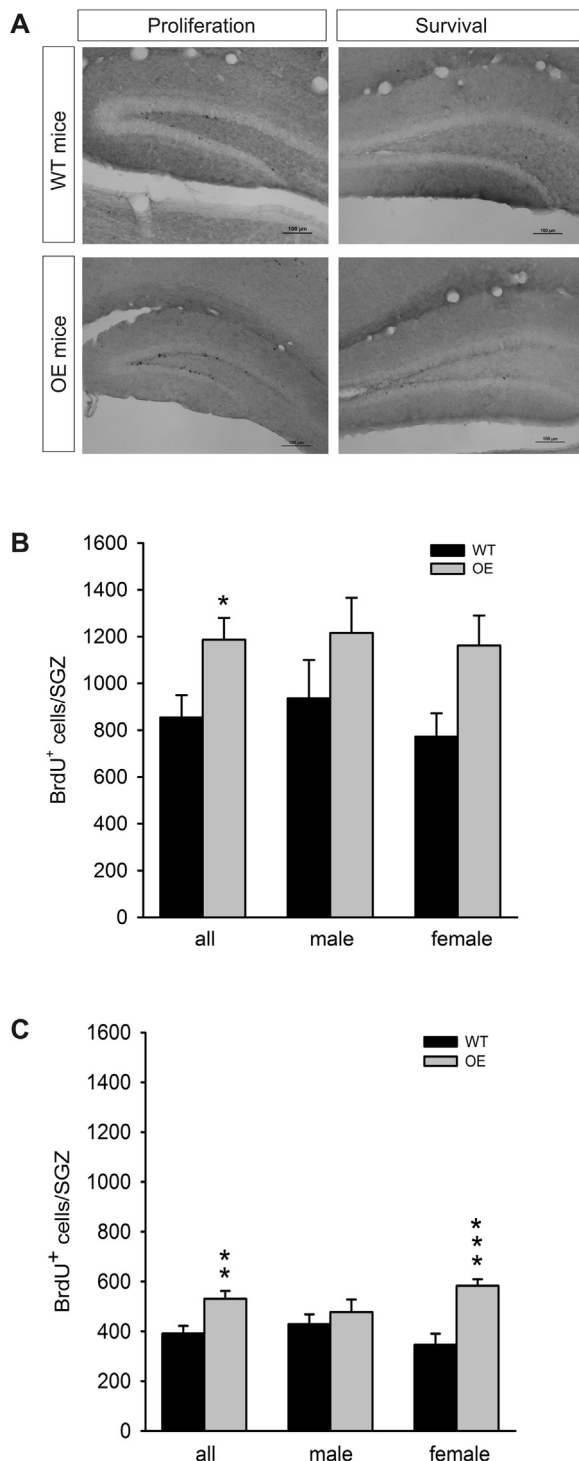
### 2.2. Immunohistochemistry

Mice were deeply anaesthetized with pentobarbital (100 mg/kg i.p.; Narcoren®, Merial GmbH) and transcardially perfused with 0.01 M phosphate-buffered saline (PBS; pH 7.4) followed by 4%-paraformaldehyde/PBS for 8 min. Brains were post-fixed for 24 h in 4%-paraformaldehyde and stored in 30%-glucose/PBS solution. The paraformaldehyde-fixed brains were cut in 40 μm coronal slices with a freezing microtome (Microm, Walldorf, Germany). Post-fixation and storage of brains or slices, respectively, was made at 4 °C.

For morphometric analyses, neurons were labelled with anti-NeuN. BrdU served as a marker for proliferating cells, type-1/2a cells were identified with the glial precursor marker Sox2 (SRY-



**Fig. 2.** Increased volumes of the DG in mice with overexpression of postsynaptic 5-HT<sub>1A</sub> receptors. Volumes of the infralimbic cortex of the PFC (A) and the dorsal DG (B) in OE mice and controls. Data are shown as means ± S.E. of 10 OE (5 male/5 female) and 10 WT (5 male/5 female) mice. Significant differences between genotypes are indicated by asterisks (\*\*p < 0.01). OE: 5-HT<sub>1A</sub>R overexpressing mice; WT: wild-type.



**Fig. 3.** Overexpression of postsynaptic 5-HT<sub>1A</sub> receptors increases proliferation and survival of newborn cells in the DG. A: Photomicrographs of DAB-stained BrdU-reactive cells in the SGZ of the DG of WT and OE mice. Immunohistochemistry was done one day (proliferation) or 21 days (survival) after the last BrdU injection. Data are shown as means  $\pm$  S.E. of 11 OE (5 male/6 female) and 10 WT (5 male/5 female; B, proliferation) or 12 OE (6 male/6 female) and 11 WT (6 male/5 female; C, survival) mice. Significant differences between genotypes are indicated by asterisks (\* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001). OE: 5-HT<sub>1A</sub>R overexpressing mice; SGZ: subgranular zone; WT: wild-type.

related HMG-box gene 2) and to analyze type-2b/3 cells the transient immature neuronal marker doublecortin (DCX) was used.

For BrdU staining, DNA was denatured in 2N HCl for 20 min at 37°C. Sections were rinsed in 0.1 M borate buffer, washed in Tris-

buffered saline (TBS) and stained free-floating with all antibodies diluted in TBS containing 3% donkey serum and 0.1% Triton X-100. Primary antibodies were applied in following concentrations: anti-NeuN (mouse, 1:1000; Merck Millipore) anti-BrdU (rat, 1:500; AbD Serotec), anti-doublecortin (anti-DCX; goat, 1:250; Santa Cruz Biotechnology), anti-Sox2 (goat, 1:250; Santa Cruz Biotechnology). For immunofluorescence, an Alexa488 conjugated secondary antibody (Dianova) was used at a concentration of 1:250. Fluorescent sections were coverslipped in Vectashield Mounting Medium (Vector Laboratories). Immunohistochemistry followed the peroxidase method with biotinylated secondary antibodies (1:250; Dako and Abcam), ABC Elite reagent (Vector Laboratories) and diaminobenzidine (DAB; Sigma Aldrich) and nickel (BrdU labelling; Sigma Aldrich) as chromogen.

### 2.3. Analysis

Analyzed brain regions were chosen in respect to their implication in hippocampal adult neurogenesis and/or depression. Adult neurogenesis is mainly restricted to the subgranular zone (SGZ) of the hippocampal DG and the subventricular zone (SVZ) of the lateral ventricle [18]. Newly born precursor cells originating from the SGZ migrate into the granular cell layer of the DG, where they get functionally integrated as principle granule neurons [32]. The infralimbic cortex as a well-defined part of the PFC was analyzed because of its relation to the serotonergic system and proposed implication in the pathophysiology of depression.

One-in-eight series of sections of each brain were stained with the peroxidase method for light microscopy (NeuN, BrdU and DCX labeling) and with immunofluorescence (Sox2 labelling). Immunoreactive cells and morphometric parameters were analyzed using a Zeiss Axioskop HBO 50/AC light microscope combined with Nikon NIS-Element Basic Research Image system or a Keyence BZ-9000 fluorescence microscope in combination with the image analysis system BZ 9000® (BZ II Viewer und Analyser, Keyence; volumes and total numbers of BrdU-reactive cells of the DG: AP –1.2 mm to 3.3 mm relative to bregma; volumes of the infralimbic area of the PFC: 1.3 mm to 2.00 mm relative to bregma). Areas were defined in accordance with the Mouse Brain Atlas from Franklin and Paxinos [12]. Numbers of the manually counted cells in the DG were multiplied by eight to obtain the total number of counted cells.

### 2.4. Statistical analysis

Statistical differences between the different genotypes and genders were evaluated by Two Way ANOVA (Sigma Plot 11.0). All values are expressed as mean  $\pm$  S.E. P values of <0.05 were considered statistically significant.

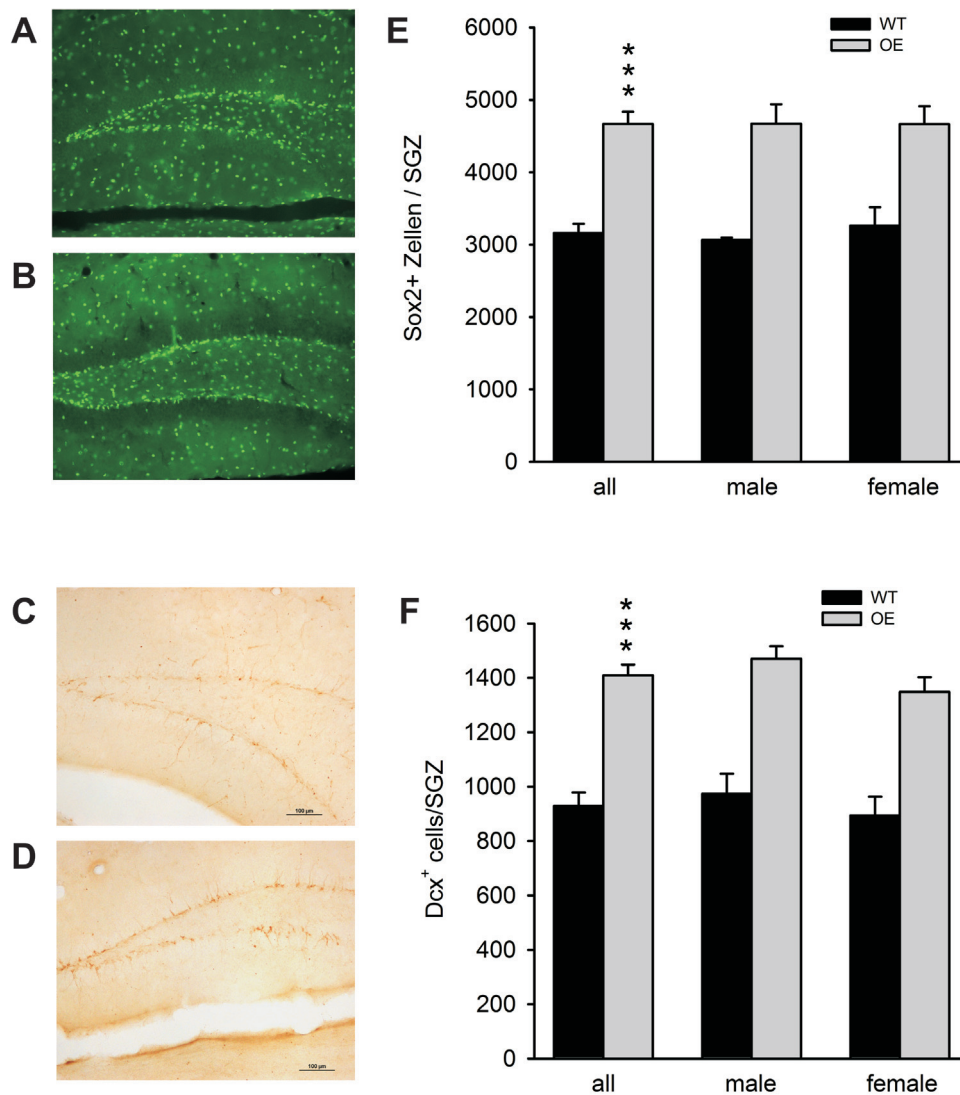
## 3. Results

### 3.1. Morphometric parameters

The volume of the DG was significantly increased in OE mice compared to controls ( $F_{(3,16)} = 10.467$ ;  $P = 0.005$ , see Fig. 2B) while the volume of the infralimbic cortex of the PFC was unaltered in OE mice in comparison to WT mice ( $F_{(3,16)} = 0.0666$ ;  $P = 0.800$ ; see Fig. 2A). In both regions, there were no gender-specific differences detected.

### 3.2. Proliferation and survival of newborn cells

The number of proliferating cells in the DG of young-adult (10 and 13 weeks) mice one day (proliferation) and 21 days (survival) after the last BrdU injection was significantly increased



**Fig. 4.** Overexpression of postsynaptic 5-HT<sub>1A</sub> receptors increases the number of stem and precursor cells in the DG. Photomicrographs of coronal brain slices of mice, showing the DG with the included SGZ (A–D). Sections were labelled with the precursor marker Sox2 (A: WT; B: OE) and the transient immature neuronal marker DCX (C: WT; D: OE). Data are shown as means ± S.E. of 8 OE and WT (each 4 male/4 female, (E)) or 10 OE (5 male/5 female) and 9 WT (4 male/5 female, (F)) mice. Significant differences between genotypes are indicated by asterisks (\*\*\*)  $p < 0.001$ . DCX: doublecortin; OE: 5-HT<sub>1A</sub>R overexpressing mice; SGZ: subgranular zone; WT: wild-type. Magnification: (A + B) 60-fold, (C + D) 50-fold.

in OE mice compared to WT controls (see Fig. 3). There were 38.86% more BrdU-reactive cells one day ( $F_{(3,17)} = 5.901$ ;  $P = 0.027$ ; Fig. 3B) and 33.06% more newborn cells 21 days ( $F_{(3,19)} = 12.160$ ;  $P = 0.002$ ; Fig. 3C) after the last BrdU injection in OE mice. According to previously observed sex differences, we could detect notably more survival cells in the DG of OE mice of the female subgroup ( $P < 0.001$ ), while no changes in cell survival were observed in male OE vs. male WT mice ( $P < 0.397$ ). These gender specific changes were not observed in the number of newly generated cells.

### 3.3. Differentiation of newborn cells

Next, we quantified the number of stem/progenitor cells in the DG of adult (10 weeks) OE and WT mice. To determine type-1/2a cells brain slices were stained with the precursor marker Sox2 (Fig. 4A (WT) and B (OE)), to analyze type-2b/3 cells the transient immature neuronal marker DCX was used (Fig. 4C (WT) and D (OE)). The number of Sox2-reactive cells was significantly increased (+47.58%) in OE mice compared to controls (Fig. 4E;  $F_{(3,12)} = 45.278$ ;  $P < 0.001$ ). There were also 51.61% more DCX-reactive cells in mice

overexpressing the postsynaptic 5-HT<sub>1A</sub>R (Fig. 4F;  $F_{(3,15)} = 61.072$ ;  $P < 0.001$ ). Both findings were not confirmed in the male and female subgroups.

## 4. Discussion

An altered adult neurogenesis is thought to be involved in the pathogenesis of various affective disorders [5]. However, the regulation of this phenomenon by different neurotransmitters, e.g. serotonin [3], and receptor types has to be further elucidated. In the present study, we used a transgenic mouse line to examine the role of postsynaptic 5-HT<sub>1A</sub>R in this context. Proliferation and survival of newborn cells was significantly increased in mice with a permanent overexpression of the postsynaptic 5-HT<sub>1A</sub>R. In accordance with these findings, larger volumes of the dentate gyrus of OE mice in comparison to controls were detected. Additionally, transgenic mice showed increased numbers in type-1/2a cells and progenitor cells of the neuronal lineage. Our results are in line with previous studies, which demonstrated a proneurogenic effect and increased numbers of neuronal precursor cells after systemic administration

of the selective 5-HT<sub>1A</sub>R agonist 8-OH-DPAT [6,19]. We assume that especially increased activation of the postsynaptic 5-HT<sub>1A</sub>R may be involved in mediation of this effect. Furthermore, our studies emphasize a pivotal role of this receptor in adult hippocampal neurogenesis and support the hypothesis of a 5-HT<sub>1A</sub>R-modulated differentiation of newborn cells in the hippocampus [19].

Depression in patients has been shown to be related to reduced volumes of the PFC and hippocampus while treatment with SSRIs increases adult hippocampal neurogenesis or cortical gliogenesis [7,11,31]. We therefore hypothesized that overexpression of the postsynaptic 5-HT<sub>1A</sub>R in our transgenic mice might have an impact on the volume of the PFC. For our morphometric analysis, we choose the infralimbic cortex, which represents the neuroanatomical equivalent of the human subgenual PFC. This region is known to play a role in the antidepressant efficacy of drugs, in depressive-like behavior in rodents [21] and has been found to be altered in depressive patients [8]. However, in our study the volume of the infralimbic cortex of the PFC was unaltered in OE mice compared to WT controls. Despite the assumption, that function of the postsynaptic 5-HT<sub>1A</sub>R is not directly linked to morphologic alterations of the PFC this finding should not be overrated. The examined mice have a permanent overexpression of the postsynaptic 5-HT<sub>1A</sub>R and original influences might be masked by compensation mechanisms. For instance, it is known that early postnatal treatment with the SSRI fluoxetine damages the morphology of 5-HT neurons in young adult rats [30]. Additionally, the PFC is strongly influenced by other neurotransmitters, e. g. it is known that antidepressant effects can also be mediated by modification of the glutamatergic or cholinergic inputs to this brain region [35]. Altered volumes of the PFC are therefore probably a result of interplay of various altered neurotransmitter systems and/or mediators.

Interestingly, our study revealed gender differences in adult hippocampal neurogenesis, with increased survival of newborn cells in the female but not in the male OE mice. Gender-dependent alterations in OE mice have been demonstrated earlier, e.g. female OE mice show a surplus expression of 5-HT<sub>1A</sub>R in the CA2 area of the hippocampus, the parietal cortex and the hypothalamus compared to male OE mice [14]. Studies in rats as well as studies in humans also demonstrated increased densities of postsynaptic 5-HT<sub>1A</sub>R in females compared to males [15,24]. At least in humans, this may be related to decreased 5-HT synthesis rates in women, which have been reported earlier [22,27]. These observations are thought to have an impact on the vulnerability to develop depression, which is greater in female compared to male individuals, but also on the treatment success with therapeutics targeting the serotonin system [17,33]. In turn, the detected higher densities of postsynaptic 5-HT<sub>1A</sub>R in female OE mice compared to males might enforce a negative feedback mechanism for the 5-HT neurons of the raphe nuclei [29], which could explain the observed lack of an antidepressant-like behavior in the forced swim test in female OE mice in contrast to males previously [14]. In the same study, treatment with the SSRI citalopram revealed a clear antidepressant effect in female OE mice, which might correspond to the good response of female depressive patients to serotonergic drugs [17]. The results of our study propose an interlinkage of gender-related modified postsynaptic 5-HT<sub>1A</sub>R function and adult neurogenesis. In view of previous studies, relations to depressive-like behavior may also be drawn. The here investigated OE mice represent a unique opportunity to further elucidate these connections. Complex behavioral experiments are underway to advance this issue.

However, early precursor type-1/2a cells and progenitor cells of the neuronal lineage were increased in both genders. We therefore conclude that promotion of differentiation of newborn cells might be a gender-independent proneurogenic effect of postsynaptic 5-HT<sub>1A</sub>R. In contrary survival of hippocampal newborn cells is possibly triggered by female sex hormones or other gender-specific

physiological differences, which are suggested to play a role in the distinctions concerning prevalence of depression and response to antidepressive drugs in males and females [17]. Furthermore, survival of newborn cells seems to be strongly influenced by local synaptic activity [9], which is driven by inputs from other brain regions. The regions which are found to have a surplus overexpression of 5-HT<sub>1A</sub>R in female OE mice [14] are probably more relevant for this step of adult neurogenesis.

The results of our study suggest a pivotal role for the postsynaptic 5-HT<sub>1A</sub>R in adult hippocampal neurogenesis. In view of gender-dependent differences in depressive patients, the detected sex-related alteration in survival of adult-born neurons might initiate further critical insights for future developments of antidepressive therapeutics.

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