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Mice with ablated adult brain neurogenesis are not impaired in antidepressant response to chronic fluoxetine

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ABSTRACT

The neurogenesis hypothesis of major depression has two main facets. One states that the illness results from decreased neurogenesis while the other claims that the very functioning of antidepressants depends on increased neurogenesis. In order to verify the latter, we have used cyclin D2 knockout mice (cD2 KO mice), known to have virtually no adult brain neurogenesis, and we demonstrate that these mice successfully respond to chronic fluoxetine. After unpredictable chronic mild stress, mutant mice showed depression-like behavior in forced swim test, which was eliminated with chronic fluoxetine treatment, despite its lack of impact on adult hippocampal neurogenesis in cD2 KO mice. Our results suggest that new neurons are not indispensable for the action of antidepressants such as fluoxetine. Using forced swim test and tail suspension test, we also did not observe depression-like behavior in control cD2 KO mice, which argues against the link between decreased adult brain neurogenesis and major depression.

1. Introduction

Major depression is a common mental disorder, however, its pathophysiology remains poorly understood. Stress, the main cause of depression, most severely affects hippocampal formation (McEwen et al., 2012), while hippocampal subgranular zone (SGZ) of the dentate gyrus (DG) is a region where new neurons originate throughout mammalian adulthood. Moreover, stress leads to significant decrease of adult neurogenesis (for review see Warner-Schmidt and Duman, 2006), implicating a role of this decrease in the onset of depression-like symptoms, however, this notion remains controversial (for reviews see Sahay and Hen, 2007; Balu and Lucki, 2009; Eisch and Petrik, 2012).

Hippocampal neurogenesis has also been proposed as a crucial process involved in the therapeutic efficacy of chronic antidepressants (ADs) treatment (*e.g.*, Santarelli et al., 2003). This hypothesis is supported by the fact that recovery from depression requires several weeks – the timescale that overlaps with the time-course of AD-stimulated neurogenesis and is also paralleled with the time needed for differentiation and incorporation of newborn neurons into existing neuronal hippocampal networks (Sahay and Hen, 2007; Balu and Lucki, 2009). Moreover, majority of the antidepressant approaches elevate neurogenesis by increasing proliferation rate and/or by enhancing newborn cells survival (for review see Samuels and Hen, 2011). Indeed, it was shown that animals with blocked adult neurogenesis do not recover from depression-like behavior when chronically administered with ADs (Santarelli et al., 2003; Surget et al., 2008; David et al., 2009; Onksen et al., 2011; Perera et al., 2011). However, recent studies suggest both neurogenesis-dependent and independent mechanisms underlying ADs action, as more studies show none or only partial effect of reducing neurogenesis on restoration of behavioral homeostasis by ADs (Meshi et al., 2006; David et al., 2007; Holick et al., 2008; Surget et al., 2008; Bessa et al., 2009a; David et al., 2009; Singer et al., 2009; Nollet et al., 2012).

Herein, we have employed cyclin D2 knockout (cD2 KO) mice showing lack of adult brain neurogenesis. We showed before that mice with mutated cyclin D2 gene display largely impaired

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proliferation of neuronal precursors in SGZ (Kowalczyk et al., 2004; Jaholkowski et al., 2009; Jedynak et al., 2012). Notably, cyclin D2 mutant mice show deficits in some hippocampal-dependent behaviors (Jedynak et al., 2012), but not in learning in general (Jaholkowski et al., 2009; Jedynak et al., 2012; Urbach et al., 2013), while selected cognitive functions are impaired (Ben Abdallah et al., 2013; Garthe et al., 2014).

In the present study, we set out to test the reaction of cD2 KO mice to chronic fluoxetine administration following chronic stress. We have chosen unpredictable chronic mild stress (UCMS) as known to cause ADs-reversible behavioral changes in rodents that parallel symptoms of major depression (Willner, 2005). This paradigm was also used in the original study suggesting a causal link between ADs efficacy and adult neurogenesis (Santarelli et al., 2003). Also, we have chosen forced swim test (FST) as one of the most widely used test of ADs action (Petit-Demouliere et al., 2005; Krishnan and Nestler, 2011), which results in reduced immobility following their acute (Porsolt et al., 1977a, 1977b) and chronic delivery (*e.g.*, Detke et al., 1997; Dulawa et al., 2004; Holick et al., 2008; Lin and Wang, 2014) as well as in reversal of increased immobility following UCMS (Willner, 2005). Finally, we have used fluoxetine as most commonly used AD in adult neurogenesis-depression studies, *e.g.*, in the original Santarelli et al. (2003) publication.

2. Materials and methods

2.1. General experimental design

A cohort of animals (cD2 KO, $n = 30$; WT, $n = 30$) was subjected to unpredictable chronic mild stress (UCMS) and chronically administered with fluoxetine. When stress and fluoxetine administration ended, all animals were tested behaviorally in the forced swim test (FST) and cell proliferation was assessed in DG using bromodeoxyuridine (BrdU) injection and immunocytochemistry. There was also an open field control test performed 24 h after the last fluoxetine administration. The overall design of experimental procedures is shown in Fig. 1.

2.2. Animals

Cyclin D2 mutant mice (Kowalczyk et al., 2004) were backcrossed into C57BL/6 background over 10 generations and kept as cyclin D2 heterozygotes (+/−). Their homozygous progeny, cD2 KO (−/−) and WT (+/+) littermates, were used. If not stated otherwise, the animals were kept under a natural light/dark cycle in Plexiglas cages with water and food provided *ad libitum*. To ensure proper care and use of laboratory animals, the national rules according to the Animal Protection Act, ensured by the permission from the First Warsaw Local Ethics Committee for Animal Experimentation, were strictly followed. The animals were males, 2–4 months old at the beginning of the experiments, with their age carefully matched between WT and cD2 KO mice. Experimenters were always unaware of the genotype of the mice.

2.3. Unpredictable chronic mild stress (UCMS) and chronic fluoxetine treatment

The mice (cD2 KO, $n = 29$; WT, $n = 29$) were divided into three age-matched groups (non-stressed, $n = 10$; stressed-vehicle, $n = 10$; stressed-fluoxetine, $n = 9$). UCMS protocol was described before (Bisaz et al., 2011; Bisaz and Sandi, 2012) and used with some modifications. Non-stressed and stressed groups were housed in separated and closed housing racks located in the same room during the duration of the stress procedure. As the experiment started, non-stressed groups were left undisturbed. Stressed groups were exposed to UCMS procedure which consisted of different kinds of stressors: cage tilting, damp sawdust, housing in an empty cage, pairing with another stressed animal, cold room, water or food deprivation, inversion of the light/dark cycle, lights on for a short period of time during the dark phase and switching cages amongst stressed animals. One or two of these stressors were applied daily at different times and following a semi-random schedule. Starting from the beginning of the 4th week of UCMS, stressed mice were given either 10 mg/kg/day (comp. Santarelli et al., 2003; Bessa et al., 2009a) fluoxetine hydrochloride (Sigma, PL), dissolved in water and prepared freshly before use, or water. The treatment lasted for 3 weeks and was provided *via* oral gavage (Fine Science Tools Inc., USA). The drug concentration was adjusted weekly from the average body weight of mice to achieve the desired doses.

2.4. Forced swim test (FST)

In order to eliminate the effects of acute fluoxetine injections, FST was performed 3 days after the last delivery of the drug. The test was conducted as described by Porsolt et al. (1977a, 1977b) by placing mice into a glass cylinder (25 cm height, 10 cm diameter) containing 1250 ml of water maintained at 24–25 °C. The test lasted for 6 min. Starting from the 3rd minute of the test, mice were rated for immobility defined as the absence of active, escape-oriented behaviors such as swimming, jumping, climbing, or diving. Two animals were tested simultaneously.

2.5. Tail suspension test (TST)

TST procedure was previously described (Steru et al., 1985). Mice were suspended by the tail temporarily attached to a metal bar using adhesive scotch tape. Total duration of immobility was counted during a 6 min test. Three animals were tested simultaneously. One WT animal climbing its tail was excluded from the experiment.

2.6. Open field

The test was performed 24 h after the termination of UCMS and fluoxetine treatment, it was done as described previously (Jedynak et al., 2012). The apparatus was a wooden floor (50 cm × 50 cm) surrounded by 34 cm high walls. Gray walls and floor as well as

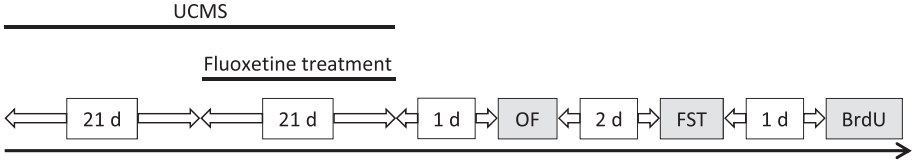

Fig. 1. Schematic plan of experimental procedures and timetable. Mice were subjected to unpredictable chronic mild stress (UCMS) for 6 weeks. Fluoxetine or water was given during the 4th–6th week of UCMS. After 3 days interruption, depression-like behavior was evaluated using forced swim test (FST), then the animals were injected with BrdU and perfused for immunocytochemistry. Control open field (OF) was performed 24 h after the last fluoxetine treatment.

normal room illumination were used to prevent suppression of activity and exploration behaviors by the anxiety response. Animals' behavior was monitored by a video camera placed above the center of the apparatus. Mice were put individually in one corner of the open field facing the wall and were allowed to explore freely for 5 min. Four open fields were employed to test four animals simultaneously. The floor of the apparatus was cleaned with 5% ethanol after each session. Data were analyzed using EthoVision 8.0 system (Noldus Information Technology, The Netherlands), and the total distance moved was acquired.

2.7. Perfusion and sectioning

To assess changes in cell proliferation in DG after chronic stress and chronic fluoxetine treatment, 24 h after completion of behavioral testing, mice were injected once with bromodeoxyuridine (150 mg/kg, i.p., Sigma, prepared in PBS, pH 7.4). Two hours after injection, mice were anesthetized and killed by cardiac perfusion (4% paraformaldehyde). Brains were then post-fixed overnight in 4% paraformaldehyde at 4 °C then transferred into a 30% sucrose/0.1% sodium azide cryoprotectant until the brains sank. Brains were embedded in tissue freezing medium, and sectioned using a cryostat to cut 50 μm coronal sections. Six to eight sections from each brain were analyzed, −1.28 to −2.75 mm from the bregma.

2.8. Bromodeoxyuridine immunohistochemistry

BrdU detection was performed as described elsewhere (Kowalczyk et al., 2004), with modifications. Free-floating sections were rinsed with PBS, then incubated in 2 N HCl for 30 min at 37 °C and neutralized by immersing in 0.1 M sodium tetra-borate buffer for 10 min. Sections were rinsed again in PBS and PBS-TX containing 0.01% Triton x-100 and then blocked with 10% normal donkey serum (NDS) for 1 h. Primary antibody (sheep anti-BrdU in 1% NDS, 1:500, Abcam, UK) was applied with overnight incubation. The following day, sections were washed of primary antibody, and incubated for 1 h with Alexa 488 secondary antibody (donkey anti-sheep in PBS, 1:500, Abcam, UK). Sections were mounted with Fluoromount-G containing DAPI nuclear marker (Southern Biotech., USA) and coverslipped. The mean numbers of BrdU+ cells per section in SGZ of DG were calculated, using ×40 magnification of an Olympus fluorescent microscope.

2.9. Data analysis

Statistical analyses were performed with GraphPad Prism 5.00 (GraphPad Software, USA). Data are reported as mean + SEM (if distributed normally) or as median with inter-quartile range (IQR), minimum and maximum values (when not distributed normally). When possible, data were submitted to ANOVA or a non-parametrical Mann–Whitney test was used (indicated in the text).

3. Results

3.1. Mutant cD2 KO mice did not show increased immobility

Basal depression-like behavior, behavioral response to UCMS as well as chronic fluoxetine treatment were assessed in FST (Fig. 2A). Two-way ANOVA of no stress and stress-vehicle groups revealed effect of both the stress ($p < 0.001$, $F(1,39) = 24.0$) and genotype ($p < 0.01$, $F(1,39) = 9.3$). The latter effect was confirmed by one-way ANOVA: cD2 KO mice showed decreased time [%] spent immobile compared to WT animals (cD2 KO mice, 38.6 ± 3.4; WT mice, 51.5 ± 3.5; $F(1,19) = 6.9$, $p < 0.05$). We further confirmed this observation by testing immobility in FST and tail suspension test (TST) using separate groups of mice (not shown). In both tests, non-stressed, naïve cD2 KO mice presented reduced immobility when compared to WT mice. The decrease in immobility duration [%] was observed in FST (cD2 KO mice, 35.5 ± 6.4, $n = 9$; WT mice, 53.7 ± 3.1, $n = 11$; $F(1,19) = 3.5$, $p < 0.05$, one-way ANOVA) and TST (cD2 KO

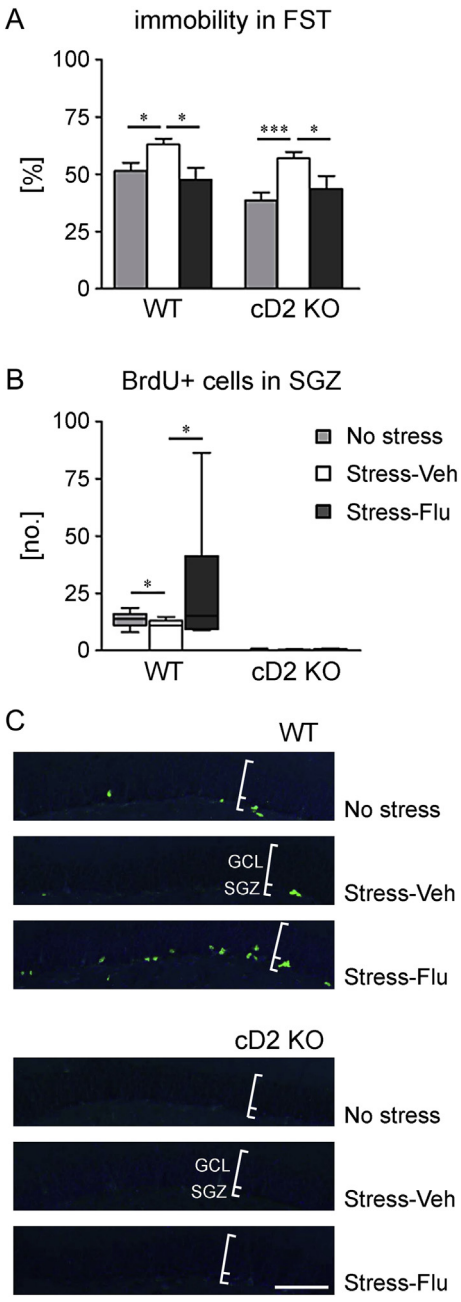

Fig. 2. Behavioral and cellular response to chronic stress and fluoxetine treatment in cD2 KO and WT mice. In FST (A), behavioral effects of chronic stress (Stress−Veh) were manifested by increased immobility time in both cD2 KO and WT animals. Chronic fluoxetine treatment (Stress−Flu) abolished this difference independently of the genotype as both cD2 KO and WT animals administered with fluoxetine showed immobility time similar to non-stressed mice. In WT mice, chronic stress caused significant decrease in the number of BrdU+ cells in the SGZ of the hippocampal formation (B, C). The effect was prevented by chronic fluoxetine treatment. There was no effect on neurogenesis in cD2 KO animals in any conditions. The mean number of new cells per slice (B) is shown along with representative parts of DG granule cell layer (GCL) with the SGZ and GCL areas indicated (C). Data shown represent the means + SEM (A) or medians with inter-quartile range, minimum and maximum values (B). *$p < 0.05$; ***$p < 0.001$; scale bar, 100 μm.

mice, 25.7 ± 1.6, $n = 6$; WT mice, 37.3 ± 3.9, $n = 6$, $F(1,11) = 7.5$, $p < 0.05$, one-way ANOVA).

3.2. Mutant mice show depression-like behaviors after stress and respond to fluoxetine treatment

The effect of stress was also confirmed as UCMS resulted in an increase of immobility time [%] in both cD2 KO (57.0 ± 2.7, $p < 0.001$, $F(1,19) = 17.5$) as well as WT mice (63.0 ± 2.5, $p < 0.05$, $F(1,19) = 7.1$, one-way ANOVA). Interestingly, cD2 KO and WT animals responded also equally well to chronic fluoxetine administration. Two-way ANOVA revealed effect of fluoxetine treatment ($p < 0.01$, $F(1,37) = 12.0$) but no effect of genotype. Both groups of fluoxetine-treated mice showed reduced immobility time [%] compared with vehicle-treated animals (cD2 KO mice, 43.6 ± 5.6, $p < 0.05$, $F(1,17) = 4.9$; WT mice, 47.6 ± 5.2, $p < 0.05$, $F(1,17) = 7.5$; one-way ANOVA).

Overall influence of fluoxetine treatment on animals activity was examined in the open field. One-way ANOVA analysis of the total distance moved reveal no effect of treatment in any group (cD2 KO mice, $F(2,28) = 0.2$, $p = 0.8$; WT mice, $F(2,28) = 1.35$, $p = 0.3$). Importantly, chronic fluoxetine did not increase the total distance [m] in cD2 KO mice (26.5 ± 2,0) when compared to vehicle-treated group (28.1 ± 1.9) and non-treated mice (28.5 ± 2.0). Fluoxetine treatment had a similar effect also in WT control animals (23.6 ± 1.7; 26.3 ± 2.1; 23.5 ± 1.4; respectively).

3.3. Neurogenic effect of chronic fluoxetine administration observed only in control animals

Detailed stereological study of adult neurogenesis in naïve cD2 KO mice in comparison to WT littermates was described elsewhere (Jaholkowski et al., 2009). In the present study, we confirmed that non-stressed cD2 KO mice showed almost complete lack of BrdU+ cells in SGZ of DG (0.0; 0.0–0.2, IQR) compared to WT mice (13.9; 13.2–15.5, IQR; $p < 0.001$, Mann–Whitney, $U = 0$; Fig. 2BC). Chronic stress decreased numbers of BrdU+ cells in WT animals (11.0; 0.2–12.6, IQR), $p < 0.05$, Mann–Whitney, $U = 18$) when compared to non-stressed ones, and had no effect in cD2 KO mice (0.1; 0.0–0.3, IQR; $p > 0.05$, Mann–Whitney). Chronic fluoxetine treatment caused significant increase of the number of proliferating cells in SGZ of WT mice when compared to vehicle-treated WT animals (15.2; 9.6–31.0, IQR, $p < 0.05$, Mann–Whitney, $U = 17$). There was no neurogenic effect of chronic fluoxetine administration in cD2 KO animals (0.3; 0.0–0.5, IQR; $p > 0.05$, Mann–Whitney).

4. Discussion

Despite numerous publications, the exact function of new neurons in the etiology and treatment of mood disorders remains elusive. In the present study, we used cD2 KO mice with ablated adult neurogenesis to investigate the role of this process in ADs action. We have found that fluoxetine treatment was efficient in the FST independently of the presence of newborn cells in the SGZ of mice. These data suggest that neurogenesis-independent mechanisms underlie, at least in some specific circumstances, the behavioral effect of fluoxetine, and that the increase in adult neurogenesis observed in WT animals is rather an epiphenomenon not necessary for the drug's direct effect.

There is limited direct evidence supporting reduced hippocampal neurogenesis in the pathophysiology of depression. Although reduced neurogenesis and depression-like phenotypes arise in tandem following various chronic stressors (*e.g.,* Surget et al., 2008; Bessa et al., 2009a), a requirement for reduced neurogenesis in the development of a depression-like phenotype has apparently been demonstrated only by Conboy et al. (2011). In our hands, however, cD2 KO mice, devoid of adult brain neurogenesis, did not show increased immobility in FST and TST tests. On the contrary, the immobility was reduced in these mice. We have observed before cD2 KO animals to be more active and explorative (Jedynak et al., 2012; Ben Abdallah et al., 2013). Please note, we were still able to increase immobility in mutant mice by UCMS and again reduce it through chronic fluoxetine treatment. The latter effect was apparently not a false positive result of an overall increase in mobility since chronic fluoxetine had no effect on the total distance moved in the open field.

Numerous observations suggest the role of adult brain neurogenesis in the treatment of depression-like behaviors (see Introduction). Importantly, in cD2 KO mice, we did not observe any increase in cell proliferation in the SGZ following chronic fluoxetine administration. It can be explained by the lack of cells targeted by fluoxetine or their proliferation impairment, as it is not clear whether the disruption of cyclin D2 gene causes developmental ablation of amplifying neural progenitors or their inability for divisions. The fact that suppression of depression-like symptoms occurred in both WT and cD2 KO mice, independently of the presence or lack of adult hippocampal neurogenesis points out a neurogenesis-independent mechanism behind chronic fluoxetine action. As concluded before by Bessa et al. (2009a), although temporal coincidence in the occurrence of impaired neurogenesis and depression-like symptoms and their reversal by ADs treatment are evident, a direct cause–effect relationship between neurogenesis and the alleviation of signs of depression-like behavior is missing. It was proposed that ADs, irrespectively of their mechanisms of action, trigger neuronal remodeling and synaptic plasticity and the increase in adult neurogenesis is rather an epiphenomenon (Bessa et al., 2009a). This notion is supported by recent finding that increase of adult hippocampal neurogenesis causes neither an anxiolytic nor antidepressant behavioral effect (Udo et al., 2008; Sahay et al., 2011). There is also accumulating evidence that other brain regions including amygdala, nucleus accumbens, or cingulate cortex are also involved in AD-like activity. It is possible as well that adult neurogenesis outside of the hippocampus may play a role in the effects of fluoxetine (Kokoeva et al., 2007). Nevertheless, our study supports a hippocampal neurogenesis-independent mechanism of fluoxetine-mediated behavioral effect.

It was suggested that fluoxetine acts independently of neurogenesis especially when tested by FST and TST, while other tests, *e.g.*, novelty-suppressed feeding (NSF) and novelty-induced hypophagia (NIH), are neurogenesis-dependent. However, this quality of FST and TST, when compared to other set of tests, was demonstrated only by one group, following rarely-used chronic corticosterone treatment (David et al., 2009; Mendez-David et al., 2014). Moreover, the treatment had no basal effect as corticosterone injections did not increase the immobility levels measured by FST (David et al., 2009, Fig. 1D) and TST (Mendez-David et al., 2014; Fig. 3P) in control mice, nor were acute vs. chronic effects of fluoxetine-delivery investigated. Furthermore, it is known that glucocorticoids affects food intake (*e.g.*, Solano and Jacobson, 1999) which is a crucial element of most of the postulated neurogenesis-dependent tests, *i.e.*, NSF, NIH. Also, using parallel FST and NSF protocols, the same group showed that behavioral effects of chronic fluoxetine in BALB/cJ mice do not require adult neurogenesis (Holick et al., 2008). Finally, Bessa et al. (2009a) demonstrated that reduction of adult neurogenesis in rats did not change the effectiveness of several ADs in both FST and sucrose preference test, while their efficacy was blocked in NSF. This observation added to the notion that NSF shows anxiolytic rather than antidepressant properties of investigated drugs (discussed in Bessa et al., 2009a;

Bessa et al., 2009b; David et al., 2009). Finally, the prevailing perception in the literature, suggests FST to be a proper test to show neurogenesis-dependence of antidepressant efficacy. Several groups described X-irradiation to block antidepressant effects using FST (*e.g.*, Zhu et al., 2010; Garza et al., 2012) while correlative observations linking antidepressant treatment effects in FST and adult hippocampal neurogenesis have been reported numerous times (Liu et al., 2008; Silva et al., 2008; Schmidt and Duman, 2010; Wainwright et al., 2011; Wang et al., 2011; Jiang et al., 2012; Lin and Wang, 2014; Lu et al., 2014).

Our experiment was designed to investigate solely the results of chronic fluoxetine treatment, since antidepressant effects of the drug can be observed following acute or short term use, *e.g.*, when administered 30 min before FST testing in mice (Holick et al., 2008). Therefore, 3 days interval between the last fluoxetine delivery and FST testing was introduced (Fig. 1) while even 1 day interval is frequently used and considered sufficient to eliminate short-term fluoxetine effects in rodents (*e.g.*, Castro et al., 2010). On the other hand, fluoxetine-induced adult-born neurons require weeks to become fully functional (Sahay and Hen, 2007), therefore we used 3 weeks plus 3 days between the introduction of the and FST testing. Three weeks of fluoxetine treatment of mice were shown before to have behavioral effects apparently dependent on adult brain neurogenesis (Santarelli et al., 2003; Surget et al., 2008; David et al., 2009). It was also shown that FST model provides a valid assessment of chronic, and not acute, fluoxetine action in mice (Holick et al., 2008).

Finally, we realize that our model and, hence, our findings have limitations. Mutant cD2 KO mice are constitutive knockout animals with the phenotype not limited to adult brain neurogenesis but also including reduction in size of several brain structures. There is also possibility of compensatory mechanisms to operate in these mice (discussed in Jaholkowski et al., 2009), however, the model is recognized as valuable and adequate tool in the field (for review see Frankland, 2013). The results obtained with the use of cD2 KO mice suggested the lack of relevance of adult brain neurogenesis for learning and memory in general (Jaholkowski et al., 2009) – a view which is now widely accepted (*e.g.*, Sahay et al., 2011) – while suggested and/or confirmed the role of this process in particular aspects of learning (Ben Abdallah et al., 2013), smell detection (Jaholkowski et al., 2009), species-typical behaviors (Jedynak et al., 2012), and alcohol consumption (Jaholkowski et al., 2011). Most of transgenic models with manipulated adult hippocampal neurogenesis, in contrast to our model, do not achieve complete ablation of newborn neurons. In case of depression etiology and treatment studies, partial suppression of adult neurogenesis can be considered as an advantage, as such model has the potential to imitate the rate of neurogenesis during aging in humans, which is reduced only partially (Balu and Lucki, 2009). On the other hand, the complete reduction of neurogenesis may prevent compensative effects from residual newborn neurons and potential intra- and inter-group variability. In conclusion, our studies do not support the role of adult neurogenesis in pharmacological treatment of depression with fluoxetine as well as put in question a strategy to search for novel therapeutic approaches aiming at cell-cycle stimulation and induction of adult neurogenesis (comp. Patricio et al., 2013).

Conflict of interest

All authors declare that they have no conflicts of interest.

Contributors

LK, RKF, and PJ designed the study. TK and CS introduced FST and UCMS techniques, respectively, into the laboratory. PJ performed the experiments and undertook the statistical analysis. PJ and RKF wrote the manuscript and prepared the figures. All authors contributed to and have approved the final manuscript.

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