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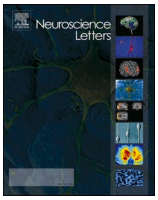

Fluoxetine increased adult neurogenesis is mediated by 5-HT3 receptor

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ABSTRACT

Adult neurogenesis is an aspect of structural plasticity that remains active during adulthood in some brain regions. One of them is the subgranular zone (SGZ) of the dentate gyrus of the hippocampus. Adult neurogenesis is reduced by different factors and in disorders of the CNS, including major depression. Antidepressant treatments, such as chronic fluoxetine administration, recover the normal level of adult neurogenesis. Fluoxetine treatment increases the free concentration of the neurotransmitter serotonin and this monoamine is implicated in the regulation of the neurogenic process; however, the target of the action of this neurotransmitter has not been fully elucidated. In this study, we have tried to determine the relevance of the serotonin receptor 3 (5-HT3) in the hippocampal neurogenesis of adult rats. We have used fluorescent immunohistochemistry to study the expression of the 5-HT3 receptor in different neurogenesis stages in the SGZ, identifying its expression in stem cells, amplifying neural progenitors and immature neurons. Moreover, we have studied the impact of a 5-HT3 antagonist (ondansetron) in the fluoxetine-induced adult neurogenesis. We observed that fluoxetine alone increases the number of both proliferating cells (ki67 positive) and immature neurons (DCX positive) in the SGZ. By contrast, co-treatment with ondansetron blocked the increase in proliferation and neurogenesis. This study demonstrates that the activation of 5-HT3 receptors is necessary for the increase of adult neurogenesis induced by fluoxetine.

1. Introduction

Adult neuronal structural plasticity is defined as the capacity of the central nervous system to perform adaptive morphological changes [1]. This phenomenon includes cell proliferation, cell migration, growth and remodeling of axons and dendrites or the formation of new synaptic contacts. In adulthood, structural plasticity is restricted to some specific regions, such as the hippocampus, prefrontal cortex, amygdala or olfactory bulb [2]. One of the few regions where adult neurogenesis remains is in the subgranular zone of the dentate gyrus of the hippocampus [3–5].

Hippocampal adult neurogenesis is a complex process modulated by physiological stimuli and can be altered under pathophysiological conditions. Hippocampal adult neurogenesis is increased by physical exercise, environmental enrichment and the action of steroids and several neurotransmitters [6]. Interestingly, exercise-induced neurogenesis seems to be mediated by an increase in the concentration of the neurotransmitter serotonin [7]. In fact, serotonin is known to directly induce hippocampal neurogenesis [8]. Hippocampal adult neurogenesis can be reduced by conditions such as chronic stress [9], age or some mood disorders such as major depression [10].

The presence of adult neurogenesis in the subgranular zone of the rodent hippocampus has been well described [5,11]. In this region, there is a population of stem cells (type 1), which can generate, by asymmetric division, a population of amplifying neural progenitors (type 2). These progenitors start a mitotic process, generating neuroblasts that migrate and differentiate into mature granule neurons in the upper layers of the dentate gyrus. These populations of cells have been characterized neurochemically and every stage of development can be identified attending to the expression of specific markers [11].

Several mood disorders have been related to alterations in structural plasticity, and particularly in adult neurogenesis. One of them is major

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depression, which in some cases has been related to decreases in the concentration of serotonin and, specifically in animal models, to reductions in adult hippocampal neurogenesis. In fact, many antidepressant treatments are based on the modulation of serotonin levels. Among them, one of the most prevalent is fluoxetine (commercially known as Prozac). Fluoxetine is a serotonin reuptake inhibitor, which leads to an increase in free serotonin concentration [12]. Previous studies in our laboratory and in others have demonstrated that fluoxetine induces changes in the expression of molecules related to structural plasticity, such as the Polysialylated form of the Neural Cell Adhesion Molecule (PSA-NCAM) [13,14]. Chronic treatment with fluoxetine induces an increment in cell activity in cortical regions [15], as shown by c-fos expression, and increased neurogenesis in the SGZ [16]. The mechanism of action of fluoxetine on PSA-NCAM expression revealed that it was mostly mediated by the ionotropic receptor for serotonin 5-HT3, since this receptor is expressed by PSA-NCAM positive neurons in cortical regions, such as the medial prefrontal cortex [14]. Moreover, the pharmacological manipulation of these receptors with fluoxetine and ondansetron induced changes in the expression of PSA-NCAM in the mPFC [14].

Recent studies have pointed to the possibility that, in some experimental conditions, adult neurogenesis could be mediated by 5-HT3 receptors. Therefore, we wondered whether the increased adult hippocampal neurogenesis observed after chronic treatment with fluoxetine may be also mediated by these receptors. To test this hypothesis, we have studied the expression of 5-HT3 receptors in the different cell phenotypes representing successive stages of adult hippocampal neurogenesis:

a) Stem/type 1 cells, using two intermediate filaments: the glial fibrillary acidic protein (GFAP) and nestin.
b) Proliferating cells, using the kinase dependent cycline of S-phase Ki67.
c) Immature neurons, using the microtubule associated protein doublecortin, DCX.
d) Mature granule neurons (using the mature neuron marker NeuN).

The aim of this study is to determine the role of 5-HT3 receptor activation in the increment of adult neurogenesis induced by serotonin. To achieve this, l, we have analyzed the effect on the different stages of adult hippocampal neurogenesis using fluoxetine, the specific 5HT3 antagonist ondansetron or the combination of the two molecules.

2. Material and methods

2.1. Animal treatments and histology

Twenty-four male Sprague–Dawley rats (4 months old, 320 ± 50 g Harlan Iberica) were used in this experiment. All animal experimentation was conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).Rats were divided into four groups (n = 6) and were treated (once a day) for 14 consecutive days, as follows. The first group received fluoxetine (10 mg/kg, intraperitoneal (i.p.), Sigma) and saline (30 min later). The second group received fluoxetine (10 mg/kg, i.p.) and the 5-HT3 receptor antagonist ondansetron (2 mg/kg, i.p., generous gift of Glaxo-Smithkline), 30 min later. The third group received a saline injection and ondansetron (2 mg/kg 30 min later). Finally, the fourth group received two injections of saline separated by 30 min. The volume injected in every i.p. injection was 500 μl. All drugs used were dissolved in saline. After treatment, rats were transcardially perfused under deep anesthesia, with saline solution followed by 4 % paraformaldehyde in sodium phosphate buffer 0.1 M, pH 7.4 (PB). After perfusion, the brains were extracted and cryoprotected in 30 % sucrose in PB. Coronal sections (50 μm, 10 per hemisphere) were obtained with a sliding microtome and stored at −20 °C in 30 % glycerol, 30 % ethylene glycol and 40 % PB until used.

2.2. Double immunofluorescence

Double immunofluorescence was performed to characterize the expression of the 5-HT3 receptor in the different steps of the neurogenic process and to characterize the effect of the different treatment in the number of proliferating cells and immature neurons in the subgranular zone of the dentate gyrus of the hippocampus. Control sections were used for the phenotypic characterization of 5-HT3 receptor expression. For the characterization of the effects of fluoxetine and ondansetron in adult neurogenesis, sections were coded prior to processing, the immunostaining was made in parallel with all sections to prevent effects of the incubation process and the code was maintained until finishing the cell quantification.

Tissue was processed 'free-floating' for immunofluorescence as follows. Briefly, sections were incubated for 1 min in an antigen unmasking solution (0.01 M citrate buffer, pH 6) at 100 °C. After cooling down the sections to room temperature, they were incubated for 1 h with 5 % normal donkey serum (NDS) (Jackson Laboratories) in PBS with 0.2 % Triton-X100 (Sigma) and were incubated overnight at room temperature with one of the following combinations:

a) Rabbit polyclonal anti −5-HT3 receptor (1:250, Calbiochem) and mouse monoclonal anti-GFAP (1:500, Sigma-Aldrich)
b) Rabbit polyclonal anti-5-HT3 receptor (1:250, Calbiochem) and mouse monoclonal anti-nestin (1:500, DSHB)
c) Rabbit polyclonal anti-5-HT3 receptor (1:250, Calbiochem) and mouse monoclonal anti-Ki67 (1:100, Novocastra).
d) Rabbit polyclonal anti-5-HT3 receptor (1:250, Calbiochem) and goat polyclonal anti-DCX (1:100, Santa Cruz).
e) Rabbit polyclonal anti −5-HT3 receptor (1:250, Calbiochem) and mouse monoclonal anti-NeuN (1:100, Chemicon).
f) Finally, for the quantification of cell proliferation and neurogenesis we have used mouse monoclonal anti-ki67 (1:100, Novocastra) and goat polyclonal anti-DCX (1:100, Santa Cruz) respectively.

After washing, sections were incubated with donkey anti-rabbit IgG, donkey anti-mouse IgG or donkey anti-goat IgG secondary antibodies conjugated with Alexa 488 or Alexa 555.

The companies of origin have previously tested the specificity of the different antibodies. Moreover, their specificity in rat tissue has been confirmed previously [17]. Overnight incubation of 5-HT3 antibody with an excess of its immunogenic peptide resulted in a total absence of 5-HT3 immunostaining in the hippocampus, as previously reported in the mPFC [14]. Additional controls for the immunohistochemical procedure were carried out in our laboratory by omitting the primary or secondary antibodies in each step of the immunohistochemical protocol.

2.3. Characterization of the phenotype of cells expressing the 5-HT3 receptor

All sections processed for fluorescent immunohistochemistry were mounted on slides and coverslipped using Dako Citomation mounting medium (Dako). Then, sections were observed under a confocal microscope (Leica TCS-SP2). Z-series of optical sections from the dentate gyrus (1 μm apart) were obtained using sequential scanning mode. These stacks were processed using ImageJ software.

Cells expressing the 5-HT3 receptor in the dentate gyrus were first identified using conventional fluorescence microscopy. Then, a stack of confocal images covering all their three-dimensional extension was taken to confirm the phenotype of these cells in every double immunofluorescence (GFAP, nestin, Ki67, DCX and NeuN).

2.4. Quantification of proliferating cells (Ki67 immunoreactive) and immature neurons (DCX immunoreactive) in the dentate gyrus of adult rats.

The number of proliferating cells or immature neurons in the dentate gyrus of the rat was estimated using a modified version of the fractionator method [18], as described before [19]. We counted cells covering 100 % of the sample area (granular layer of the dentate gyrus). The fractionator sampling scheme refers to the methodology of examining one out of every 10 brain sections. Thus, our modification of the optical dissector combined with a 1:10 fractionator sampling is truly a modification of the optical fractionator method. 1:10 systematic-random series of sections covering the whole rostral to caudal extension of this structure were viewed on an Olympus CX41 microscope. Cell somata were identified and counted with a 40X objective. Cells appearing in the upper focal plane were omitted to prevent counting cell caps. The volume of the different analyzed areas was determined for each animal using the Cavalieri's principle [20]. Means were determined for each experimental group and the data (after checking homocedasticity and normality) were subjected to one-way ANOVAs followed by Student–Newman–Keuls post hoc tests.

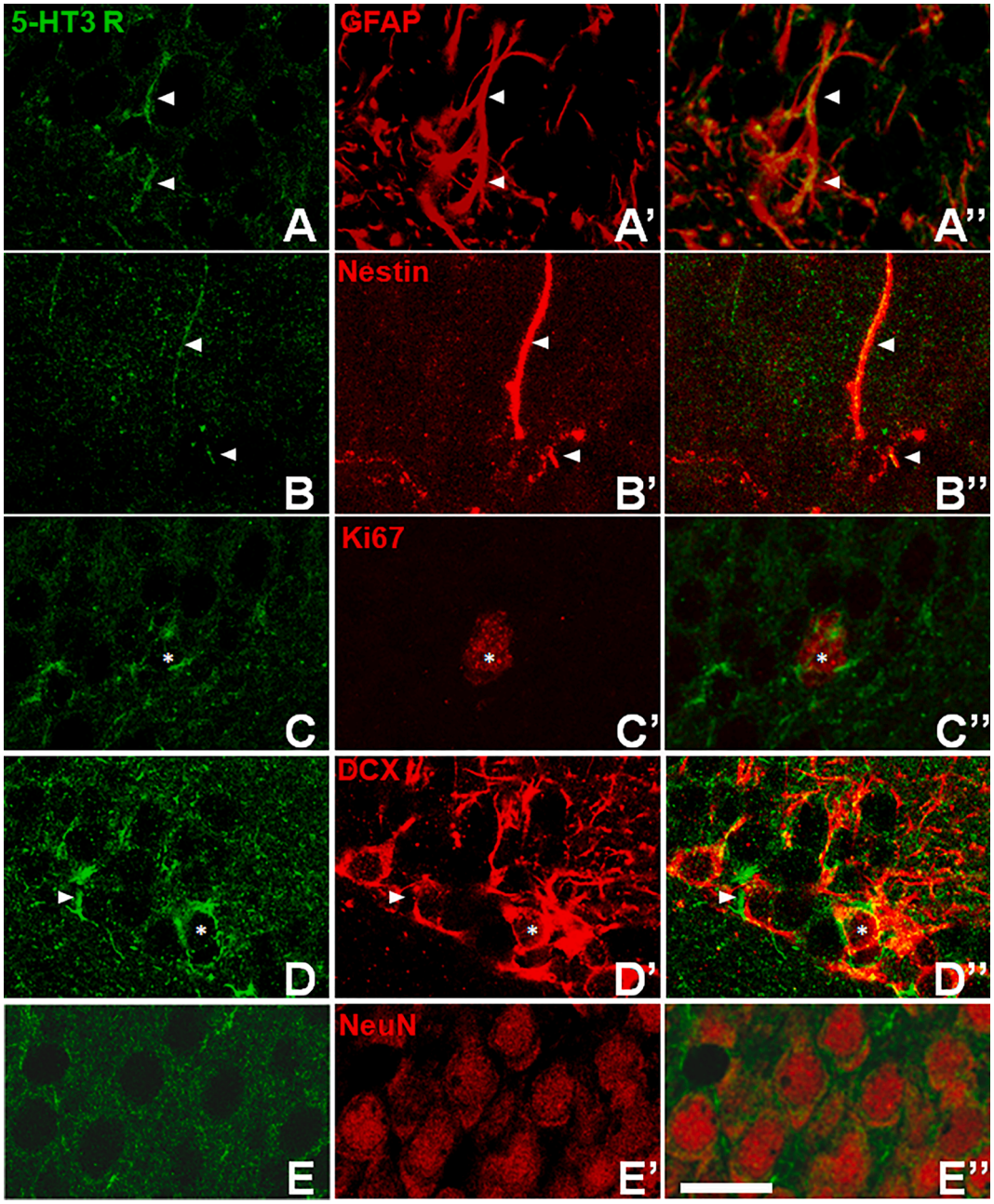

Fig. 1. Expression of the 5-HT3 receptor in stem/type 1cells and proliferating cells in the subgranular zone of the dentate gyrus of the hippocampus of adult rats. A. Expression of the 5-HT3 receptor in stem cells. Double labeling for 5-HT3 receptor (green) and Glial Fibrillary Acidic Protein (red), arrowheads indicate double-labeled cells. B. Double labeling for 5-HT3 receptor (green) and nestin (red), arrowheads point to processes positive for nestin, which also show 5-HT3 receptor expression. C. Expression of the 5-HT3 receptor in proliferating cells. Double labeling for 5-HT3 receptors (green) and Ki67 (red) in a proliferating cell. D. Expression of the 5-HT3 receptor in immature neurons. Double labeling for 5-HT3 receptor (green) and doublecortin (DCX, red). Asterisk marks an immature neuron soma expressing the 5-HT3 receptor. Arrowhead points to a 5-HT3 expressing cell lacking DCX labeling. E. Lack of expression of the 5-HT3 receptor in mature neurons. Double labeling for 5-HT3 receptor (green) and NeuN (red). Scale bar represents 20 μm.

3. Results

First, we analyzed the presence of the 5-HT3 receptor in the different stages of neurogenesis in the subgranular zone of the adult rat dentate gyrus (Fig. 1). We observed an intense expression of the receptor in stem/type 1 cells (Fig. 1A and B).These cells, located in the subgranular zone, express the intermediate filament GFAP and display a clear unipolar morphology (Fig. 1A). To confirm the nature of these cells, we performed double labeling with nestin antibodies, observing 5-HT3 receptor expression in nestin positive cells (Fig. 1B).The 5-HT3 receptor was also present in the proliferating cells in the SGZ, showing a Ki67-immunoreactive nucleus (Fig. 1C). Immature neurons (DCX-immunoreactive) also expressed the 5-HT3 receptor (Fig. 1D). Finally, mature neurons (NeuN-immunoreactive) lacked 5-HT3 receptor expression (Fig. 1E).

Considering the presence of 5-HT3 receptor at early stages of the adult hippocampal neurogenesis, we analysed whether fluoxetine-induced neurogenesis was mediated by 5-HT3 receptor. We administered fluoxetine, ondansetron and their combination in adult rats. We then quantified the number of proliferating cells (Ki67) (Fig. 2A), and the number of immature neurons (DCX) (Fig. 2B). Chronic fluoxetine treatment induced a significant increase in the number of proliferating cells in the dentate gyrus (1502 ± 86 cells in control rats vs 1995 ± 93 cells in fluoxetine treated rats, $p < 0.01$, F-value 6,042; DF 3; Power 0,922). By contrast, the co-treatment with fluoxetine and ondansetron reduced the number of proliferating cells to basal conditions (1557 ± 119 cells in fluoxetine + ondansetron treated rats, n.s. difference with control). The treatment with ondansetron alone had no effect over cell proliferation. When analyzing the number of immature neurons, the results were similar to those observed in proliferating cells (Fig. 2B). Chronic treatment with fluoxetine induced a significant increase in the number of immature neurons (17540 ± 1396 immature neurons in control vs 22860 ± 2352 immature neurons in fluoxetine treated rats, $p < 0.05$; F-value 8,552; DF 3; Power 0,985) and this effect was reverted when ondansetron was co-administered with fluoxetine (14750 ± 434 immature neurons in fluoxetine + ondansetron treated rats, n.s. difference with control).

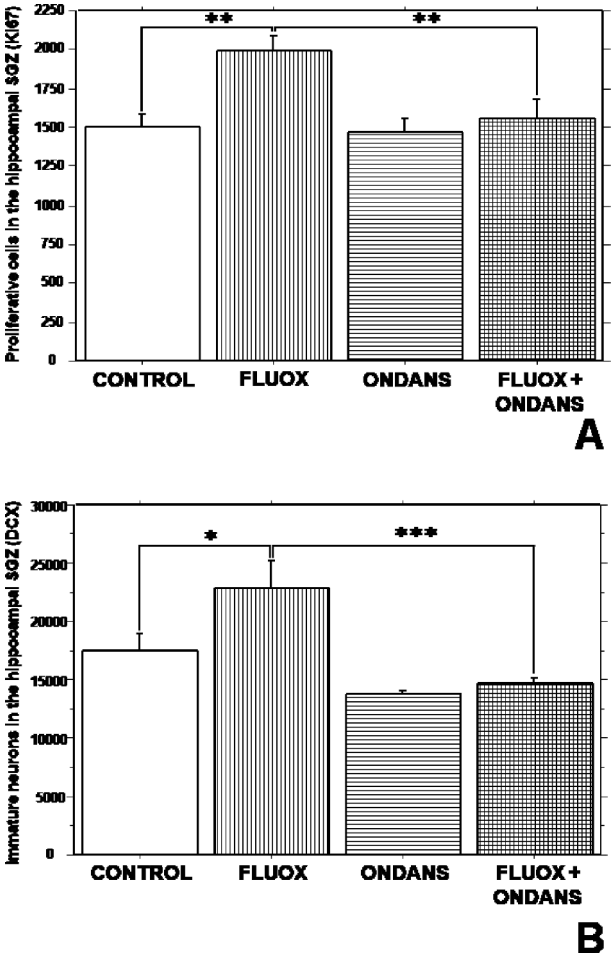

Fig. 2. Cell proliferation and neurogenesis in the hippocampus of adult rats after chronic fluoxetine, ondansetron and fluoxetine + ondansetron treatments. A. Graph representing the number of cells expressing Ki67 in the hippocampal sugranular zone (SGZ) in the different groups: control (white bar), fluoxetine (vertical striped bar), ondansetron (horizontal striped bar) and fluoxetine + ondansetron (squared bar). B. Graph representing the number of cells expressing Doublecortin in the hippocampal subgranular zone in the different groups: control (white bar), fluoxetine (vertical striped bar), ondansetron (horizontal striped bar) and fluoxetine + ondansetron (squared bar). (* $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$). Data represent mean ± SEM.

4. Discusion

In this study, we have analysed the expression of the 5-HT3 receptor in the SGZ of the dentate gyrus of adult rats. We have observed that this receptor is present in stem/type 1 cells, proliferating cells and to a lesser extent in immature neurons, but it is absent in mature granule neurons. We have observed a progressive decrease in the expression of the receptor, being higher in cells with mitotic activity (stem cells and proliferating cells) whereas it is reduced in immature neurons. Mature granule neurons did not express this receptor. Chronic treatment with fluoxetine increased the number of proliferating cells (Ki67+) and the number of immature neurons (DCX+). Co-treatment with an antagonist of 5-HT3, ondansetron, blocked the fluoxetine-dependent increase in proliferation and neurogenesis.

The neurotransmitter serotonin has a high impact over adult hippocampal neurogenesis. In fact, treatments that induce an increase in free serotonin concentration, such as fluoxetine, highly increase the number of proliferating cells in the SGZ of the dentate gyrus [11,21]. Previous studies have analysed the effect of fluoxetine treatment on structural plasticity showing its ability to increase the expression of a key marker of structural plasticity, the Polysialylated form of the Neural Cell Adhesion Molecule (PSA-NCAM), in several brain regions including the hippocampus [13,14].

Some studies have analysed the distribution of the different serotonin receptors in the hippocampus, observing expression of the 5-HT1A receptor in stem cells and neural amplifying progenitors, 5-HT2A receptor was observed in the hilus, mostly associated to glial cells, and, by contrast, 5-HT2B and 5-HT2C receptors were observed in mature granule neurons [22,23]. 5-HT3 receptor expression was described in hilar interneurons [22]. Recent studies have indicated the importance of 5-HT3 receptor in neurogenesis, describing that the increase in adult hippocampal neurogenesis induced by exercise was mediated by these receptors [7].

Previous studies from our group have observed a pivotal role for 5-HT3 receptors in the effects of fluoxetine on the induction of structural plasticity [14]. In a previous study, it was demonstrated that chronic treatment with fluoxetine increased the expression of PSA-NCAM in the rat mPFC. Moreover, PSA-NCAM was expressed in this region by interneurons, and these neurons expressed the 5-HT3 receptor. Finally, blocking the function of 5-HT3 receptor, by the use of the antagonist ondansetron, inhibited the increase of PSA-NCAM expression induced by fluoxetine [14]. PSA-NCAM is also expressed by newly born neurons in the SGZ.

In line with above mentioned evidence, we have analysed the 5-HT3 receptor expression in the SGZ of the adult rat dentate gyrus. We have found that the receptor is expressed by the initial stages of the neurogenic process (stem cells and early immature neurons). A previous study using a transgenic model expressing EGFP under the 5-HT3A receptor

promoter have shown the presence of the receptor in immature neurons in the subventricular zone and the rostral migratory stream, although they did not observe the SGZ [24]. In the hippocampus, former reports have observed a high expression of 5-HT3 receptors in the dentate gyrus [25]. A recent study using the same transgenic mice as in [24] has demonstrated also a high expression of the receptor in the dentate gyrus and found that positive cells displayed a morphology compatible with that of stem/type 1 cells and immature neurons [26]. However, this study lacks a neurochemical characterization of these cell populations.

In our study, we have observed that the presence of the 5HT3 antagonist ondansetron blocks fluoxetine-induced increase in neurogenesis. Our results are in accordance with previous studies that have observed an important role of 5-HT3 receptor in the adult hippocampal neurogenesis induction by serotonin mediated by physical exercise [7,27]. Chronic treatment with agonists of 5-HT3 receptors induces increases in neurogenesis and reverts depressive-like behaviors [27]. These results support partially our findings. The main difference is that the authors observed an additional increase in cell proliferation independent from fluoxetine treatment [27] suggesting that the activation of this receptor could be induced by mechanism no directly related to fluoxetine. It is interesting to note that in a mice model that lacks the 5-HT3A receptor, the treatment with fluoxetine was unable to increase the levels of neurogenesis [7].These results add weight to our conclusion that 5-HT3 receptors are fundamental for fluoxetine-induced neurogenesis, and that pharmacologically blocking 5-HT3 receptors appears to be enough to block the increase in neurogenesis mediated by fluoxetine. However, some studies have shown antidepressant effects of 5HT3 receptor antagonists, such as ondansetron [28–30], while some other studies found them ineffective [31]. In these studies, a chronic treatment with a low dose of ondansetron reverted depressive-like behaviors (0.5 mg/kg), but a higher dose (2.0 mg/kg), equal to the one used in our study, did not exert this effect [28,29]. The action of the newly multimodal antidepressant drug vortioxetine (which blocks serotonin reuptake, acting as an agonist for 5-HT1A and 5-HT1B receptors and partially antagonizing 5-HT1C, 5-HT3A and 5-HT7 receptors) [32,33] could be controversial with our results. First, increased adult neurogenesis is not the only feature of antidepressant treatment, structural plasticity [13] and cellular activity [15] can be also affected. Vortioxetine has multiple effects over the serotoninergic system, making to isolate the effect on 5-HT3 receptors, complicated. Moreover, studies analyzing the effect of vortioxetine over 5-HT3 receptors has shown that it initially induces a brief agonistic response followed by inhibition [34]. That could explain the apparent discrepancy.

Adult neurogenesis is a late component in the generation of neurons in the brain. In fact, the vast majority of new neurons are generated pre- and *peri*-natally. We wonder how serotonin, and specifically, the 5-HT3 receptor may affect the generation of neurons is these early stages. The presence of serotonin is low during embryogenesis. In rodents, serotonin initially reaches fetuses only from the placenta. From E16-17 serotonin begins to be produced in the fetus itself [35]. Serotonin induces cell proliferation in embryos [36]. An increased concentration of serotonin decreases the migratory speed of newly generated neurons [37]. Regarding the 5-HT3 receptor, its stimulation seems to have no effect on cell proliferation in this period [38], but have a crucial role on dendritic development of the embryonic newly generated neurons [39]. The difference with adult neurogenesis might be that neuronal progenitors in the embryo are fully activated whereas the neural progenitors during the adulthood remain quiescent and activation of 5-HT3 receptors can be necessary to activate them. Nevertheless, the difference can be originated by the fact that progenitors of granule are different type of progenitor from those generating other neuron types and therefore they could have different serotonin receptor expression.

In conclusion, in this study we have observed 5-HT3 receptor expression in the early stages of adult hippocampal neurogenesis in rats. We have observed that chronic treatment with fluoxetine increases neurogenesis and how ondansetron blocks such increase. From this study-two main ideas arise: a) 5-HT3 receptor activation is essential for the increase of adult hippocampal neurogenesis induced by fluoxetine treatment, which is likely induced by the increase of free serotonin; and b) 5-HT3 receptor activation is responsible for different aspects of the structural plasticity mediated by fluoxetine in the adult brain (affecting both adult neurogenesis and PSA-NCAM expression).

CRediT authorship contribution statement

I. Olivas-Cano: Investigation, Visualization, Writing – review & editing. **J.M. Rodriguez-Andreu:** Investigation, Writing – review & editing. **J.M. Blasco-Ibañez:** Visualization, Writing – review & editing. **C. Crespo:** Formal analysis, Writing – review & editing. **J. Nácher:** Conceptualization, Methodology, Writing – review & editing. **E. Varea:** Conceptualization, Methodology, Formal analysis, Visualization, Investigation, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors are unable or have chosen not to specify which data has been used.

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