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ORIGINAL ARTICLE

A novel 5HT3 receptor–IGF1 mechanism distinct from SSRI-induced antidepressant effects

M Kondo, Y Koyama, Y Nakamura and S Shimada

Depression is a common mental disorder affecting around 350 million people worldwide. Although selective serotonin reuptake inhibitors (SSRIs) are the most widely used antidepressants, a significant proportion of depressed patients do not achieve remission with SSRIs. In this study, we show that a serotonin type 3 receptor (5HT3R) agonist induces antidepressant effects as well as hippocampal neurogenesis independent of fluoxetine (a commonly used SSRI). Notably, our histological analysis reveals that 5HT3R and insulin-like growth factor 1 (IGF1) are expressed in the same neurons in the subgranular zone of the hippocampal dentate gyrus. Furthermore, our *in vivo* microdialysis analysis shows that 5HT3R regulates hippocampal extracellular IGF1 levels, and we also show that 5HT3R-dependent hippocampal neurogenesis is mediated by increased IGF1 levels. Altogether, our findings suggest a novel 5HT3R–IGF1 mechanism that is distinct from fluoxetine-induced responses and that provides a new therapeutic target for depression, especially bringing significant benefits for SSRI-resistant depressed patients.

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INTRODUCTION

Depression is a common mental disorder. According to a World Health Organization report, around 350 million people of all ages suffer from depression globally.[1] Selective serotonin reuptake inhibitors (SSRIs) are the most widely used antidepressants. However, a significant proportion of depressed patients do not achieve remission with SSRIs.[2] Therefore, development of novel and effective antidepressants is highly desirable, particularly for SSRI-resistant patients.

Adult neurogenesis occurs in the subgranular zone of the hippocampal dentate gyrus in a large number of animal species including humans.[3,4] Adult neurogenesis involves several steps and includes proliferation of adult neural stem cells or progenitors, differentiation into immature neurons, survival, neuronal maturation and integration into existing neural circuitry.[4] Previous studies have shown that adult hippocampal neurogenesis is influenced by various factors and stimuli, with its modulation suggested to foster behavioral responses by regulating certain brain functions, such as cognition and mood, under physiological and pathological conditions.[4–7] Further, antidepressant treatment increases hippocampal neurogenesis, while the importance of neurogenesis for the behavioral effects of antidepressants has been demonstrated.[4–10] Indeed, SSRI treatment results in increased proliferation of neural precursor cells in the adult dentate gyrus,[8] and enhanced hippocampal neurogenesis is required for the behavioral effects of SSRI treatment.[6,9,10] Moreover, a recent study found that serotonin type 1 A receptor (5HT1AR) in the hippocampal dentate gyrus is necessary for SSRI fluoxetine-induced hippocampal neurogenesis and its antidepressant response.[11]

The serotonin type 3 receptor (5HT3R) is the only ionotropic receptor in the 5HTR subfamily and is expressed in the limbic system, including the hippocampus, amygdala and prefrontal cortex.[12] 5HT3R is reported to play important roles in cognitive and emotional function,[13,14] although possible roles of 5HT3R in behavioral and neurogenic responses to SSRI treatment have not been defined. Exercise provides neurogenic and antidepressant effects,[15,16] and we recently demonstrated that 5HT3R is essential for exercise-induced hippocampal neurogenesis and antidepressant effects.[17] Therefore, to provide improved understanding of therapeutic targets for SSRI-resistant depressed patients, we sought to determine the mechanisms of 5HT3R action on neurogenic and antidepressant effects in comparison with fluoxetine, a commonly used SSRI.

MATERIALS AND METHODS

Mice

5HT3AR subunit-deficient (*Htr3a*$^{-/-}$) mice[18] and 5HT3AR-EGFP transgenic mice[19] were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and Mutant Mouse Regional Resource Center (Chapel Hill, NC, USA), respectively. *Htr3a*$^{-/-}$ mice were backcrossed with C57BL/6J mice for at least 10 generations. Here, 7- to 9-week-old male wild-type (C57BL/6J) and *Htr3a*$^{-/-}$ mice and 10- to 12-week-old male 5HT3AR-EGFP transgenic mice were used. Previous studies have shown that EGFP expression reflects normal 5HT3AR expression in 5HT3AR-EGFP transgenic mice.[19–22] All mice received standard lab chow and water *ad libitum*. All animal protocols were approved by the Institutional Animal Care and Use Committee at Osaka University Medical School.

Tail suspension test

The tail suspension test was performed as previously described.[17] A selective 5HT3 receptor agonist, SR 57227A (4-amino-1-(6-chloro-2-pyridyl)-piperidine hydrochloride; Sigma, St Louis, MO, USA),[23] or fluoxetine (Sigma) was intraperitoneally administered. Thirty minutes after single administration (acute) or following 3 weeks' daily drug treatment (chronic), mice were suspended by adhesive tape placed approximately 2 cm from the tip of the tail, and the tail was taped to a piece of suspended tubing. Immobility time was recorded during a 6-min test session. Data

Department of Neuroscience and Cell Biology, Graduate School of Medicine, Osaka University, Suita, Osaka, Japan. Correspondence: Dr M Kondo, Department of Neuroscience and Cell Biology, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan.
E-mail: mkondo@anat1.med.osaka-u.ac.jp
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acquisition and analysis were performed using CompACT FSS Ver.2 software (Muromachi Kikai, Tokyo, Japan).

Assessment of spontaneous activity

Spontaneous activity of mice was measured for 10 min using a Supermex and photocell beam system (Muromachi Kikai), as previously described.[17]

Experimental timeline in neurogenesis analysis

Mice were intraperitoneally administered with drugs or saline for 21 days as follows—drug doses: SR 57227A, 5 mg kg^{-1} and fluoxetine, 20 mg kg^{-1}; groups: Sal, saline (days 1–21); Sal+SR, saline (days 1–18) and SR 57227A (days 19–21); SR, SR 57227A (days 1–21); Sal+Flx, saline (days 1–18) and fluoxetine (days 19–21); Flx, fluoxetine (days 1–21); Flx+SR, fluoxetine (days 1–21) and SR 57227A (days 19–21). Bromodeoxyuridine (BrdU; Sigma) (50 mg kg^{-1}) was intraperitoneally injected two times, 2 h apart on day 21. Mice were killed 24 h or 4 weeks after the first BrdU injection for neurogenesis analysis (Figure 2).

For the drug microinjection study, mice were intraperitoneally administered with saline, SR 57227A (5 mg kg^{-1}), or fluoxetine (20 mg kg^{-1}), as well as 1 µl of vehicle or AG 1024 (1 mM; Selleck, Houston, TX, USA) microinfused into the hippocampus. Three hours after drug treatment, BrdU was intraperitoneally injected (100 mg kg^{-1}) and mice were killed 24 h after BrdU injection for neurogenesis analysis (Figure 5).

SR 57227A at 5 mg kg^{-1} was selected as the minimal dose providing maximal neurogenic effects[17] and antidepressant effects in our behavioral tests (Figure 1). Fluoxetine at 20 mg kg^{-1} was selected as the dose providing maximal antidepressant effects with no effect on spontaneous activity in our behavioral tests (Figure 1). Additionally, serum fluoxetine levels following chronic treatment with this dose were found to be towards the high end of plasma levels found in patients taking 20–80 mg per day fluoxetine, which is the clinical range used to treat major depressive disorders.[24–26]

Immunohistochemistry

Mice were deeply anesthetized and perfused with 4% paraformaldehyde in phosphate-buffered saline, or with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer. Brains were then removed, further fixed overnight at 4 °C and cryoprotected in sucrose solutions. Coronal sections of the entire hippocampus (20 µm for neurogenesis analysis; 30 µm for others) were prepared using a cryostat. For immunohistochemical detection of BrdU, sections were incubated in 2 M HCl at 37 °C for 30 min, and then neutralized in 0.1 M borate buffer (pH 8.5) for 10 min, as previously described.[27] After blocking with 3% bovine serum albumin in phosphate-buffered saline containing 0.1% Triton X for 30 min (neurogenesis analysis) or 0.3% Triton X for 1 h (others), sections were incubated with primary antibodies overnight at 4 °C, followed by incubation with the corresponding Alexa-labeled secondary antibodies at room temperature for 1 h. For neurogenesis analysis, 1 in 12 (Figure 2) or 1 in 4 (Figure 5) hippocampal dentate gyrus sections from each brain were quantified (Figure 2, plates 43–62; Supplementary Figure S1, dorsal, plates 43–56; ventral, plates 56–62; Figure 5, plates 56–60; using the Mouse Brain atlas[17,28]). Results were multiplied by 12 or 4, respectively, to obtain the absolute number, as previously described.[17] Images were acquired using a FV1000D confocal laser-scanning microscope (Olympus, Tokyo, Japan). For primary antibodies, anti-BrdU (ab6326, rat; Abcam, Cambridge, UK; 1:200), anti-neuronal nuclei (NeuN) (MAB377, mouse; Millipore, Billerica, MA, USA; 1:200; mature neuronal marker), anti-doublecortin (DCX) (sc-8066, goat; Santa Cruz Biotechnology, Dallas, TX, USA; 1:150; immature neuronal marker), anti-GFP (ab13970, chicken; Abcam; 1:500), anti-insulin-like growth factor 1 (IGF1) (sc-9013, rabbit; Santa Cruz Biotechnology; 1:50), anti-glial fibrillary acidic protein (G3893, mouse; Sigma; 1:100; astrocyte marker), anti-sex determining region Y-box 2 (Sox2) (sc-17320, goat; Santa Cruz Biotechnology; 1:100; neural progenitor marker) and anti-brain lipid-binding protein (ab32423, rabbit; Abcam; 1:100; neural progenitor marker) were used. For secondary antibodies, Alexa 488-labeled goat/donkey anti-rat (A11006 and A21208), Alexa 488-labeled goat anti-chicken (A11039), Alexa 568-labeled goat anti-mouse (A11031), Alexa 568-labeled donkey anti-goat (A11057), Alexa 594-labeled donkey anti-rabbit (A21207) and

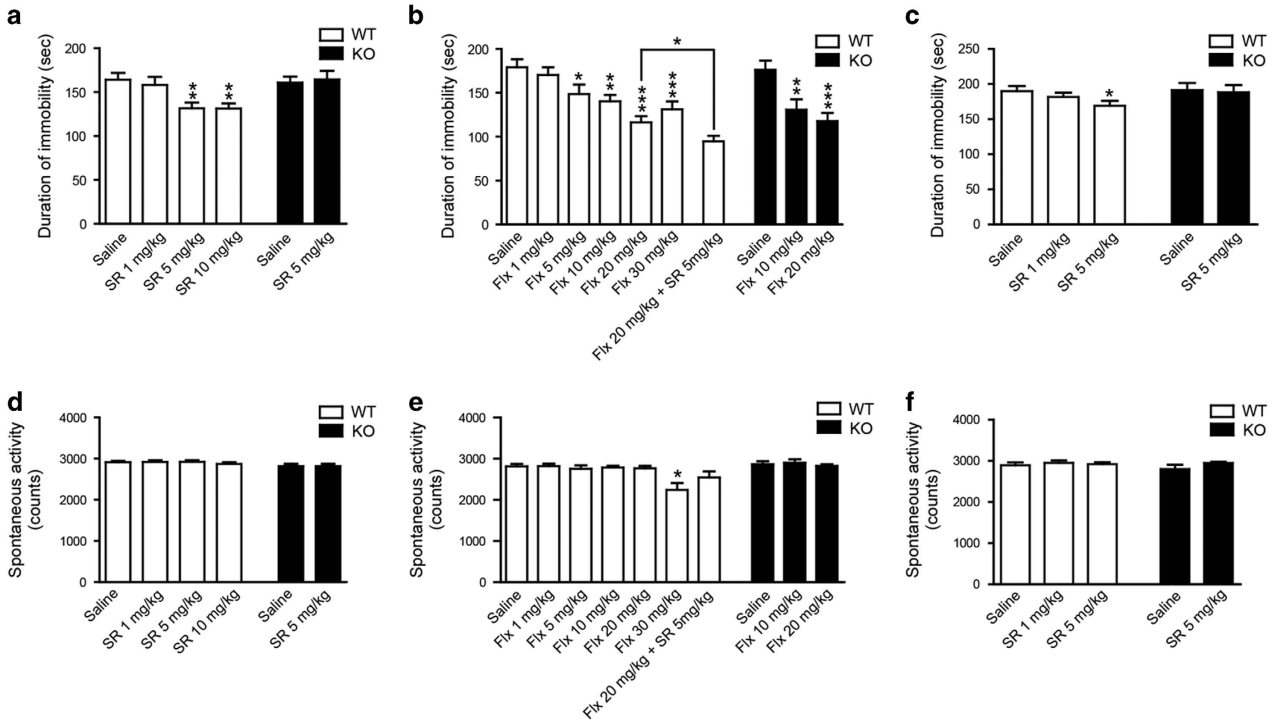

Figure 1. 5HT3R agonist induces antidepressant effects independent of fluoxetine. (**a**–**c**) Tail suspension test. Duration of immobility time in wild-type (WT) and *Htr3a*$^{-/-}$ (KO) mice after acute (**a**, **b**) or chronic (**c**) drug treatment (**a**: WT, $n = 21$; KO, saline, $n = 14$; SR 5 mg kg^{-1}, $n = 12$; **b**: WT, $n = 18$; KO, saline, Flx 10 mg kg^{-1}, $n = 12$; Flx 20 mg kg^{-1}, $n = 11$; **c**: WT, saline, $n = 14$; SR 1 mg kg^{-1}, SR 5 mg kg^{-1}, $n = 16$; KO, saline, $n = 9$; SR 5 mg kg^{-1}, $n = 8$). (**d**–**f**) Spontaneous activity of WT and KO mice after acute (**d** and **e**) or chronic (**f**) drug treatment ($n = 8$ mice). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (two-tailed *t*-test). Mean ± s.e.m. in all histograms.

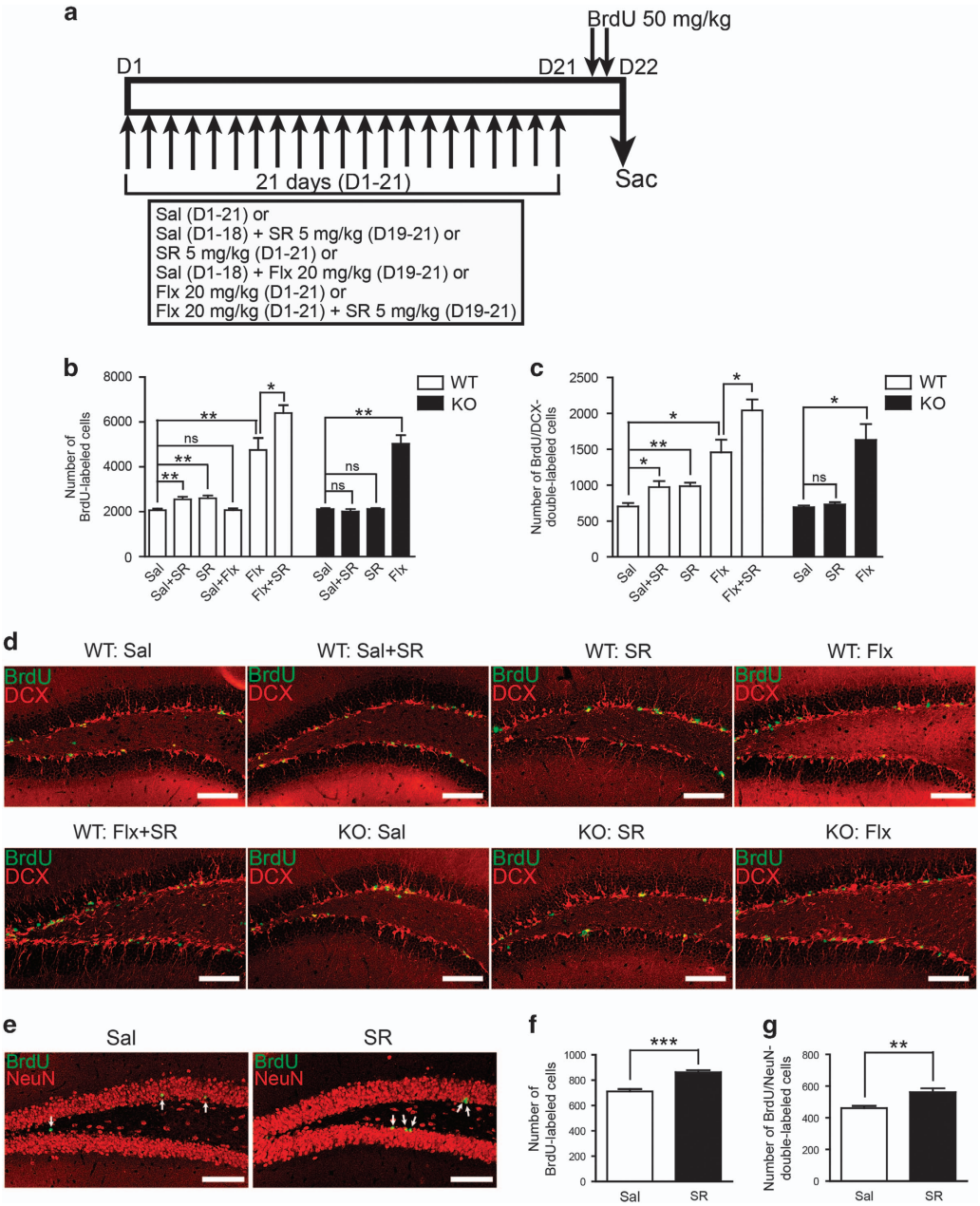

Figure 2. 5HT3R agonist increases hippocampal neurogenesis independent of fluoxetine. (**a**–**g**) Neurogenesis analysis in the hippocampal dentate gyrus. (**a**) Experimental time course. (**b**, **c**) Quantification of BrdU-labeled cells (**b**) and BrdU/DCX-double-labeled cells (**c**) in the dentate gyrus (WT: Sal, Flx, $n = 6$; other groups, $n = 5$; KO, $n = 5$). Sal, saline (days 1–21); Sal+SR, saline (days 1–18) and SR 57227A (days 19–21); SR, SR 57227A (days 1–21); Sal+Flx, saline (days 1–18) and fluoxetine (days 19–21); Flx, fluoxetine (days 1–21); Flx+SR, fluoxetine (days 1–21) and SR 57227A (days 19–21). Drug doses: SR 57227A, 5 mg kg^{-1}; fluoxetine, 20 mg kg^{-1}. (**d**) Representative images of the dentate gyrus, double-stained for BrdU and DCX. Wild-type (WT) and *Htr3a*$^{-/-}$ (KO) mice with respective drug treatments are shown. Scale bars, 100 μm. (**e**) Representative images of BrdU/NeuN double-labeled cells are indicated by arrows. Scale bars, 100 μm. (**f**, **g**) Quantification of BrdU-labeled cells (**f**) and BrdU/NeuN double-labeled cells (**g**) in the dentate gyrus ($n = 5$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS, not significant (two-tailed *t*-test). Mean ± s.e.m. in all histograms.

Alexa 594-labeled donkey anti-mouse (A21203) (Invitrogen, Carlsbad, CA, USA; 1:500) were used.

In situ hybridization

cDNA fragments of mouse 5HT3AR (NM_013561.2; 520–1208) and IGF1 (NM_010512.5; 373–700) were obtained by PCR from brain cDNA of wild-type mice, and used as templates for probe synthesis. Digoxigenin (DIG)-labeled riboprobes were prepared by *in vitro* transcription. Mice were deeply anesthetized and the brains immediately removed and frozen on dry ice. Serial mirror-image coronal sections (14 μm) containing the hippocampus were prepared using a cryostat. *In situ* hybridization was performed, as previously described.[22,29] Sections were fixed with 4% formaldehyde in 0.1 M phosphate buffer, treated with 0.1% activated diethyl pyrocarbonate and equilibrated with 5× saline sodium citrate buffer. After prehybridization at 58 °C for 2 h in hybridization buffer (50% formamide, 5× saline sodium citrate buffer, 40 μg ml^{-1} salmon sperm DNA), hybridization was performed in hybridization buffer containing DIG-labeled cRNA probe at 58 °C for 2 days. Next, sections were washed with 2× saline sodium citrate buffer at 65 °C for 1 h and 0.1× saline

sodium citrate buffer at 65 °C for 1 h. Subsequently, sections were incubated with alkaline phosphatase-conjugated anti-DIG antibody (sheep; Roche, Mannheim, Germany; 1:5000) in Tris-buffered saline buffer (100 mM Tris-HCl pH 7.5, 150 mM NaCl) containing 0.5% blocking reagent (Roche) at room temperature for 2 h, followed by incubation with NBT/BCIP solution (100 mM Tris-HCl pH 9.5, 150 mM NaCl, 50 mM $MgCl_2$, 3.5 μl ml^{-1} BCIP (Roche), 4.5 μl ml^{-1} NBT (Roche)) for a colorimetric reaction, which was stopped by adding TE solution (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Images were acquired using a BX53 light microscope (Olympus). For mirror-image sections, false-colored images (5HT3AR, green; IGF1, red) and their merged images were obtained using Photoshop software (Adobe Systems, San Jose, CA, USA). The number of 5HT3AR-positive cells, IGF1-positive cells and double-positive cells in the hippocampal dentate gyrus were quantified. *In situ* hybridization using mirror-image sections (mirror-ISH) is a highly specific method to examine expression of two molecules in the same cell.[22,29] Because of cross-sectional surface loss and imbalanced volume of divided cells in both mirror sections, approximately 60% was used as the co-positive rate for perfect matching of mirror-ISH.[22]

In vivo microdialysis

In vivo microdialysis was performed as previously described.[30] Mice were anesthetized with sodium pentobarbital (60 mg kg^{-1}, i.p.) and fixed to a stereotaxic frame (Narishige, Tokyo, Japan). A microdialysis guide cannula (PEG-4; Eicom, Kyoto, Japan) was placed in the ventral hippocampus (A − 3.1 mm, L 2.5 mm from bregma, V − 1.3 mm from dura[28]) and fixed to the skull. After surgery, mice were individually housed and allowed to recover for at least 2 days. Before the experiment, a microdialysis probe (PEP-4-3, membrane length 3 mm; Eicom) was inserted into the hippocampus through a guide cannula. To obtain stable baseline recordings, the probe was inserted and perfused with 0.15% bovine serum albumin (Wako, Osaka, Japan) in Ringer's solution (147 mM NaCl, 4.0 mM KCl, 2.3 mM $CaCl_2$) in a push-pull manner using a micropump at 10 μl min^{-1} for at least 3 h before baseline sample collection. Next, the probe was perfused at 1 μl min^{-1}, and samples were collected at 60 min intervals to obtain baseline measurements. Mice were then intraperitoneally administered saline or drugs. After the experiment, the probe position was histologically verified. IGF1 content in dialysate samples was analyzed using the Quantikine mouse/rat IGF1 Immunoassay ELISA kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Data were calculated as percentage change from basal dialysate concentrations, with 100% defined as the average of two samples before drug administration.

Drug microinjection procedure

Mice were anesthetized with sodium pentobarbital (60 mg kg^{-1}, i.p.) and fixed to a stereotaxic frame (Narishige). For drug microinjection, a guide cannula (AG-4; Eicom) was placed in the ventral hippocampus (A − 3.1 mm, L 2.2 mm from bregma, V − 1.1 mm from dura[28]) and fixed to the skull. After surgery, mice were individually housed and allowed to recover for 1 week. Before drug administration, a microinjection cannula (AMI-4.5; Eicom) was inserted into the hippocampus through the guide cannula to a depth of 0.5 mm lower than the guide cannula. Solution (1 μl vehicle or 1 mM AG 1024 in saline containing 5% dimethyl sulfoxide) was infused over a period of 4 min at a constant flow using a microinjection pump. The microinjection cannula was left in place for an additional 1 min after drug administration was completed.

General information on methods and statistical analysis

Mice of each genotype were randomly selected for each group. Analysis of behavioral tests was automated to preclude experimenter bias. Analysis of other experiments was performed blind for genotype or treatment. Data were analyzed by two-tailed *t*-tests, or if the variance was significantly different, two-tailed *t*-tests with Welch's correction. *P*-values are shown in the results. For analysis of *in vivo* microdialysis data, two-way repeated-measures analysis of variance was used, and statistical values of treatment × time interactions shown in the results. Sample size was estimated based on previous studies.[17] Definition of error bars and number of animals used in each experiment are described in the figure legends.

RESULTS

5HT3R agonist induces antidepressant effects independent of fluoxetine

Treatment with the selective 5HT3R agonist, SR 57227A, significantly reduced immobility time in the tail suspension test in wild-type mice, indicating decreased depressive-like behavior (acute: SR 5 mg kg^{-1}, $P = 0.0030$; 10 mg kg^{-1}, $P = 0.0020$) (Figure 1a), which corroborates previous results.[31] Antidepressant-like effects were also observed after 3 weeks of SR 57227A treatment (chronic: SR 5 mg kg^{-1}, $P = 0.0481$) (Figure 1c). In contrast to wild-type mice, there was no significant reduction in immobility time in 5HT3AR subunit-deficient (*Htr3a*$^{-/-}$) mice (acute, $P = 0.7543$; chronic, $P = 0.8266$) (Figures 1a and c). Additionally, fluoxetine treatment significantly reduced immobility time in a U-shaped dose-dependent manner in wild-type mice (acute: Flx 5 mg kg^{-1}, $P = 0.0413$; 10 mg kg^{-1}, $P = 0.0024$; 20 mg kg^{-1}, $P < 0.0001$; 30 mg kg^{-1}, $P = 0.0007$) (Figure 1b). Interestingly, *Htr3a*$^{-/-}$ mice also exhibited a similar reduction in immobility time with fluoxetine treatment (Flx 10 mg kg^{-1}, $P = 0.0080$; 20 mg kg^{-1}, $P = 0.0005$) (Figure 1b). Moreover, co-treatment with 20 mg kg^{-1} fluoxetine and 5 mg kg^{-1} SR 57227 A significantly reduced immobility time in wild-type mice. This antidepressant effect was greater than single treatment of 20 mg kg^{-1} fluoxetine, which provided maximal antidepressant effects in a U-shaped dose-dependent manner ($P = 0.0238$ (vs Flx 20 mg kg^{-1})) (Figure 1b). Except for 30 mg kg^{-1} fluoxetine, there were no significant differences in spontaneous activity between different treatment paradigms (Figures 1d–f). These results suggest that 5HT3R is not required for fluoxetine-induced antidepressant effects, and independent of fluoxetine, 5HT3R agonist induces antidepressant effects in its own unique way.

5HT3R agonist increases hippocampal neurogenesis independent of fluoxetine

Previous studies have shown that enhanced hippocampal neurogenesis is required for antidepressant effects.[6,9,10,17,32,33] Therefore, we analyzed hippocampal neurogenesis after 5HT3R agonist and fluoxetine treatment (Figure 2a). Treatment with SR 57227A for both 3 days (acute) and 3 weeks (chronic) significantly increased the number of BrdU-positive cells (Sal+SR, $P = 0.0045$; SR, $P = 0.0025$) and BrdU/DCX-double-positive cells (Sal+SR, $P = 0.0170$; SR, $P = 0.0025$) in wild-type mice, while no increase was observed in *Htr3a*$^{-/-}$ mice (Sal+SR, $P = 0.3688$; SR, $P = 0.9201$) (Figures 2b–d), as we have previously described.[17] Fluoxetine treatment for 3 days (acute) did not increase the number of BrdU-positive cells (Sal+Flx, $P = 0.9728$) in wild-type mice, but treatment for 3 weeks (chronic) increased the number of BrdU-positive cells (Flx, $P = 0.0042$) and BrdU/DCX-double-positive cells (Flx, $P = 0.0142$) (Figures 2b–d), consistent with previous results.[8,9] Interestingly, *Htr3a*$^{-/-}$ mice also exhibited a significant increase in the number of BrdU-positive cells (Flx, $P = 0.0016$) and BrdU/DCX-double-positive cells (Flx, $P = 0.0134$), which was comparable to the response in wild-type mice (Figures 2b–d). Moreover, in wild-type mice, co-treatment with fluoxetine and SR 57227 A significantly increased the number of BrdU-positive cells (Flx+SR, $P = 0.0359$ (vs Flx)) and BrdU/DCX-double-positive cells (Flx+SR, $P = 0.0359$ (vs Flx)), compared with single fluoxetine treatment (Figures 2b–d). These results suggest that 5HT3R is not required for fluoxetine-induced hippocampal neurogenesis, and that 5HT3R agonist increases hippocampal neurogenesis in a fluoxetine-independent manner. Taken together, our findings indicate that 5HT3R does not contribute to fluoxetine-dependent antidepressant and neurogenic responses. Furthermore, a distinct, fluoxetine-independent, 5HT3R-mediated mechanism plays a role in antidepressant effects and hippocampal neurogenesis.

In addition, mice were allowed to survive 4 weeks after chronic treatment with SR 57227A and BrdU injection and then analyzed

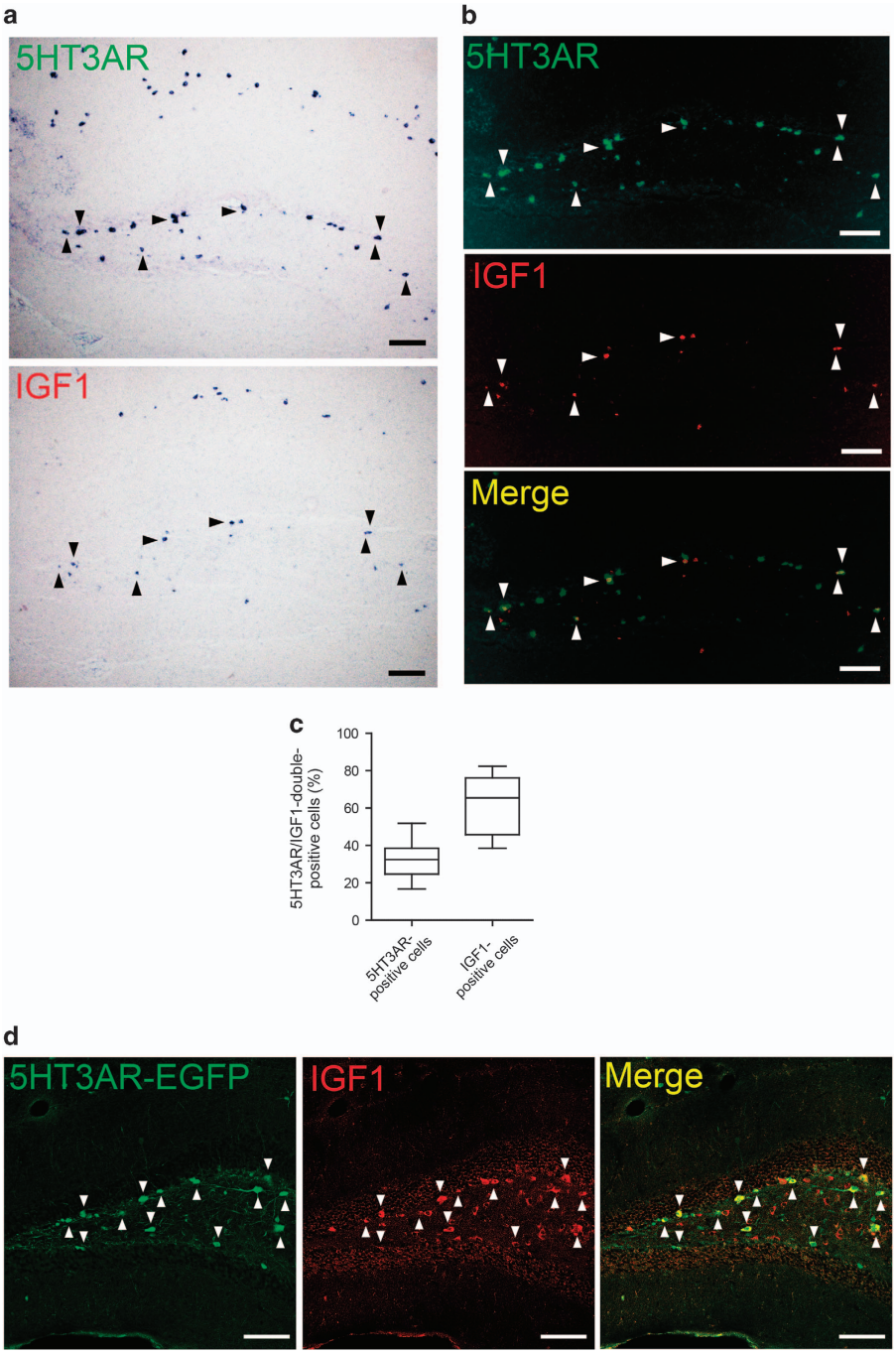

Figure 3. 5HT3R and IGF1 expression in the hippocampal dentate gyrus. (**a**–**c**) *In situ* hybridization of 5HT3AR and IGF1 in mirror-image sections of the hippocampal dentate gyrus (**a**) and false-colored images (5HT3AR, green; IGF1, red) with merged images (**b**). Arrowheads indicate cells expressing both 5HT3AR and IGF1 mRNA. Scale bars, 100 μm. (**c**) The percentage of 5HT3AR/IGF1-double-positive cells in the respective positive cells is represented by whisker-box plots. Boxes show lower, median and upper quartiles. Whiskers show minimum and maximum values ($n = 8$ mirror-image sections from three mice). (**d**) Immunohistochemical analysis in 5HT3AR-EGFP transgenic mice. Representative images of the hippocampal dentate gyrus, double-stained for EGFP and IGF1. Arrowheads indicate neurons expressing both EGFP and IGF1. Scale bars, 100 μm. Mean ± s.e.m. in all histograms. IGF1, insulin-growth factor 1.

for hippocampal neurogenesis.[4,34] Our analysis revealed that SR 57227A treatment significantly increased the number of BrdU-positive cells (SR, $P = 0.0002$) and BrdU/NeuN double-positive cells (SR, $P = 0.0069$) (Figures 2e–g), indicating that newborn neurons can survive and fully differentiate after treatment with 5HT3R agonist. Neurogenesis analysis in the dorsal and ventral hippocampus is shown in Supplementary Figures S1a–h.

5HT3R and IGF1 are expressed in the same neurons in the hippocampal dentate gyrus

The fluoxetine-independent 5HT3R-mediated mechanism remains to be determined. IGF1 is a neurotrophic factor that promotes hippocampal neurogenesis and induces antidepressant effects.[35,36] Further, IGF1 expression has been shown in the hippocampus.[37] Our *in situ* hybridization study revealed that

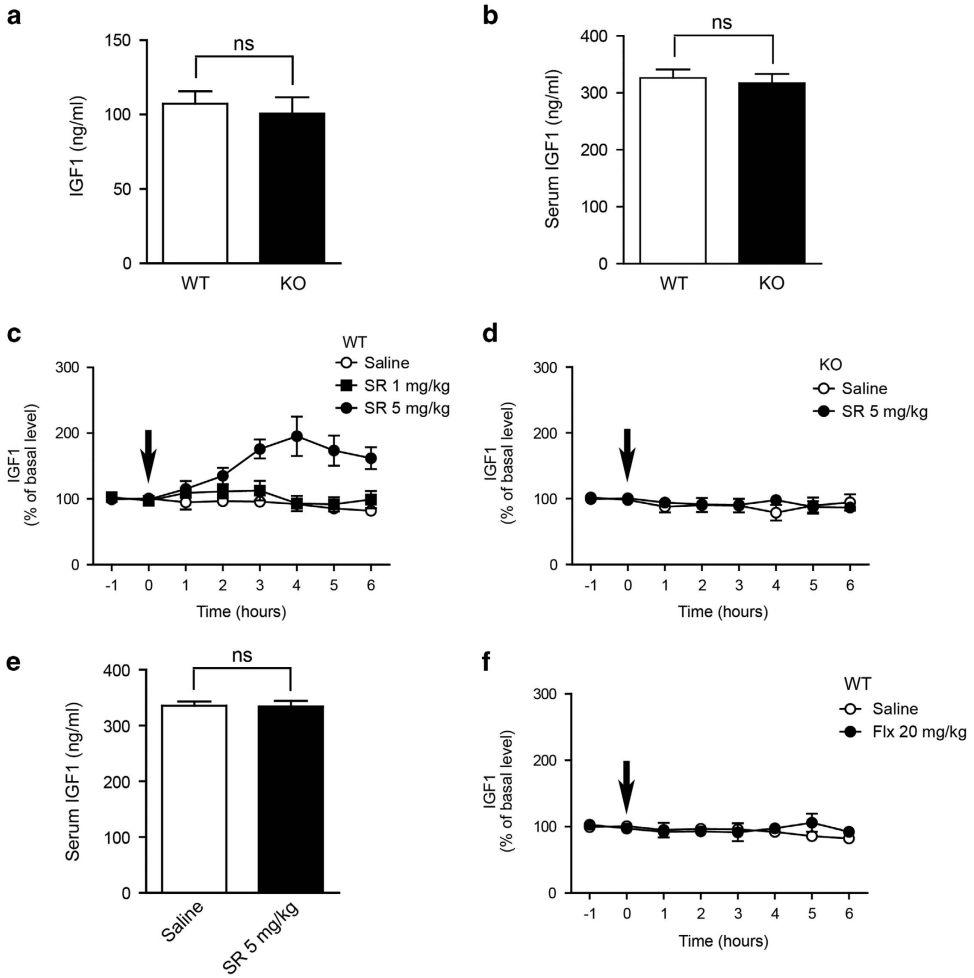

Figure 4. 5HT3R regulates hippocampal extracellular IGF1 levels. (**a**, **b**) Hippocampal extracellular (**a**) or serum (**b**) IGF1 levels in wild-type (WT) and *Htr3a*$^{-/-}$ (KO) mice at baseline (**a**: WT, $n=20$; KO, $n=10$; **b**: $n=6$). (**c**, **d**) Effect of SR 57227 A treatment on extracellular IGF1 levels in the hippocampus of WT (**c**) and KO (**d**) mice ($n=5$). Arrows indicate the injection time. (**e**) Serum IGF1 levels in WT mice 4 h after SR 57227 A treatment (5 mg kg^{-1}) ($n=6$). (**f**) Effect of fluoxetine treatment (20 mg kg^{-1}) on extracellular IGF1 levels in the hippocampus of WT mice ($n=5$). NS, not significant (two-tailed *t*-test). Mean ± s.e.m. in all histograms. IGF1, insulin-growth factor 1.

5HT3AR and IGF1 mRNA expression colocalize in the same cells in the subgranular zone of the hippocampal dentate gyrus (Figures 3a and b). Quantitative analysis revealed that most IGF1 mRNA-positive cells also express 5HT3AR mRNA (5HT3AR/IGF1-double-positive cells in IGF1-positive cells, 62.21 ± 5.710%) (Figure 3c). In addition, immunohistochemical analysis using 5HT3AR-EGFP transgenic mice confirmed that 5HT3AR is expressed in neurons (Supplementary Figures S2a–d).[12,22] Furthermore, 5HT3AR-EGFP/IGF1-double-positive neurons were identified in the dentate gyrus (Figure 3d), with a similar distribution along the dorsoventral axis (Supplementary Figures S2e and f). These data suggest a possible relationship between 5HT3R and IGF1 in the hippocampal dentate gyrus.

5HT3R regulates hippocampal extracellular IGF1 levels

Previous studies have shown that neurogenesis in the ventral dentate gyrus preferentially contributes to emotional affect.[6,38–41] Therefore, we focused on the ventral hippocampus in the following analyses. To examine the functional relationship between 5HT3R and IGF1, we collected extracellular fluid from the ventral hippocampus by *in vivo* microdialysis to analyze IGF1 levels. At basal levels, no significant difference was observed in IGF1 levels of hippocampal extracellular fluid or serum between wild-type and *Htr3a*$^{-/-}$ mice (extracellular fluid, $P=0.6431$; serum, $P=0.6940$) (Figures 4a and b). Treatment with SR 57227A significantly increased extracellular IGF1 levels in the hippocampus of wild-type mice (SR 5 mg kg^{-1}, $F(5, 40)=3.702$, $P=0.0076$) (Figure 4c). However, SR 57227A did not affect hippocampal extracellular IGF1 levels in *Htr3a*$^{-/-}$ mice ($F(5, 40)=1.119$, $P=0.3657$) (Figure 4d), and serum IGF1 levels remained unchanged after SR 57227A treatment in wild-type mice ($P=0.9224$) (Figure 4e). Importantly, fluoxetine treatment did not affect extracellular IGF1 levels in wild-type mice ($F(5, 40)=0.8127$, $P=0.5477$) (Figure 4f). Together, these results suggest that 5HT3R regulates hippocampal extracellular IGF1 levels.

5HT3R-dependent hippocampal neurogenesis is mediated by increased extracellular IGF1

Finally, we examined the role of 5HT3R-dependent increases in hippocampal extracellular IGF1 levels in the neurogenic cell response in wild-type mice (Figure 5a). SR 57227A treatment increased the number of BrdU-positive cells (SR-Veh: $P=0.0056$) and BrdU/DCX-double-positive cells (SR-Veh: $P=0.0178$) in the ventral hippocampal dentate gyrus (Figures 5b–d), as shown in Figure 2. These increases were completely blocked by hippocampal microinjection of an IGF1 receptor antagonist, AG 1024 (SR-AG:

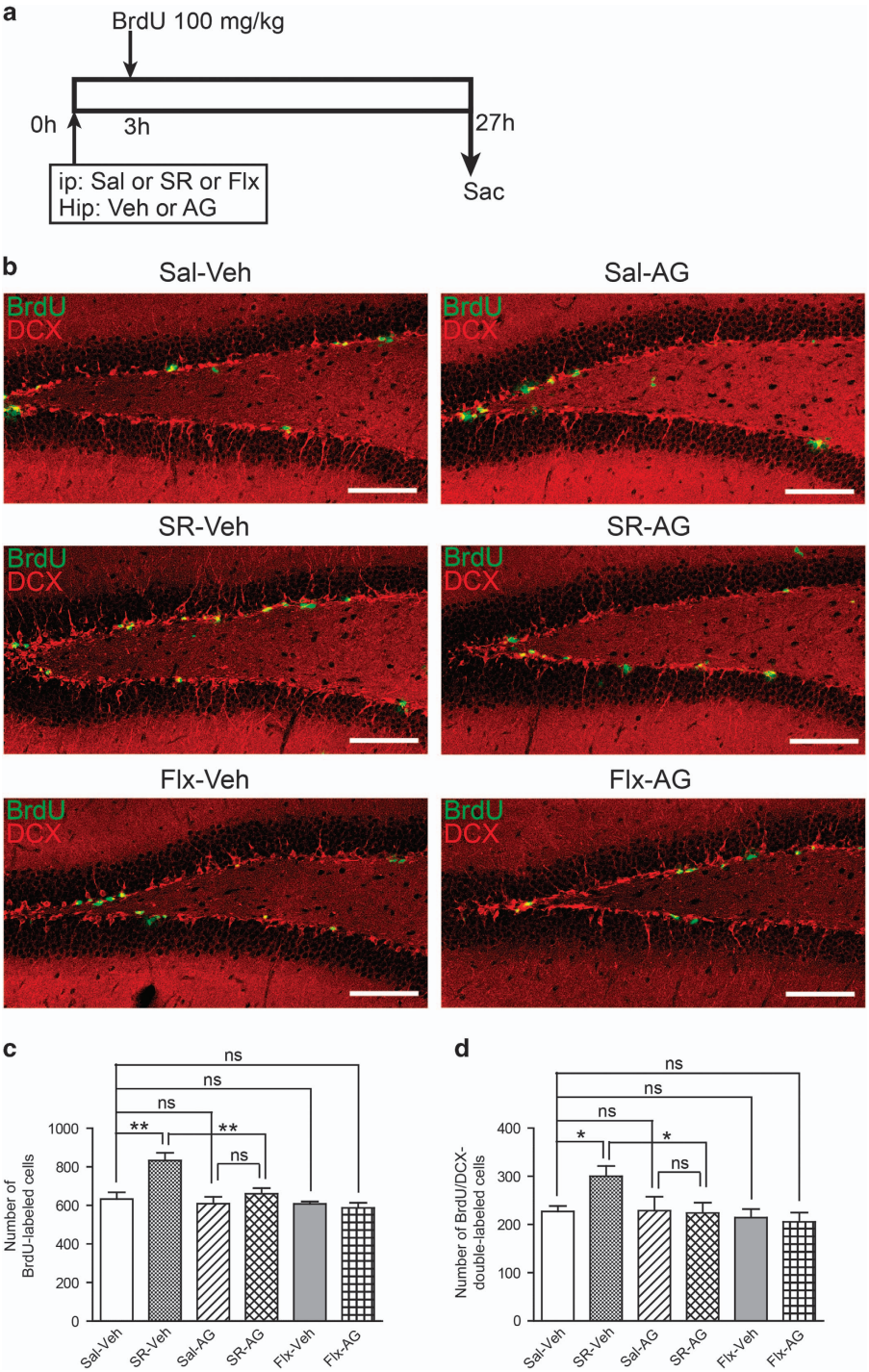

Figure 5. 5HT3R-dependent hippocampal neurogenesis is mediated by increased hippocampal extracellular insulin-growth factor 1 levels. (**a**–**d**) Effect of AG 1024 on 5HT3R-dependent neurogenic effects in the hippocampal dentate gyrus. (**a**) Experimental time course. (**b**) Representative images of the dentate gyrus double-stained for BrdU and DCX. Data for wild-type (WT) mice with respective drug treatments are shown. Scale bars, 100 μm. (**c**, **d**) Quantification of BrdU-labeled cells (**c**) and BrdU/DCX-double-positive cells (**d**) in the dentate gyrus ($n = 5$ mice). Sal-Veh, saline and vehicle; SR-Veh, SR 57227A and vehicle; Sal-AG, saline and AG 1024; SR-AG, SR 57227A and AG 1024; Flx-Veh, fluoxetine and vehicle; Flx-AG, fluoxetine and AG 1024. Drugs were injected intraperitoneally or microinjected into the hippocampus, respectively. Drug doses: SR 57227A, 5 mg kg^{-1}; fluoxetine, 20 mg kg^{-1}; AG 1024, 1 mM in 1 μl. *$P < 0.05$; **$P < 0.01$; NS, not significant (two-tailed *t*-test). Mean ± s.e.m. in all histograms.

BrdU, $P = 0.0083$; BrdU/DCX, $P = 0.0371$ (vs SR-Veh)) (Figures 5b–d). However, AG 1024 microinjection or fluoxetine treatment itself did not affect the number of BrdU-positive cells (Sal-AG, $P = 0.6449$; Flx-Veh, $P = 0.5266$; Flx-AG, $P = 0.3333$) and BrdU/DCX-double-positive cells (Sal-AG, $P = 0.9603$; Flx-Veh, $P = 0.5593$; Flx-AG, $P = 0.3642$) (Figures 5b–d). These results suggest that 5HT3R-dependent hippocampal neurogenesis is mediated by increased hippocampal extracellular IGF1.

DISCUSSION

5HT3R, hippocampal neurogenesis and antidepressant effects

Our neurogenesis analysis shows that a 5HT3R agonist increases cell proliferation and neuroblast generation in the hippocampal dentate gyrus. Further, newborn neurons were able to survive and fully differentiate after 5HT3R agonist treatment (Figure 2). Our findings suggest that 5HT3R agonist treatment may modulate several steps of adult neurogenesis in the hippocampus, and thus 5HT3R-dependent newborn neurons can integrate into pre-existing neural circuitry and enhance dentate gyrus neural plasticity.[4,34] Accordingly, 5HT3R-dependent modulation of neurogenesis may regulate hippocampal functions, such as cognition and mood,[4–7] which emerge as behavioral responses following 5HT3R agonist treatment. However, further studies are needed to clarify which stage of neurogenesis is important for 5HT3R agonist-induced behavioral effects.

Previous studies have shown that neurogenesis in the ventral dentate gyrus preferentially contributes to emotional affect.[6,38–42] In addition, antidepressants were recently suggested to exert their behavioral effects by increasing ventral hippocampal neurogenesis.[43,44] In our study, a 5HT3R agonist increased neurogenesis in both the dorsal and ventral dentate gyrus. Interestingly, compared with single fluoxetine treatment, enhanced neurogenesis by co-administration of 5HT3R agonist and fluoxetine was significant in only the ventral portion (Supplementary Figure S1). Hence, our data suggest that 5HT3R agonist-induced behavioral/antidepressant effects may be achieved via enhanced ventral hippocampal neurogenesis. Our findings support participation of ventral hippocampal neurogenesis controlled by 5HT3R in emotional behaviors such as antidepressant-like behavior.[6,38–44]

Based on accumulating evidence, adult hippocampal neurogenesis has gained considerable traction as a potential mechanism or substrate underlying antidepressant action.[4–6,45] Previous studies have shown that hippocampal neurogenesis is required for the behavioral effects of antidepressants.[9,10,33,46,47] Meanwhile, it is suggested that dependence of the behavioral effects of antidepressants on neurogenesis can be influenced by several factors, such as the nature of antidepressants.[4,6] Considering the pleiotropic effects of antidepressants on neural circuitry, hippocampal neurogenesis may be one of several mechanisms in the brain that antidepressants harness to exert their behavioral effects.[6,48,49] Therefore, our findings using 5HT3R agonists suggest a causal link between neurogenic responses and antidepressant effects mediated via 5HT3R, although further research is necessary to improve our understanding of this relationship.

A novel 5HT3R–IGF1 mechanism distinct from SSRI-induced responses

Our *in situ* hybridization and immunohistochemical analyses revealed that 5HT3R and IGF1 are expressed in the same neurons in the hippocampal dentate gyrus (Figure 3). Meanwhile our *in vivo* microdialysis and drug microinjection analyses showed that 5HT3R agonist treatment increases extracellular IGF1 levels in the hippocampus and, further, that IGF1 signaling is required for 5HT3R-dependent hippocampal neurogenesis (Figures 4 and 5). Importantly, the IGF1 receptor (IGF1R) is expressed in hippocampal neural progenitor cells,[35,50,51] and IGF1 has a neurogenic effect in progenitor cells.[35,52,53] Therefore, 5HT3R-dependent increased IGF1 can act through IGF1R in hippocampal progenitor cells and enhance neurogenic responses. Furthermore, previous studies have shown that IGF1 produces antidepressant behavioral effects.[36,54,55] Taken together, our findings suggest that 5HT3R-dependent IGF1 regulation in the hippocampus is important for modulation of neurogenesis and antidepressant effects, although we cannot completely deny involvement of other cell types in 5HT3 agonist-induced effects.

While fluoxetine required chronic administration for neurogenic cell responses,[8,9] 3-day treatment with 5HT3R agonist showed significant neurogenic effects in the hippocampus (Figure 2). Furthermore, IGF1-mediated neurogenic effects, which were induced by 5HT3R agonist, were not observed following fluoxetine treatment in our experiments (Figures 4 and 5). Therefore, we believe that this neurogenic effect is a characteristic feature of the 5HT3R–IGF1 pathway, which may account for the difference in time course of neurogenic effects between 5HT3R agonist and fluoxetine.

Previous reports have shown that 5HT1ARs are highly expressed in mature dentate granule cells,[11,56] while a recent study showed that 5HT1AR on dentate granule cells is critical for fluoxetine-induced hippocampal neurogenesis and the antidepressant response.[11] In contrast, 5HT3R expression in the dentate granule cell layer is little or none, but 5HT3Rs are expressed in interneurons in the subgranular layer and hilus (Figure 3), as previously described.[12,22,56–60] This difference in cellular distribution might reflect different cell types expressing 5HT1AR and 5HT3R, and supports the likelihood of distinct underlying pharmacological mechanisms between fluoxetine and 5HT3R agonist.

5HT3R as a potential target for novel antidepressant drugs

Our behavioral data show 5HT3R agonist-induced antidepressant effects in mice (Figure 1), which is consistent with previous reports.[31] In addition, we further show that a 5HT3R agonist exhibits antidepressant effects and increases hippocampal neurogenesis in mice with depressive-like behavior (Supplementary Figure S3 and Supplementary Text). In contrast, several reports have shown antidepressant effects of 5HT3R antagonists,[61–63] although ineffective 5HT3R antagonist responses have also been reported.[63,64] Some previous studies have shown biphasic dose–response profiles of 5HT3R antagonists, producing antidepressant effects at low doses but with no effect at high doses.[61,62] Thus, dose–effect characteristics of drugs, species differences and variability in experimental protocols may partially account for these varied behavioral responses to drugs acting on 5HT3R.[63] Nonetheless, our and other studies have undoubtedly identified 5HT3R as a potential target for antidepressants, but further investigation is needed to establish the exact role of 5HT3R in antidepressant action of 5HT3R agonists/antagonists.

In conclusion, our data demonstrate that 5HT3R agonist-induced antidepressant effects and IGF1-mediated hippocampal neurogenesis are fluoxetine-independent mechanisms. Our findings suggest a novel 5HT3R–IGF1 mechanism that is distinct from fluoxetine-induced responses and that could provide a new therapeutic target for depression, especially bringing significant benefits to SSRI-resistant depressed patients.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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