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# Antidementia effects of *Enterococcus faecalis* 2001 are associated with enhancement of hippocampal neurogenesis via the ERK-CREB-BDNF pathway in olfactory bulbectomized mice

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ABSTRACT

Our previous study showed that *Enterococcus faecalis* 2001 (EF-2001) suppresses colitis-induced depressive-like behavior through the enhancement of hippocampal neurogenesis in mice. In the present study, we investigated the effect of EF-2001 on the cognitive behavior of olfactory bulbectomized (OBX) mice and its molecular mechanisms. The OBX-induced cognitive dysfunction was significantly suppressed by EF-2001. Moreover, EF-2001 also recovered the reductions in p-ERK1/2, p-CREB, BDNF and DCX levels and in neurogenesis observed in the hippocampus of OBX mice. These results suggest that EF-2001-induced antidementia effects are associated with enhanced hippocampal neurogenesis through the ERK-CREB-BDNF pathway.

## 1. Main text

Alzheimer's disease (AD) patients show cognitive impairment, and about 30 % of AD patients suffer from depression [1]. It is unclear whether depression is a risk factor for AD or vice versa. AD and depression are thought to have a common pathophysiology. For example, it has been reported that the olfactory bulb volume is smaller in depressed patients than in healthy people [2], and a similar finding was noted in AD patients by magnetic resonance imaging [3]. In animals, olfactory bulbectomy (OBX) can result in various abnormal behaviors such as impaired learning and memory [4] and depressive-like behavior [5], as well as neurochemical changes such as the reduction of cholinergic neurons, neurogenesis, monoamines, and brain-derived neurotrophic factor (BDNF) levels in the hippocampus [5–7]. BDNF is known to play an important role in neuronal survival and is believed to regulate neuroplasticity, including neurogenesis [8]. Especially, the integration of newborn neurons influences animal behaviors, such as learning and memory [9], including depressive behaviors [10]. A decrease in adult neurogenesis is observed in AD, depression and schizophrenia patients [11]. OBX mice also show diminished neurogenesis in the dentate gyrus (DG) of the hippocampus [4,5,7]. These abnormal behaviors and pathological changes in OBX mice were rescued by an antidementia drug or the chronic administration of antidepressant drugs [5,12]. Therefore, the regulation of hippocampal neurogenesis in OBX mice could lead to therapeutic strategies against the cognitive deficits that is observed in AD with depression.

A recent study suggested that microbiota can regulate neurogenesis and the expression of BDNF in the hippocampus [13]. Interestingly, heat-killed *Lactobacillus brevis* SBC8803, which is a lactic acid bacterium, improves memory dysfunction and enhances adult hippocampal

*Abbreviations:* AD, Alzheimer's disease; ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; BrdU, 5-bromo-2'-deoxyuridine; BRM, biological response modifier; CREB, cAMP-responsive element binding protein; DCX, doublecortin; DG, dentate gyrus; EF-2001, Enterococcus Faecalis 2001; ERK, extracellular signal-regulated protein kinase; OBX, Olfactory bulbectomy; p, phospho; PAT, passive avoidance test; p.o., per os; SEM, standard error of the mean; SGZ, subgranular zone

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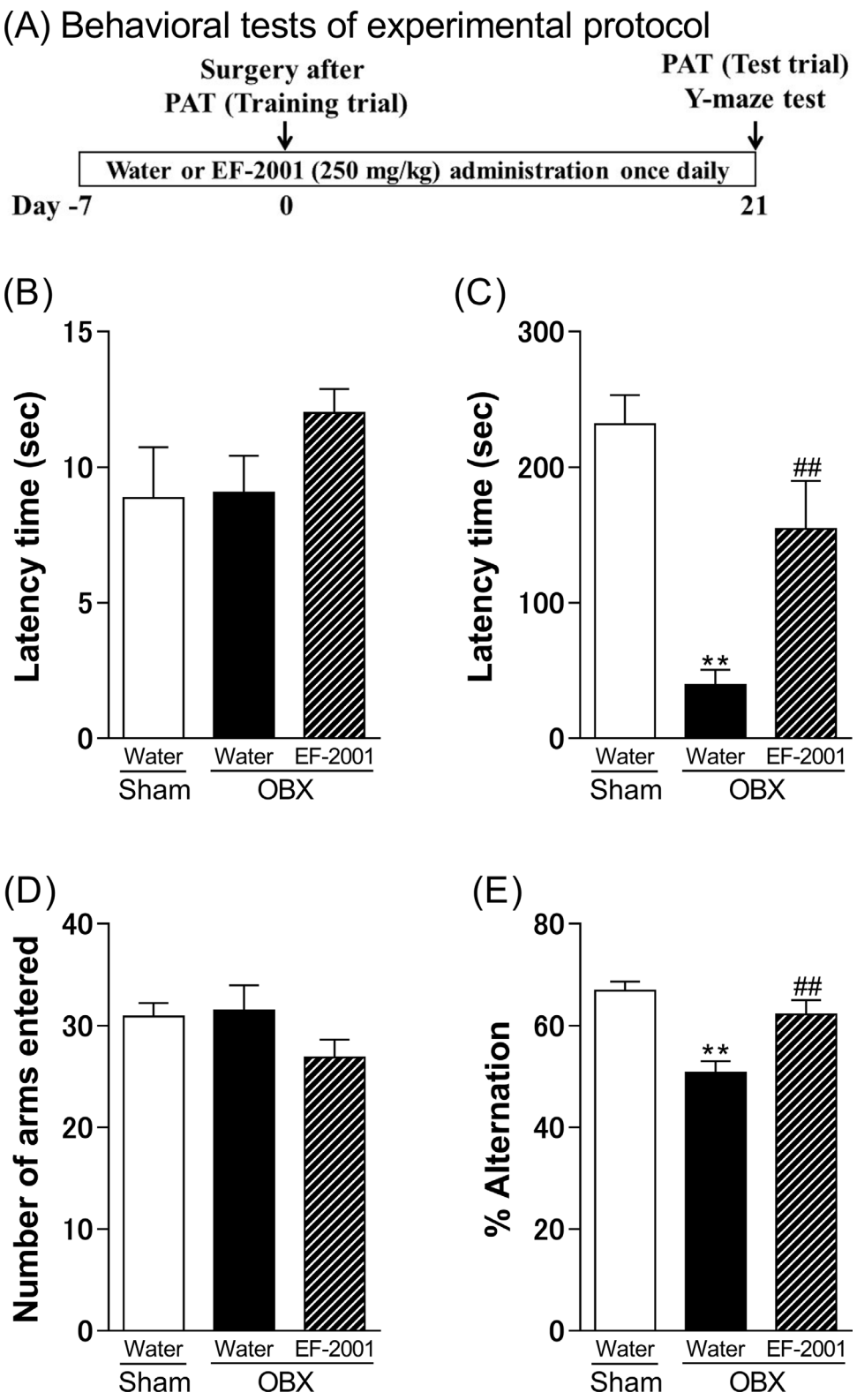


**Fig. 1.** Effects of EF-2001 treatment on OBX-induced memory deficit behaviors. A: The time course of the experimental protocol. B and C: The latency times for the different groups are shown for the training trial (B) and the test trial (C) in the passive avoidance test (PAT). D and E: Locomotor activity (D) and spontaneous alternation behavior (E) during the Y-maze test in mice. The bars represent the mean ± standard error of the mean (SEM). One-way ANOVA [$F(2, 42) = 1.511$, $p = 0.2324$, (B); $F(2, 42) = 15.25$, $p < 0.0001$, (C); $F(2, 49) = 1.639$, $p = 0.2047$, (D); $F(2, 49) = 13.59$, $p < 0.0001$, (E)]. ** $p < 0.01$, compared to the sham + water group. ## $p < 0.01$, compared to the OBX + water group.

neurogenesis in mice [14]. We recently found that *Enterococcus faecalis* 2001 (EF-2001), which is also a lactic acid bacterium that is used as a biological response modifier (BRM), suppresses colitis-induced depressive-like behavior via the enhancement of neurogenesis in the hippocampus [15]. However, it remains unknown whether EF-2001 affects OBX-induced cognitive dysfunction. Therefore, we investigated the effect of EF-2001 on OBX-induced impairment of memory and its molecular mechanisms in mice.

All experiments were approved by the Ethics Committee of Animal Experiments at the International University of Health and Welfare (Ohtawara, Japan; approval number: 19015). All procedures followed the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Bethesda, MD). Efforts were made to minimize animal suffering and reduce the number of animals used.

We used male ddY mice (age, 6-7 weeks; weight, 26-28 g; Japan SLC, Shizuoka, Japan) for all experiments (total, n = 176; behavioral

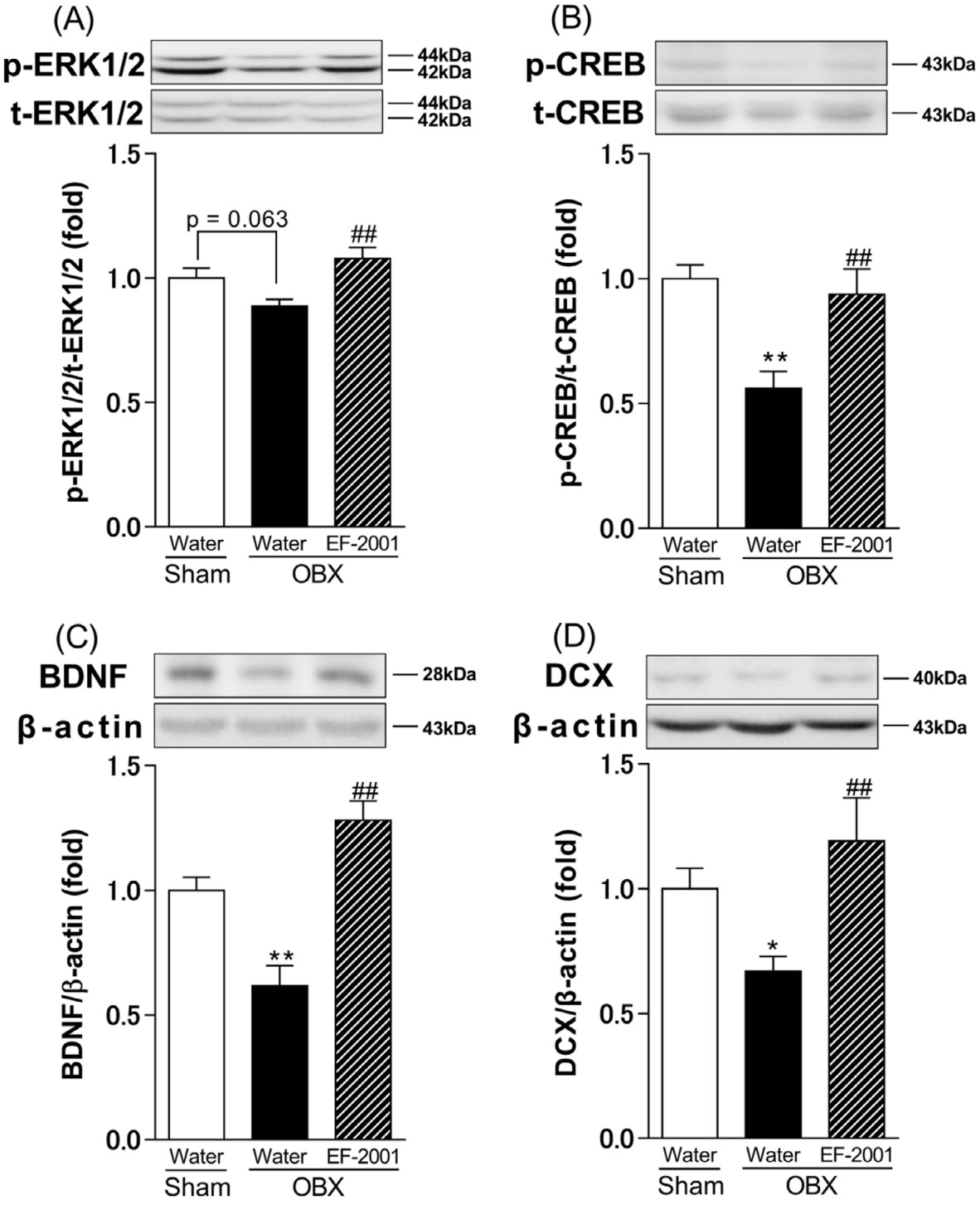


**Fig. 2.** Effects of EF-2001 on the changes in the levels of p-ERK1/2 (A), p-CREB (B), BDNF (C) and DCX (D) in the hippocampi of OBX mice. The bars represent the mean ± SEM. One-way ANOVA [$F(2, 36) = 6.394$, $p = 0.0042$, (A); $F(2, 38) = 12.69$, $p < 0.0001$, (B); $F(2, 37) = 20.01$, $p < 0.0001$, (C); $F(2, 34) = 6.876$, $p = 0.0031$, (D)]. * $p < 0.05$ and ** $p < 0.01$, compared to the sham + water group. ## $p < 0.01$, compared to the OBX + water group.

test, n = 113; western blotting analysis, n = 40; and immunohistochemical study, n = 23). The mice were housed in cages containing five to six mice, and were subjected to steady conditions (temperature, 23 ± 1°C; humidity, 55 ± 5%, and 12/12 hour light-dark cycle with lights on at 7:00). Behavioural tests were performed between 10:00 and 17:00.

OBX surgery was performed as described previously [7]. All mice were euthanized at the end of the experiment and we visually confirmed that two-thirds of the OB had been lesioned. Mice were excluded from the data if the lesion did not extend to more than two-thirds of the OB or if it extended to the cortex. Sham operations followed the same surgical procedure without removal of the OB.

Commercially available heat-treated EF-2001 was originally isolated from healthy human feces. It was supplied as a heat-killed, dried powder by Nihon BRM Co. (Tokyo, Japan). EF-2001 (250 mg/kg) was dissolved in drinking water (vehicle) and administered orally (per os [p.o.]) once a day in a volume of 0.1 mL/10 g mouse body weight using a 1 mL syringe with an oral probe from 7 days before the OBX operation

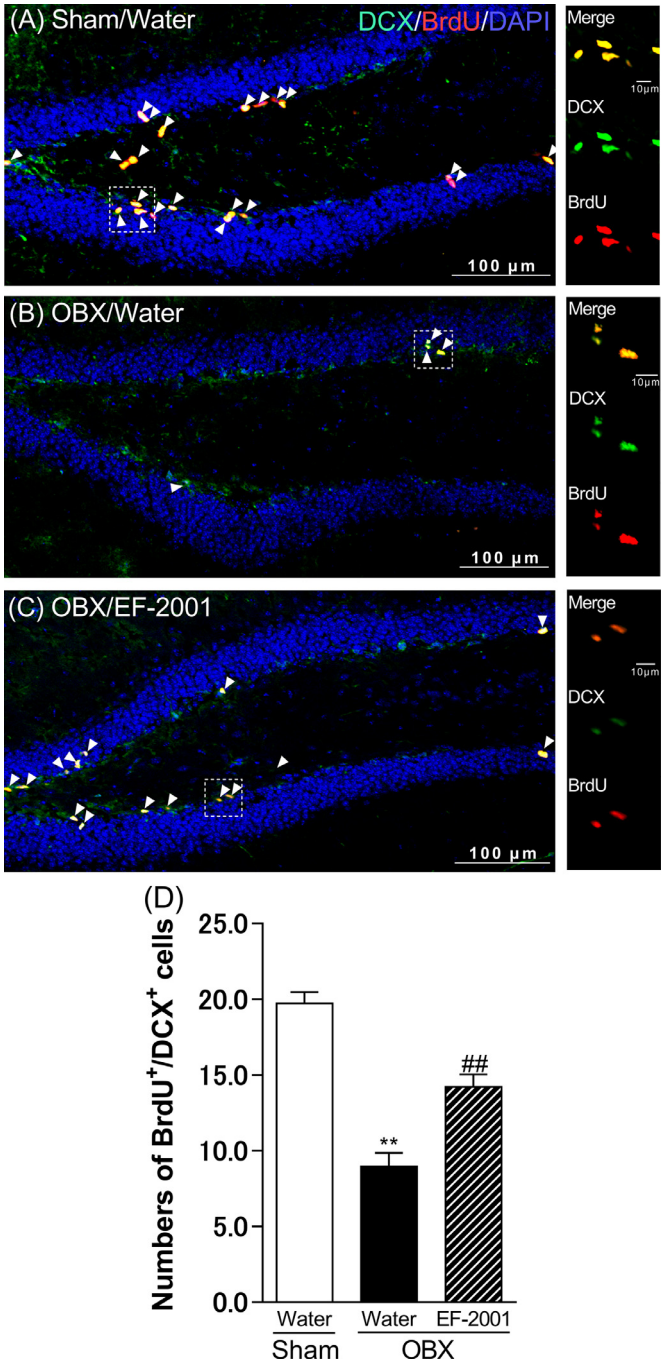


**Fig. 3.** Effect of EF-2001 on the OBX-induced reduction of neurogenesis in the hippocampal DG. A-C: The confocal images of brain slices are stained with DAPI (blue), BrdU (red), and DCX (green). The arrowheads indicate triple-positive cells in the subgranular zone (SGZ). Sham treated with water (A); OBX treated with water (B); OBX treated with EF-2001 (C). D: Quantitative analysis of the number of $BrdU^+/DCX^+$ cells in the SGZ. The bars represent the mean ± SEM. One-way ANOVA [$F(2, 20) = 47.14$, $p < 0.0001$, (D)]. ** $p < 0.01$, compared to the sham + water group. ## $p < 0.01$, compared to the OBX + water group.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

for 28 days.

The behavioral tests, western blotting and immunohistochemical study were performed as previously described [4,16]. Extracellular signal-regulated protein kinase (ERK; 1:1000, Cell Signaling Technology, MA, USA), phosphorylated (p)-ERK (1:1000, Cell Signaling Technology), cAMP response element-binding protein (CREB; 1:1000; Cell Signaling Technology), p-CREB (1:200, Cell Signaling Technology), BDNF (1:1000; Abcam Ltd., Cambridge, UK), doublecortin (DCX; 1:50; Santa Cruz Biotechnology, CA, USA), β-actin (1:1000; Santa Cruz Biotechnology) and horseradish peroxidase-conjugated secondary antibody (1:10000; Jackson Immuno Research Laboratories, Inc., PA, USA) were obtained from commercial sources. Double-staining immunohistochemical analysis used primary monoclonal antibodies against rat anti-BrdU (1:200; Abcam Ltd., Cambridge, UK) and mouse DCX (1:50; Santa Cruz Biotechnology), and secondary monoclonal antibodies against goat anti-rat IgG Alexa fluor 568 (1:200; Molecular Probes, OR, USA) and goat anti-mouse IgG Alexa fluor 488 (1:200; Molecular Probes), and DAPI (1:100; Wako Pure Chemical Industries, Ltd, Osaka, Japan) to identify nuclei.

Results are expressed as the mean ± standard error of the mean. Normality and homoscedasticity assumptions were verified prior to the use of any parametric test (Shapiro-Wilk normality test and equality of variances F-test). The significance of differences was determined by a one-way analysis of variance (ANOVA) to compare groups, followed by a Tukey-Kramer test to correct for multigroup comparisons; a significant difference was defined as $p < 0.05$. All statistical analyses were performed by investigators other than the experimenters to avoid bias and to ensure blinding.

We examined the effects and underlying mechanisms of EF-2001 on the impairment of memory-related behavior in OBX mice in the present study. Previous studies reported that OBX rodents exhibit not only depressive-like behaviors [5], but also cognitive dysfunction [4]. Our previous studies have shown that OBX induces memory impairment in the Y-maze and passive avoidance tests, which are used to evaluate short- and long-term memory, respectively [5,16]. The present study found that OBX mice exhibited impairment of memory-related behavior in both tests, and these changes were prevented by the administration of EF-2001 [Fig. 1 (B-E)]. OBX has been reported to induce hyper-locomotor activity in the dark phase [17]. Our previous study suggested that there was no difference in locomotor activity in the light phase between OBX and Sham mice [4]. The present behavioral tests were performed between 10:00 and 17:00. Thus, we consider that the results of behavioral tests in the present study are not influenced by locomotor activity. The present findings therefore suggest that EF-2001 has an antidementia effect in OBX mice.

Several studies have suggested that microbiota regulates hippocampal BDNF by mediating the cholinergic system in the vagus nerve [13,18]. Acetylcholine stimulates the ERK1/2/CREB/BDNF signaling pathway via $M_1$ receptor [19]. A decrease in the BDNF expression level was confirmed in an AD patient's brain, particularly in the hippocampus and cerebral cortex [20]. Additionally, it was also revealed that CREB is important for mediating neuronal plasticity and BDNF transcription factors, and the activation of CREB is involved in learning and memory formation [21]. Previous studies reported reduced levels of p-ERK1/2, p-CREB, BDNF, and DCX, a marker for immature neurons, in the hippocampus of OBX mice [5,7], suggesting that these changes may be related to the reduction of neurogenesis in the hippocampal dentate gyrus (DG). In the present study, the p-CREB, BDNF, and DCX immunocontent was significantly lower in OBX mice than in sham controls (p-ERK1/2 levels in the OBX group were slightly decreased ($p = 0.063$) compared to those in the sham group), while EF-2001 administration significantly increased p-ERK1/2, p-CREB, BDNF, and DCX levels in the hippocampus of OBX mice [Fig. 2 (A-D)]. This suggests that the activation of cholinergic system and ERK1/2/CREB/BDNF signaling pathways could regulate the effects of EF-2001.

Neurogenesis in the hippocampal DG significantly contributes to central neuroplasticity mechanisms such as learning and memory [22]. To determine the change in hippocampal neurogenesis in OBX mice, the animals were injected with BrdU. Anti-DCX antibody was then used to identify immature neurons in the DG area. The incorporation of BrdU into a cell indicated that it was cycling at the time of BrdU injection. OBX mice had a significantly lower number of BrdU/DCX-positive cells

than the control group, consistent with our previous study [7], and the administration of EF-2001 protected against this change [Fig. 3 (A-D)]. Likewise, it has been reported that neurogenesis is reduced in the hippocampus of patients with depression [23] and AD [24]. Moreover, an increase in adult-born neurons is associated with an improvement in spatial memory-related behavior [25]. Considering these reports, the present finding suggests that the enhancement of neurogenesis in the hippocampus by EF-2001 may be associated with the improvement of memory-related behavioral impairment in OBX mice.

Besides the hippocampus, the prefrontal cortex also affects memory function [26]. In this study, we examined the mechanisms of the antidementia effects of EF-2001 focusing only on the hippocampus. Moreover, we hypothesize that the antidementia effect of EF-2001 may be regulated by hippocampal neurogenesis via the cholinergic system in the vagus nerve, but this hypothesis has not yet been established. Hence, further experiments are needed to examine the relationship between the prefrontal cortex and the effects of EF-2001, as well as the contribution of the cholinergic system to the effects of EF-2001.

In conclusion, the present results indicate that the antidementia effect of EF-2001 may involve the enhancement of neurogenesis via the activation of ERK1/2/CREB/BDNF signaling pathways. EF-2001 may have the potential to prevent cognitive dysfunctions in neuropsychiatric disorders.

## Author contribution

Kohei Takahashi: Conceptualization, Methodology, Formal analysis, Investigation, Writing-original draft, Visualization, Funding acquisition. Kazuhiro Kurokawa: Investigation. Kazuya Miyagawa: Investigation. Atsumi Mochida-Saito: Investigation. Yukio Nemoto: Supervision. Hiroyuki Iwasa: Resources. Osamu Nakagawasai: Writingreview & editing. Takeshi Tadano: Supervision. Hiroshi Takeda: Supervision. Minoru Tsuji: Conceptualization, Writing-review & editing, Project administration. All authors critically reviewed the manuscript and approved the final version for publication.

## Declaration of Competing Interests

Hiroyuki Iwasa is an employee of Nihon Berm Co., Ltd. All other authors declare that they have no competing interests.

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## References

[1] D. Enache, B. Winblad, D. Aarsland, Depression in dementia: epidemiology, mechanisms, and treatment, Curr. Opin. Psychiatry 24 (2011) 461–472.
[2] S. Negoias, et al., Hummel T. Reduced olfactory bulb volume and olfactory sensitivity in patients with acute major depression, Neuroscience 169 (2010) 415–421.
[3] P.A. Thomann, et al., Reduced olfactory bulb and tract volume in early Alzheimer's disease–a MRI study, Neurobiol. Aging 30 (2009) 838–841.
[4] K. Takahashi, et al., Time-dependent role of prefrontal cortex and hippocampus on cognitive improvement by aripiprazole in olfactory bulbectomized mice, Eur. Neuropsychopharmacol. 27 (2017) 1000–1010.
[5] K. Takahashi, et al., Memantine ameliorates depressive-like behaviors by regulating hippocampal cell proliferation and neuroprotection in olfactory bulbectomized mice, Neuropharmacology 137 (2018) 141–155.
[6] S. Hozumi, et al., Characteristics of changes in cholinergic function and impairment of learning and memory-related behavior induced by olfactory bulbectomy, Behav. Brain Res. 138 (2003) 9–15.
[7] T. Odaira, et al., Mechanisms underpinning AMP-activated protein kinase-related effects on behavior and hippocampal neurogenesis in an animal model of depression, Neuropharmacology 150 (2019) 121–133.
[8] E.G. Waterhouse, et al., BDNF promotes differentiation and maturation of adult-born neurons through GABAergic transmission, J. Neurosci. 32 (2012) 14318–14330.
[9] J.S. Snyder, et al., A role for adult neurogenesis in spatial long-term memory, Neuroscience 130 (2005) 843–852.
[10] J.S. Snyder, et al., Adult hippocampal neurogenesis buffers stress responses and depressive behaviour, Nature 476 (2011) 458–461.
[11] S.M. Hussaini, et al., Wnt signaling in neuropsychiatric disorders: ties with adult hippocampal neurogenesis and behavior, Neurosci. Biobehav. Rev. 47 (2014) 369–383.
[12] A. Saitoh, et al., Effects of milnacipran and fluvoxamine on hyper-emotionalbehaviors and the loss of tryptophan hydroxylase-positive cells in olfactory bulbectomized rats, Psychopharmacology (Berl.) 191 (2007) 857–865.
[13] O.F. O'Leary, et al., The vagus nerve modulates BDNF expression and neurogenesis in the hippocampus, Eur. Neuropsychopharmacol. 28 (2018) 307–316.
[14] R. Ishikawa, et al., Dietary heat-killed Lactobacillus brevis SBC8803 (SBL88™) improves hippocampus-dependent memory performance and adult hippocampal neurogenesis, Neuropsychopharmacol. Rep. 39 (2019) 140–145.
[15] K. Takahashi, et al., Effect of Enterococcus faecalis 2001 on colitis and depressive-like behavior in dextran sulfate sodium-treated mice: involvement of the brain-gut axis, J. Neuroinflamm. 16 (2019) 201.
[16] O. Nakagawasai, et al., BE360, a new selective estrogen receptor modulator, produces antidepressant and antidementia effects through the enhancement of hippocampal cell proliferation in olfactory bulbectomized mice, Behav. Brain Res. 297 (2016) 315–322.
[17] J.P. Kelly, A.S. Wrynn, B.E. Leonard, The olfactory bulbectomized rat as a model of depression: an update, Pharmacol. Ther. 74 (1997) 299–316.
[18] H.M. Corpuz, et al., Oral administration of Okara soybean by-product attenuates cognitive impairment in a mouse model of accelerated aging, Nutrients 11 (2019) E2939.
[19] C.H. Jin, et al., Fustin flavonoid attenuates beta-amyloid (1-42)-induced learning impairment, J. Neurosci. Res. 87 (2009) 3658–3670.
[20] H.S. Phillips, et al., BDNF mRNA is decreased in the hippocampus of individuals with Alzheimer's disease, Neuron 7 (1991) 695–702.
[21] S. Kida, A functional role for CREB as a positive regulator of memory formation and LTP, Exp. Neurobiol. 21 (2012) 136–140.
[22] F. Massa, et al., Conditional reduction of adult neurogenesis impairs bidirectional hippocampal synaptic plasticity, Proc. Natl Acad. Sci. USA 108 (2011) 6644–6649.
[23] N.A. DeCarolis, A.J. Eisch, Hippocampal neurogenesis as a target for the treatment of mental illness: a critical evaluation, Neuropharmacology 58 (2010) 884–893.
[24] O. Lazarov, R.A. Marr, Neurogenesis and Alzheimer's disease: at the crossroads, Exp. Neurol. 223 (2010) 267–281.
[25] S.B. Oh, et al., Baicalein attenuates impaired hippocampal neurogenesis and the neurocognitive deficits induced by γ-ray radiation, Br. J. Pharmacol. 168 (2013) 421–431.
[26] E. Ankudowich, S. Pasvanis, M.N. Rajah, Age-related differences in prefrontal-hippocampal connectivity are associated with reduced spatial context memory, Psychol. Aging 34 (2019) 251–261.