

Chronic Fluoxetine Treatment Reverses Depressive-like Behaviors in Mice via Enhancing Neuroplasticity

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Abstract

Objectives: Depression remains a refractory psychiatric disorder. Fluoxetine is a preferred class of antidepressant medication due to restrain retaking of biogenic monoamines. There was a new mechanism discovery that neuroplasticity was considered to underlie clinical antidepressant effects. However, reports display that fluoxetine's actions on neuroplasticity still remain controversial. This study investigates fluoxetine's role in the impact of synaptic function and morphology by different durations of fluoxetine treatment and the possible mechanisms involved.

Materials and Methods: The chronic depression mice model was established by using the 7-week-old male C57BL/6 mice. Fluoxetine 10 mg/kg was treated for 7 days and 14 days. The depression-like behaviors were assessed using the tail-suspension test, forced swim test, sucrose preference, and open-field tests. Nissl staining was applied to assess hippocampus formation. Immunofluorescence and Golgi staining were used to investigate synaptic function and morphology. The hippocampal protein expression of SYP was examined using Western blotting.

Results: We found that fluoxetine treatment for 2 weeks, as opposed to just 1 week, significantly alleviated symptoms of behavioral despair, anhedonia, and anxiety in the depressive mice. Furthermore, both 7- and 14-day fluoxetine administrations resulted in reduced impairment of hippocampal neurons and a tendency to increase the dendritic spine numbers in the context of depression. Additionally, only the 14-day fluoxetine treatment promoted the expression of SYP in the hippocampus.

Conclusion: Chronic administration of fluoxetine significantly reduced depressive and anxiety-like behaviors and hippocampal impairment and enhanced synaptic plasticity in mice.

Keywords

Depression, fluoxetine, hippocampal impairment, synaptic plasticity

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Introduction

Major depressive disorder (MDD) is a ubiquitous mental disorder with a wide-reaching impact.¹ The primary symptoms are characterized by affective impairment, anhedonia, persistent sadness, and insomnia.² There are over 350 million people suffering from MDD.³ Antidepressant drugs remain the cornerstone of pharmacotherapy for MDD.⁴ The earliest discovery of drugs, such as monoamine oxidase inhibitors (MAOIs) and tricyclics, has been a secondary choice in clinic practice.⁵ Currently, selective serotonin reuptake inhibitors (SSRIs) have become the first-line medication due to their fewer side effects.⁶ However, SSRIs take weeks to produce a moderate efficacy. In addition, they also produce significant series of defects, such as delayed onset of action and limited effective rate.⁷ The therapeutic effect of SSRIs remained

elusive. Therefore, continued research on the SSRIs' effect on depression is still urgent.

It is long-term believed that the monoamine deficiency in synaptic cleft underlies MDD. All the existing antidepressants developed based on this hypothesis are primarily characterized by inhibiting the reuptake and release of monoamines.⁸ Currently, ketamine exhibits a fast-acting antidepressant and

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has been demonstrated to effectively reverse the synaptic deficits caused by depression.⁹ Molecular and cellular mechanisms in MDD revealed neuronal synapse functions impaired.¹⁰ Growing studies conducted on humans with MDD have confirmed a reduction in the number of synapses and a loss of synapse-related genes in the hippocampus.¹¹ Furthermore, animal and clinical studies have indicated that disruptions in synaptogenesis contribute to the susceptibility to depression.¹² Interestingly, most antidepressants have been determined to produce effects within the synapse.¹³ Both animal and clinical experiments have shown that neuroplasticity could serve as the primary pathogenesis of MDD. Therefore, the process of synaptic plasticity has been proposed as the fundamental mechanism for the clinical effects of antidepressant treatments.

Fluoxetine, a widely utilized SSRI, is often prescribed as the primary pharmacotherapy for depression. Most studies have shown that chronic fluoxetine treatment strengthens neuroplasticity in the adult brain,^{12,14,15} which underlies the efficacy of fluoxetine administration.¹⁴ Nevertheless, increasing data indicated that fluoxetine has no or opposite action on neuronal plasticity.^{16–19} In line with these, previous studies suggested that chronic fluoxetine treatment exhibits harm to synaptic plasticity.^{20,21} Furthermore, growing evidence from both animal and human studies indicated that fluoxetine may exacerbate depressive symptoms.²² Therefore, the fluoxetine's action on the neuroplasticity of MDD still remains to be investigated. Herein, our study's objectives aim to investigate fluoxetine treatment at different durations on depressive-like behaviors, synaptic morphology, and function via a mice model of depression. Our study will determine the strengthened synaptic plasticity of SSRIs in MDD and further verify that neuroplasticity may be the mechanism for MDD.

Materials and Methods

Animals

The male C57BL/6 mice (7 weeks; 22 ± 2 g) were obtained from Liaoning Changsheng Biological Co. Ltd., (SCXK(LIAO)2020-0001). The mice were fed under the required living environment (22°C, 12-h light–dark cycle, acquired food and water freely). This study was implemented strictly according to the recommendations in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health of the United States (NIH publication No. 80-23, revised 1996). All protocols were approved by the Institutional Animal Care and Use Committee (IACUC), Sun Yat-sen University.

Chronic Depression Animal Model and Fluoxetine Treatment

Reserpine irreversibly suppresses vesicular monoamine transporter (VMAT), which causes a depletion of monoamines.²³ The reserpine-induced chronic depression model exhibits long-term physiological and behavioral

phenotypes similar to human depression.²⁴ In brief, 7-week-old male mice were intraperitoneally injected with reserpine (0.5 mg/kg; sigma) for 14 days once daily. Mice were given intensive care including keeping warm and sugar saline oral administration.

Fluoxetine (CAS: 56296-78-7, HPLC $\geq 98\%$) was treated by intraperitoneal (i.p.) injection. According to the previous report,²⁵ 10 mg/kg of fluoxetine showed better behavioral efficacy by increasing neurogenesis and neurotrophic. Thus, the volume was prepared based on the weight of the fluoxetine and treated with 10 mg/kg.

Behavioral Tests

Tail-Suspension Test (TST)

According to the previous report,²⁶ it was extensively accepted for estimating depression-like behaviors in mice. In short, every mouse was overhung by medical tape tied up the tip of its tail (~1 cm). The immobility was regarded as the mouse completely motionless with passive suspension. The 75% alcohol was applied to eliminate the effect of feces or urine odor on behavioral measurements. The reduced immobility during TST was regarded as antidepressant action. The result is shown as the lasting of immobility (s) in TST.

Forced-Swim Test (FST)

As Porsolt's protocol,²⁷ the mouse was evaluated for 6 minutes in a glass cylinder (45 cm $\times$ 35 cm $\times$ 60 cm) full of warm water ($24 \pm 0.5^\circ\text{C}$) to 30 cm, after which 4 min of immobility were recorded. The result is shown as the lasting of immobility (s) in FST.

Open-Field Test (OFT)

The OFT was used to test mice's depression and anxiety.²⁸ Every mouse was placed in the same center of the open-field box (40 cm $\times$ 40 cm). The data was recorded and analyzed by EthoVision XT 9.0 (Wageningen, the Netherlands) for 15 min.

Sucrose Preference Test (SPT)

Sucrose preference test (SPT) is an accepted method to assess the anhedonia of depression.²⁹ In the present experiment, the sucrose preference test was performed during the dark phase. After food and water deprivation for 24 h, each mouse was always placed with tap water and 1% sucrose solution. After water consumption was tested for 24 h, the bottles were removed and weighed.

Nissl Staining Analysis and Immunofluorescence Analysis

Mice were penetrative anesthetized via 1.5% isoflurane. The thoracic cavity was opened fast to expose the heart. A catheter was penetrated into the ascending aorta, and mice were

perfused with 4% paraformaldehyde (PFA) after saline. After postfixed in 4% PFA overnight at 4°C, the brain was dehydrated, transparentized, and embedded in paraffin. Serial hippocampal coronal sections were cut and gathered sequentially.

Nissl staining was used for assessing hippocampus formation. The 1% toluidine blue was applied to stain with the paraffin slices that were deparaffinized and rehydrated. Every 15th staining slice was selected for analysis (five slices/mice). The neurons were determined and analyzed via the ImageJ/NIH image software system.

Golgi Staining

Golgi staining was applied to observe the shape of dendritic spines. The brains were cut into 100 μ m thick and mounted with glycerin gelatin. Images of hippocampal sections were obtained via a digital slice scanner. The terminal branches have been demonstrated more plastic than non-terminal branches; thus, the numbers of terminal branches of dendrite were evaluated by the localized dendritic remodeling. The dendritic segments of 10 neurons per animal were selected in the region of the dentate gyrus for counting the number of dendritic spines. The dendritic spine density was regarded as spines per unit length.

Western Blot Analysis

The samples were divided by 10% SDS-PAGE and transferred to PVDF membranes. Then, the anti-SYP (rabbit, 1:1000, CST) and anti-GAPDH (mouse, 1:10000, ZSGB-BIO) were used to incubate with membranes overnight at 4°C. The membranes were incubated with anti-rabbit (goat, 1:3000, CST) or anti-mouse (goat, 1:10000, ZSGB-BIO) for 1 h at room temperature before being washed with TBST. The blots of membranes were developed via chemiluminescent substrate. The results were obtained by Quantity One (version 4.4).

Statistical Analysis

The GraphPad Prism was used to analyze the experimental data. All the data were shown to the mean $\pm$ standard error of the mean (SEM). The one-way analysis of variance (ANOVA) method was used to evaluate the homogeneity of variances among groups, followed by multiple comparisons between two groups using the Bonferroni method. Differences were considered significant at $p < 0.05$.

Results

Chronic Fluoxetine Treatment Reduces Depressive-like Behaviors in Mice

Fluoxetine is an accepted antidepressant as SSRIs in the clinic. Further study is urgently needed to ensure its effective

timing. According to our previous study, repeated reserpine-induced mice displayed depressant-like behavior such as despair, anhedonia, and social withdrawal (Figure 1A–D).²⁴ Then, mice were exposed to fluoxetine with 10 mg/kg for 7 or 14 days, and their behaviors were evaluated by performing the TST, FST, and SPT (Figure 1A–D). Our data showed that fluoxetine treatment shortened TST immobility and increased SPT and times in the center zone with 14-day treatment rather than 7 days (Figure 1A–D). The present experiment suggested that chronic fluoxetine administration ameliorated depression-like behavior. Notably, fluoxetine was unable to reduce immobility in FST with 14-day treatment (Figure 1B). In summary, these data suggest that chronic fluoxetine administration improves depressive behavior in mice.

Chronic Fluoxetine Treatment Decreased Anxiety-like Phenotype in Depressive Mice

Furthermore, fluoxetine's onset effect on depression-induced anxiety-related behaviors was examined. The depressive mice performed anxious behavior, displaying less time in spending on the center zone and times of distance crossing (Figure 2A–D). Fluoxetine treatment decreased anxiety-like behavior with 14-day treatment (Figure 2A–D). In sharp contrast, fluoxetine increased traveled distance in the zone of depressed mice with 7-day treatment (Figure 2A–D). These results indicated chronic fluoxetine treatment was effective in ameliorating reserpine-evoked anxiety.

Fluoxetine Mitigates Depression-induced Hippocampal Neuron Impairment in Mice

Nissl staining was applied to clarify fluoxetine's neuroprotective effect in depressed mice. Our data indicated that the hippocampal pyramidal neurons in control mice were consistent and systematic and displayed integrated structures with vivid nucleoli in hippocampal CA1 and CA3 regions (Figure 3A). However, depressed mice's hippocampal CA1 regions were destroyed in the stratified pyramidal neurons' structure with an obvious neuronal decrease, and the regions of CA3 showed a chaotic arrangement with an inordinate morphology, not transparent cytoplasm, and nuclei shrinkage (Figure 3A). Both the 7- and 14-day fluoxetine treatments reduced the loss of depression-caused pyramidal neurons and mitigated the pathological changes in the hippocampal CA1 and CA3 regions (Figure 3A,B). These results revealed that fluoxetine possessed well neuroprotective effect.

Chronic Fluoxetine Treatment Increases Synapse Remodeling in Depressed Mice

Antidepressants exert incipient therapeutic effects within the synapse.¹⁰ The changes in shape and number of spines have been considered a hallmark of synaptic plasticity (PMID:35659473). Compared with control mice, the dendritic

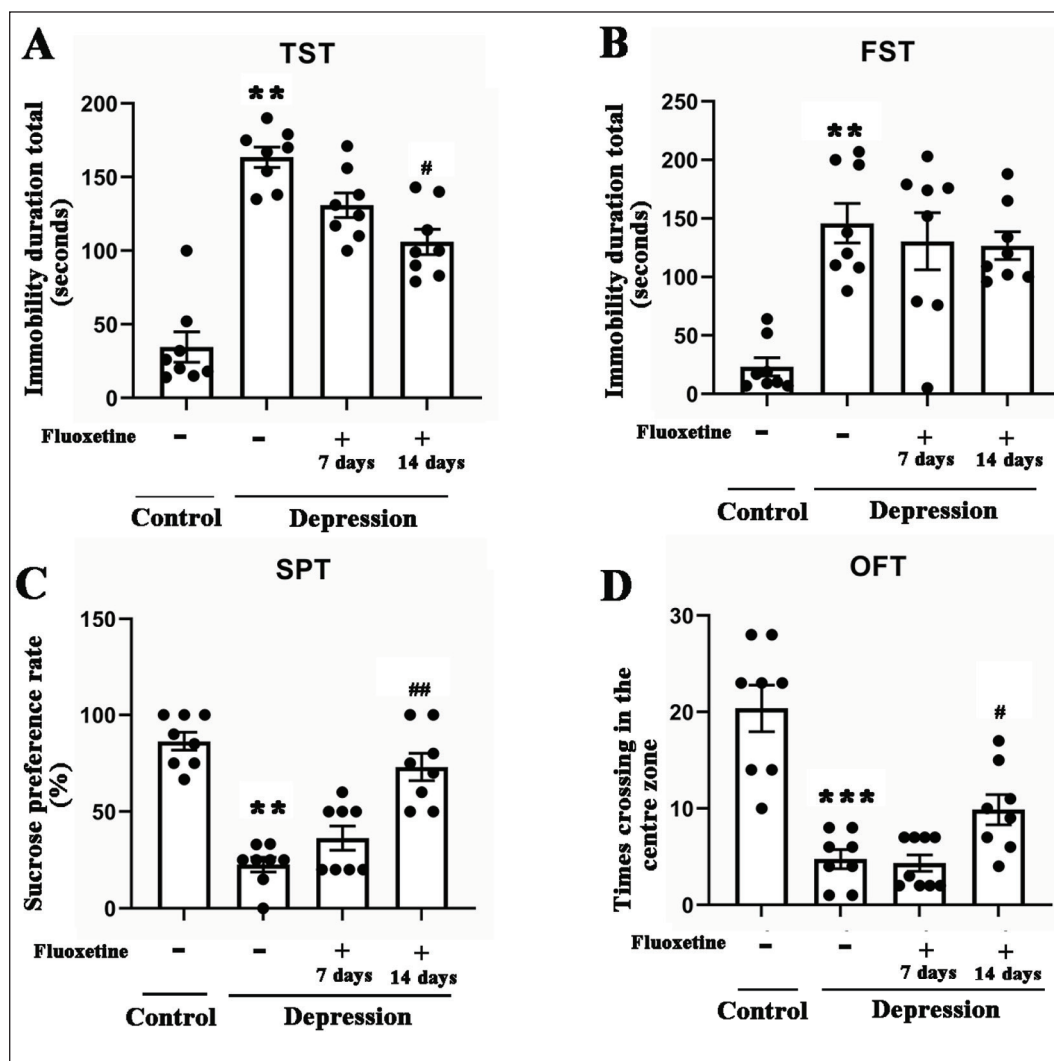


Figure 1. Fluoxetine Administration Reduced Depression-like Behavior. (A) Tail-Suspension Test. Statistical data are as described: $F(3,28) = 40.07$, $n = 8$; $**p < 0.01$ compared with the control group, $#p < 0.05$ compared with the depression group. (B) Forced Swim Test. Statistical data are as described: $F(3,28) = 11.66$, $n = 8$; $**p < 0.01$ compared with the control group. (C) Sucrose Preference Test. Statistical data are as described: $F(3,28) = 29.09$, $n = 8$; $**p < 0.01$ compared with the control group, $##p < 0.01$ compared with the depression group. (D) Open-Field Test; Time Spent in the Center Zone. Statistical data are as described: $F(3,29) = 23.26$, $n = 8$; $*p < 0.01$ compared with the control group, $###p < 0.01$ compared with the depression group. Mean $\pm$ SEM. n refers to the number of mice.

Abbreviations: TST, Tail-Suspension Test; FST, Forced Swim Test; SPT, Sucrose Preference Test; OFT, Open-Field Test.

spines' densities decreased in the hippocampus of depressive mice. However, fluoxetine treatment did not increase the number of dendritic spines with 7- or 14-day treatment (Figure 4A, B). Synaptic cytoskeletal proteins were associated with variable expression of the change's dendritic spine shape.¹⁰ Synaptophysin (SYP) is a synapse-associated structural protein. The results indicated that the expression of SYP was reduced in the hippocampus of depressed mice. While 14-day fluoxetine administration increased SYP expression (Figure 4E,F), 7-day fluoxetine treatment could not suppress these alterations (Figure 4 C, D).

Discussion

SSRIs, which inhibit the reuptake of serotonin neurotransmission, continue to be the initial pharmacotherapy for depression.³⁰ Their precise mechanisms to synapse still remain examined. Although increasing data suggested that chronic fluoxetine treatment has no or opposite action on neuronal plasticity,^{16–21} we discovered that 2-week rather than 1-week fluoxetine treatment significantly alleviated behavioral despair, anhedonia, and anxiety in depressive mice. Interestingly, both 7- and 14-day fluoxetine administration ameliorated hippocampal impairment.

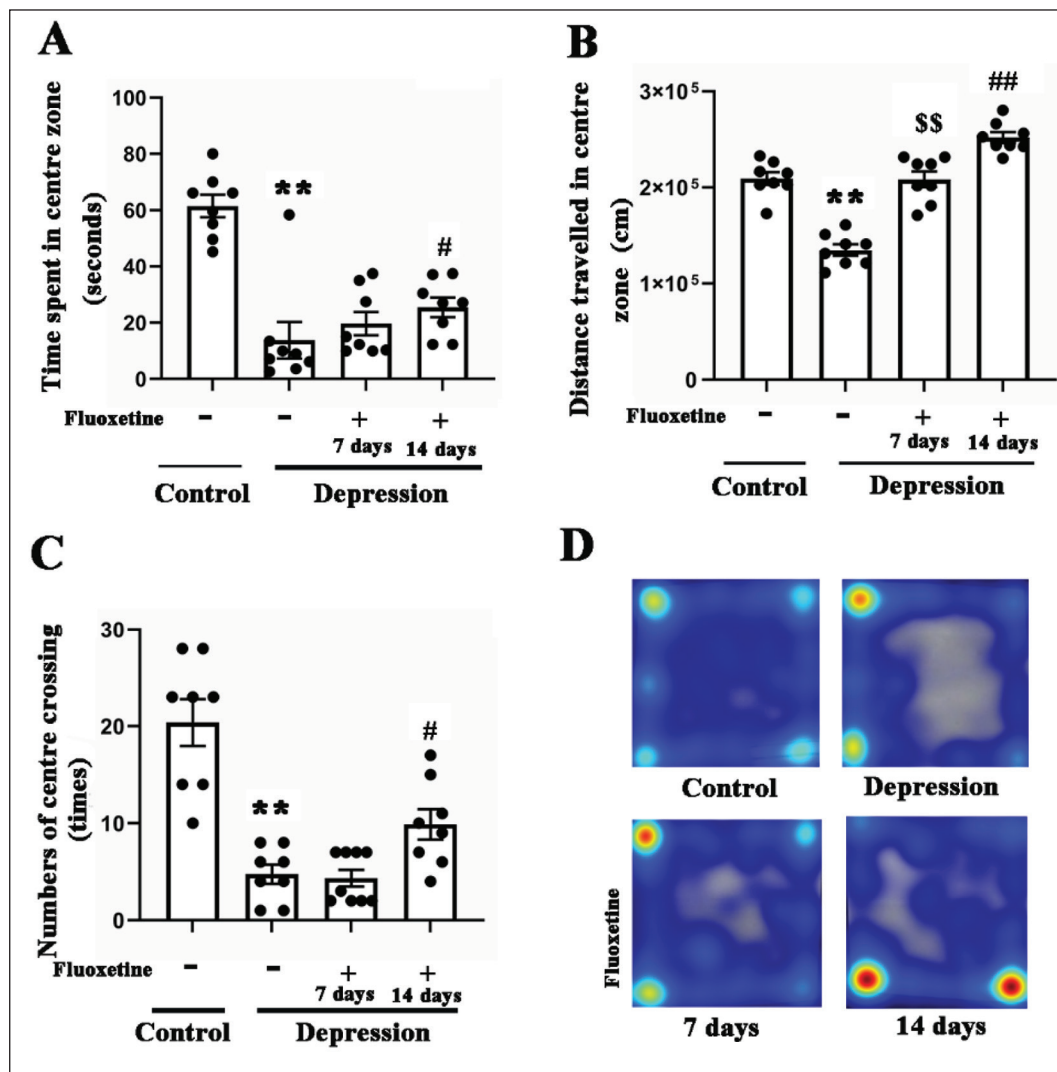


Figure 2. Fluoxetine Ameliorated Anxiety-like Behaviors in Mice. OFT Was Used to Investigate the Effects of Fluoxetine on Depression and Complications of Anxiety in Mice. (A) Time Spent in the Center Zone. Statistical data are as described: $F(3,28) = 20.96$, $n = 8$; $**p < 0.01$ compared with the control group, $#p < 0.05$ compared with the depression group. (B) Distance Traveled in the Center Zone. Statistical data are as described: $F(3,29) = 53.63$, $n = 8$; $**p < 0.01$ compared with the control group, $##p < 0.01$ compared with the depression group, $$$p < 0.01$ compared with the depression group. (C) Number of Center Zone Crossing; $**p < 0.01$ compared with the control group; Statistical data are as described: $F(3,30) = 28.93$, $n = 8$; $**p < 0.01$ compared with the control group, $#p < 0.05$ compared with the depression group. (D) The Trajectories in the Zone. Mean $\pm$ SEM, $n = 8$. n refers to the number of mice.

Abbreviation: OFT, Open-Field Test.

Furthermore, chronic (14 days) fluoxetine treatment only increased the expression of SYP without affecting dendritic spines. Therefore, we determined that long-term fluoxetine treatment alleviated depressant phenotype in mice, which may be associated with ameliorating hippocampal impairment and enhancing hippocampal neuroplasticity.

The delayed onset of action has long been considered a drawback of SSRIs used as antidepressants.⁴ SSRIs in nonhuman primates and humans have been found to widely bioeffect of actions.³¹ We found that treating mice with fluoxetine for only 14 days began to alleviate reserpine-induced anhedonia and desperate behaviors. Consistent with

previous research, SSRIs were demonstrated to take several weeks to action.³² However, the patients with long-term fluoxetine treatment were found to impair their symptoms.³³ These discrepancies seem to be dose-dependent. The actions of SSRIs on the depressant-like phenotype have been found to be driven by the individual and living environment.^{30,34} We determined that 7-day fluoxetine administration had no significant action on depression-like behaviors in mice. Delay action is still the primary defect in SSRIs.⁴ Therefore, we infer that these factors may contribute to the visible SSRIs' side effects that patients often find intolerable, such as sexual dysfunction, weight gain, nausea, and headaches. Nonetheless,

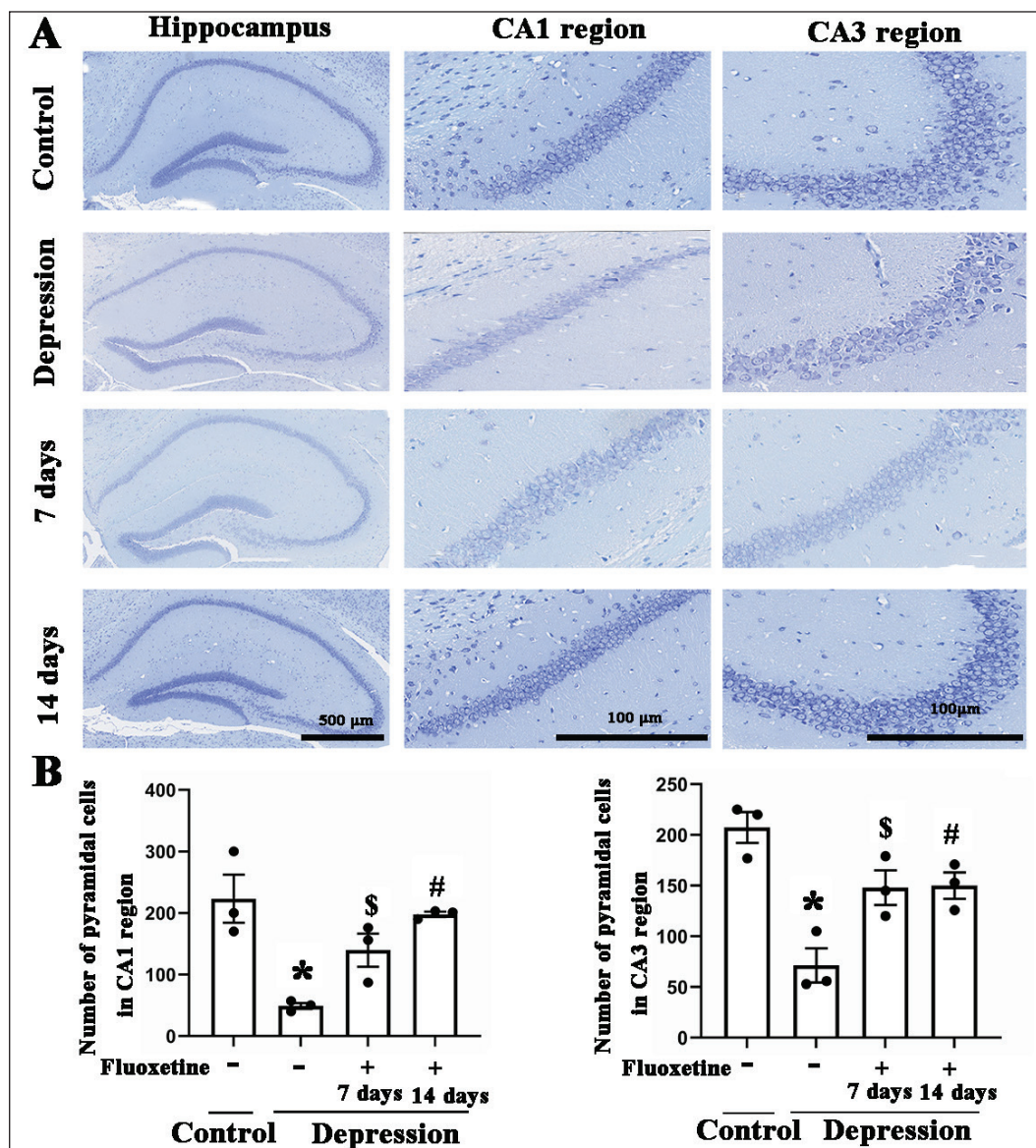


Figure 3. Fluoxetine Improved Hippocampal Impairment in Mice. Nissl Staining Was Applied to Evaluate Hippocampal Neuronal Damage. (A) The Entire Hippocampus and CA1 and CA3 Regions Were Stained by Nissl Staining. Statistical data are as described: $F(3,8) = 10.36$, $n = 3$; $*p < 0.05$ compared with the control group; $\#p < 0.05$ compared with the depression group; $\$p < 0.05$ compared with the depression group. (B) Pyramidal Neurons in CA Regions Underwent Quantitative Analysis. Statistical data are as described: $F(3,8) = 12.72$, $n = 3$; $*p < 0.05$ compared with the control group; $\#p < 0.05$ compared with the depression group; $\$p < 0.05$ compared with the depression group. Mean $\pm$ SEM, $n = 3$. n refers to the number of mice.

our research highlights the impact that fluoxetine can have on depressive symptoms and sheds light on its potential mechanisms of action.

Depression causes hippocampal impairment, which has been thought to account for anhedonia and desperate behaviors.¹⁰ Our previous study found that mice exposed to reserpine exhibited hippocampal deficits, further confirming the link between depression and hippocampus impairment.²⁴ We found that fluoxetine treatment effectively mitigated the loss of hippocampus pyramidal neurons in depressive mice. Hippocampus pyramidal neuron loss in animal models and

humans has been demonstrated to be related to the chronic treatment response to antidepressants.³⁵ Previous studies also indicated that long-term use of SSRIs increased hippocampal granule neuron number and dentate gyrus volume.³⁶ The reduction of the hippocampus volume in depression caused mature neurons atrophy and neurogenesis to decrease.³⁷ The lack of these new neurons of dentate gyrus from animal models of depression has been demonstrated to not causally participate in depression.³⁵ Neurogenesis is considered to be involved in the physiological processes and remission of the impaired brain. We found that fluoxetine treated with 7 days

improved the hippocampus pyramidal neuron loss. These may explain the latency of response to antidepressants.³⁵ Furthermore, cell proliferation, survival, and differentiation are regarded as three phenomena of neurogenesis.³⁷ Many SSRIs may mainly increase cells' early progenitor; thus, these studies have suggested that SSRIs may hinder neurogenesis or have no significant impact.^{17–19}

Synaptic plasticity underlies pathophysiological mechanisms of depression.³⁸ The alterations in morphology and number of dendritic spines have been considered a hallmark of synaptic plasticity.³⁹ We indicate that chronic fluoxetine treatment could not increase dendritic synapse numbers. Consistent with our study, fluoxetine was demonstrated to have low rates of efficacy in terms of synaptic

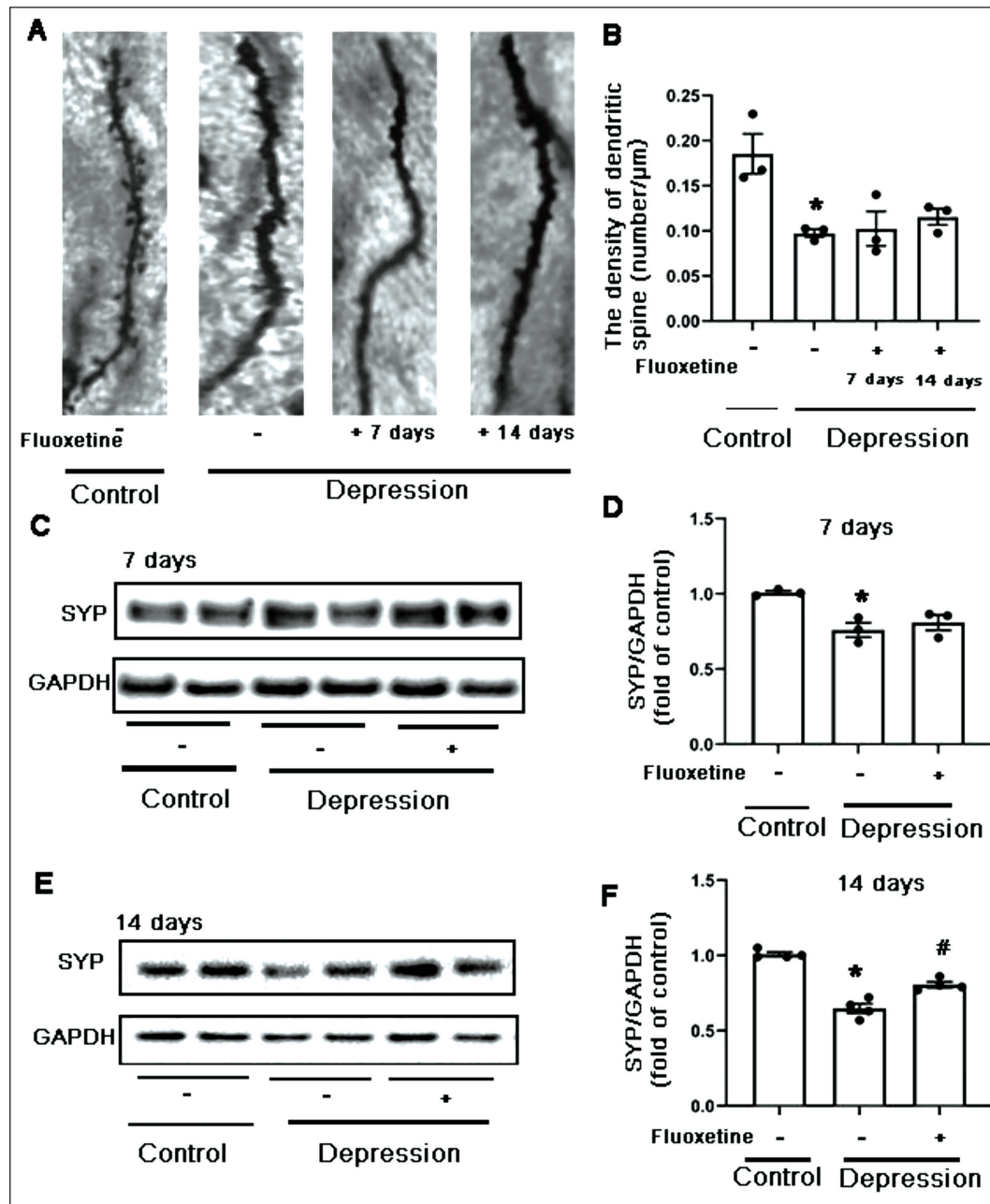


Figure 4. Chronic Fluoxetine Reversed Depression-caused Synapse Loss. (A) The Density of Dendritic Spines in the Dentate Gyrus of the Hippocampus. (B) Quantitative Analysis of the Densities of Dendritic Spines. Statistical data are as described: $F(3,8) = 6.985$, $n = 3$; * $p < 0.05$ compared with the control group. (C) Western Blot Analysis of the Expression of SYP and PSD95 in the Hippocampus. (D) Quantitative Analysis of the Expression of SYP. Statistical data are as described: $F(2,6) = 10.37$, $n = 4$; * $p < 0.05$ compared with the control group. (E) Western Blot Analysis of the Expression of SYP in the Hippocampus. (F) Quantitative Analysis of the Expression of SYP. Statistical data are as described: $F(2,9) = 61.55$, $n = 4$; * $p < 0.05$ compared with the control group; # $p < 0.05$ compared with the depression group. Mean $\pm$ SEM, $n = 3-5$.

structural and connectivity modifications.³⁸ In contrast, ketamine's fast antidepressant effect has been demonstrated to cause rapid changes in neuroplasticity.⁹ This may explain why short-term fluoxetine treatment has a marginal effect on depressant-like behaviors. In addition, the synaptic plasticity's integral functions have been defined to associate with synaptic structural protein levels.^{40,41} Synaptophysin, a vesicular protein, plays a role in synaptogenesis and synaptic plasticity.^{42,43} We discovered that chronic treatment with fluoxetine increased the expression of synaptophysin in the hippocampi of depressive mice. In accordance with ours, long-term fluoxetine treatment has been shown to enhance synaptic plasticity in mice.⁴⁴ Indeed, most antidepressants take weeks to months of treatment to promote neuroplasticity.⁴⁵ Hence, this may reveal the underlying reason that conventional antidepressants possess delayed onset and limited efficiency. Interestingly, long-time fluoxetine treatment was found to have positive or negative impaired neuroplasticity.^{20,21,46} This may be that the effect of fluoxetine on neuroplasticity strongly depended on the living environment.^{30,47,48} However, further research is necessary to elucidate a novel mechanism underlying the efficacy of fluoxetine in treating depression.

In summary, fluoxetine demonstrates the potential to alleviate depression and anxiety-related behavioral abnormalities. Notably, chronic fluoxetine administration was demonstrated to mitigate hippocampal impairment and enhance neuronal plasticity, which may generate novel synapses in depression-sensitive brain regions. Given these positive attributes, fluoxetine constitutes a promising antidepressant agent with a favorable safety profile, warranting further investigation in the field of psychopharmacology.

Conclusion

Long-term SSRI treatment is still the first-line medication for depression due to its superiority. We determine that chronic fluoxetine administration exerts a significant reduction in depressive and anxiety-like behaviors in mice. Importantly, fluoxetine treatment reduces hippocampal impairment for a short time. Moreover, chronic fluoxetine administration increases SYP expression. Therefore, these findings provide evidence that chronic fluoxetine treatment strengthens hippocampal neuron plasticity and promotes synaptogenesis to alleviate depression.

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Authors' Contribution

XQ, YZ, and H-JT designed the study. XQ performed the experiments and wrote the manuscript. YZ and H-JT conceived the project and revised the manuscript.

Data Availability

The original data used in this study are contained in the paper, and further requests can be directed to the corresponding authors (qianx5@mail2.sysu.edu.cn).

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Statement of Ethical Approval and Informed Consent

The ethics committee of the Animal Research Committee, Sun Yat-sen University, approved all experiment protocols (Approval No. SYSU-IACUC-2022-B0067).

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