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Original Article

Neonatal exposure to fluoxetine and fluvoxamine alteres spine density in mouse hippocampal CA1 pyramidal neurons

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Abstract: Some women in childbearing ages take selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine and fluvoxamine for treating psychiatric symptoms. However, these compounds may cause some side effects to their children. It has been identified that early life exposure to SSRIs increased the chance of developing mood disorders and the biological basis is still unclear. Here, we studied the effects of neonatal exposure to SSRIs on neuronal morphology. We used GFP-transgenic mice to investigate the acute and long-lasting effects of early life exposure to SSRIs on dendritic spine density of CA1 neurons. We found that 18-day drug applications of fluoxetine and fluvoxamine significantly reduced spine density of basal dendrites at postnatal day 22 (P22), but only fluvoxamine caused a reduction of spine density of apical dendrites. Interestingly, compared with the control group, the spine densities of basal dendrites after fluoxetine and fluvoxamine exposure and the spine density of apical dendrites after fluoxetine exposure increased in adult mice at the age of P90. We also observed impaired locomotor activity in adult mice after exposure to SSRIs. Our findings demonstrated that neonatal exposure to SSRIs was capable of influencing the morphological plasticity of excitatory synapses. It raised the caution for clinical use of SSRIs.

Keywords: SSRI, dendritic spine density, neuronal development, fluoxetine, fluvoxamine

Introduction

Depression is a major psychiatric disorder with a global prevalence of about 4%, and about 8-20% women may suffer from depression at one point in their lives, usually during childbearing years [1]. Antidepressants include monoamine oxidase inhibitors (MAOIs), selective serotonin reuptake inhibitors (SSRIs), and serotonin norepinephrine reuptake inhibitors (SNRIs). The SSRIs are the most widely used, as they are associated with fewer anticholinergic and cardioarrhythmic side effects [2, 3]. Newborn babies of the women using SSRIs may develop neonatal abstinence syndromes such as behavioral abnormalities [4, 5], and have a higher risk of persistent pulmonary hypertension (PPHN) [6]. In animal models, early-life exposure to antidepressants induced long-lasting alteration of some behaviors such as the rapid eye movement sleep [7] and depression-like behaviors [8, 9]. These studies suggested that maternal use of SSRIs may be harmful to the development of the infants' nervous system. However, the neuronal morphological basis for these behavioral changes is still unevaluated.

Serotonins plays important neurotrophic roles during brain development. The cell bodies of serotonergic neurons, which are located in raphe nuclei in brain stem, project widely to other brain regions. The activities of tyrosine and tryptophan hydroxylases are increased in a critical period of brain development, in which the synaptogenesis undergoes extensive development [10]. Moreover, during this period, high-level serotonins by the treatment of monoamine oxidase A inhibitors could impair the formation of

barrel cortex in mouse [11]. Moreover, gene knockout mice of serotonin transporter exhibited abnormal emotional behaviors, which mimicked the behavioral phenotype of mice treated with SSRIs during early life [12]. Therefore, we hypothesized that the disruption of serotonin homeostasis in early life by SSRIs may affect neuronal morphogenesis. We focused on dendritic spines, as the changes of spine morphology were implicated in the synaptic plasticity [13, 14], and abnormal morphogenesis of dendritic spines was associated with some neurological disorders such as neurodegenerative diseases and mental retardation [15, 16]. We examined locomotor activity in adult mice treated with SSRIs during neonatal period. Furthermore, we examined locomotor activity in adult mice treated with SSRIs during neonatal period.

Material and Methods

Animals and experiment designs

We used GFP-transgenic mice for morphological observation, and wild-type C57 mice for behavioral tests. Male mouse pups in each litter were randomly divided into three groups in a blinded manner, regardless of their body weight, locomotor activity and other appearance before the first injection. In order to avoid the influences about feeding and body weight gain of the mouse pups, we keep the amount of pups in each litter 5-6 in total.

Slice preparation and confocal imaging

Mice were deeply anesthetized before sacrificed, and perfused with 37℃ saline, then perfused with ice-cold 4% paraformaldehyde (PFA). The brain tissues were removed and post-fixed in 4% PFA overnight, and then dehydrated in 15% and 30% sucrose solutions in dark. After that, brain tissues were cut into 50 μm coronal sections. Then slices were mounted with a mixture of glycerol and PBS (V: V = 9:1), and stored at -20℃ until use. The confocal images were obtained using a Zeiss confocal microscope. First, low magnification images of a single GFP neuron in CA1 hippocampal region were taken using a 10X objective lens (10X, NA 0.3, 488 nm laser, LSM 510). Then a low magnification image of a signle neuron was divided into several squares and optical serial sections of 0.5 μm were taken for each square under high magnification using a 100X objective lens (100X, NA 1.40, 488 nm laser, LSM 510) (**Figure 1B**, upper panel). After the collection of images, the serial sections of each high magnification image were used for spine density analysis using software Neurolucida (version 6.0). The total spine amount on each traced dendrites was counted. For each animal, one or two neurons were randomly selected for image collection and 4-7 basal or apical dendrites were traced. At last, the whole traced segment could be reconstructed to yield a complete 3-D image by software NeuroExplorer (**Figure 1C**).

Open field test

The open field test was carried out in a room with controlled temperature, noise and light. A open field chamber is a box with dimentions of 30 cm x 30 cm x 40 cm (length x width x height, TruScan). Mice were first allowed to adapt the light and temperature of the room environment in their home cage for at least 2 hours before the test. After that, mice were individually put into the middle bottom of the chambers to free explore the apparatus for 60 min. The total moving distance in the arena, moving time and time spent in central zone were automatically recorded by TruScan system.

Statistic analysis

The dendritic spines were analyzed using the software Neurolucida (**Figure 1C**). Dendritic spines on secondary or third class dendrites were chosen for analysis. For each dendrite, the spine density was calculated as the average number of spines per millimeter of the dendrite. More than five basal or apical dendrites in each neuron were traced. For each animal, one or two neurons were collected. At least four independent animals in each treatment were used for statistical analysis. Statistical significance was determined by *post hoc* analysis using the least significant difference (LSD) test as elsewhere [17] (neuronal morphology and body weight) and Student's *t* test (locomotor activity).

Experimental procedures

Newborn male GFP-transgenic mice [18] or wild-type C57 mice were given daily intraperitoneal (i.p.) injections of vehicle saline or two SSRIs, fluoxetine (10 mg/kg/day, 0.5 mg/ml, Sigma) and fluvoxamine (10 mg/kg/day, 0.5 mg/ml,

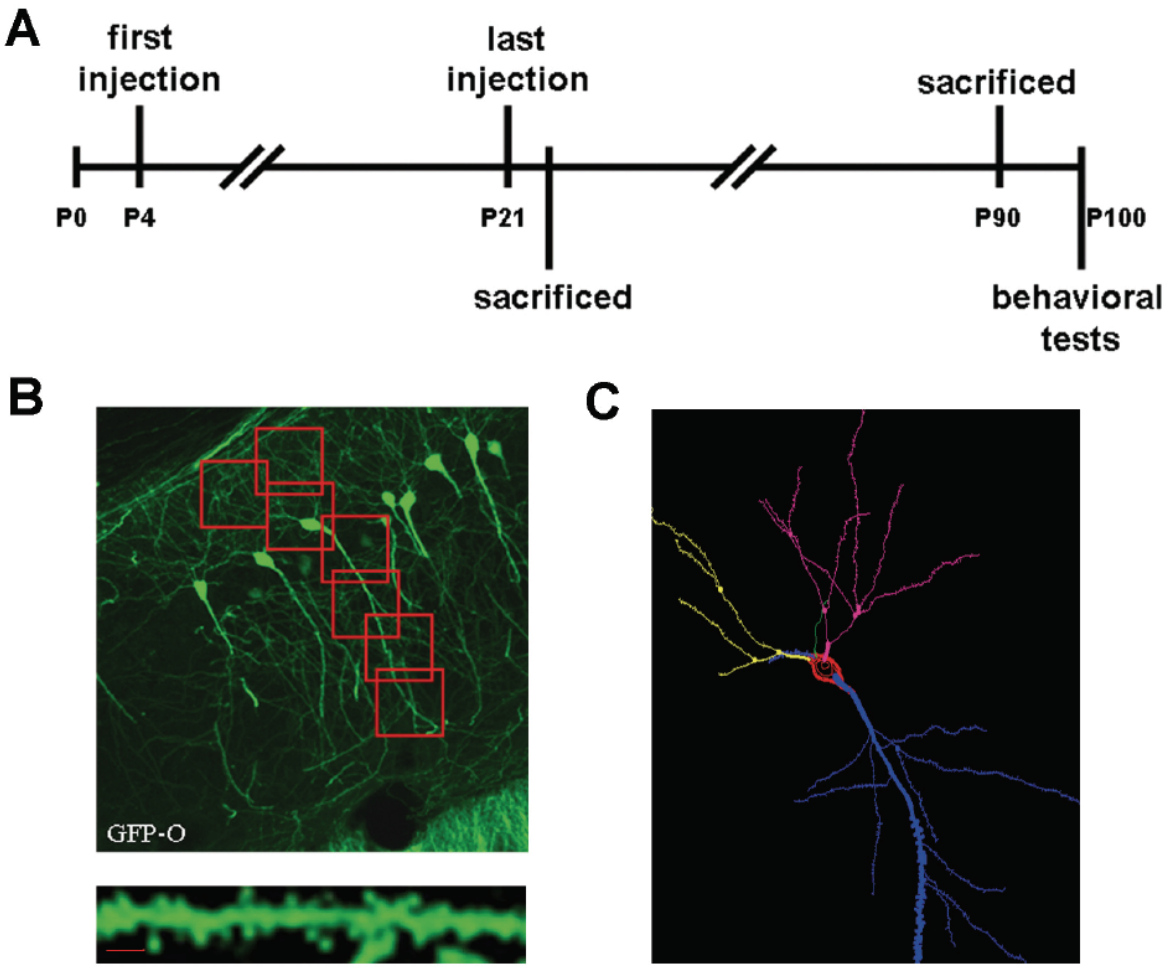

Figure 1 Schematic diagram of experimental design and morphological analysis. (A) GFP transgenic mice received daily injection (i.p.) of SSRIs or vehicle from P4 to P21. The drug dose is 10 mg/kg for both of the two SSRIs, fluoxetine and fluvoxamine, while control group received similar volume of saline. For acute morphological analysis, mice were sacrificed at P22, 20-24 h after the last injection. For behavioral tests, mice were separated by sex on P30 and housed under 12:12 h light/dark cycle. At the age of P90, part of the male mice was used for open field test, while others were sacrificed for evaluation of long-term consequence of SSRI treatment on neuronal morphology. (B) Image data were collected by Zeiss confocal microscope. *upper panel*: hippocampal CA1 pyramidal neurons of GFP-O transgenic mice in low magnification (10X, NA 0.30, 488 nm laser, LSM 510); *lower panel*: dendritic spines under high magnification (100X, NA 1.40, 488 nm laser, LSM 510) on secondary dendrites. Scale bar, 5 μm. (C) Neurites and dendritic spines were traced with software Neurolucida (version 6.0) and the spine density was analyzed by NeuroExplorer (version 4.0).

Sigma) from postnatal day 4 (P4) to P21 as described previously [12]. Male mice in control group received similar volume of solvent saline. Part of the male GFP-transgenic mice at the age of P22, 24 hr after last injection, was sacrificed for the study of short-term effects of SSRIs on neuronal morphology. Mice were weaned at P22 and housed under 12:12 h light/dark cycle. At the age of 13 weeks, the GFP-transgenic male mice were used for the long-term observation of neuronal morphogenesis, while the wild-type C57 male mice were for behavioral tests. The experiment design was illustrated in **Figure 1A**. All experiments were under the approval of the Animal Experiment Committee of Shanghai Institutes for Biological Sciences, and were carried out in accordance with the the National Research Council guide for the care and use of

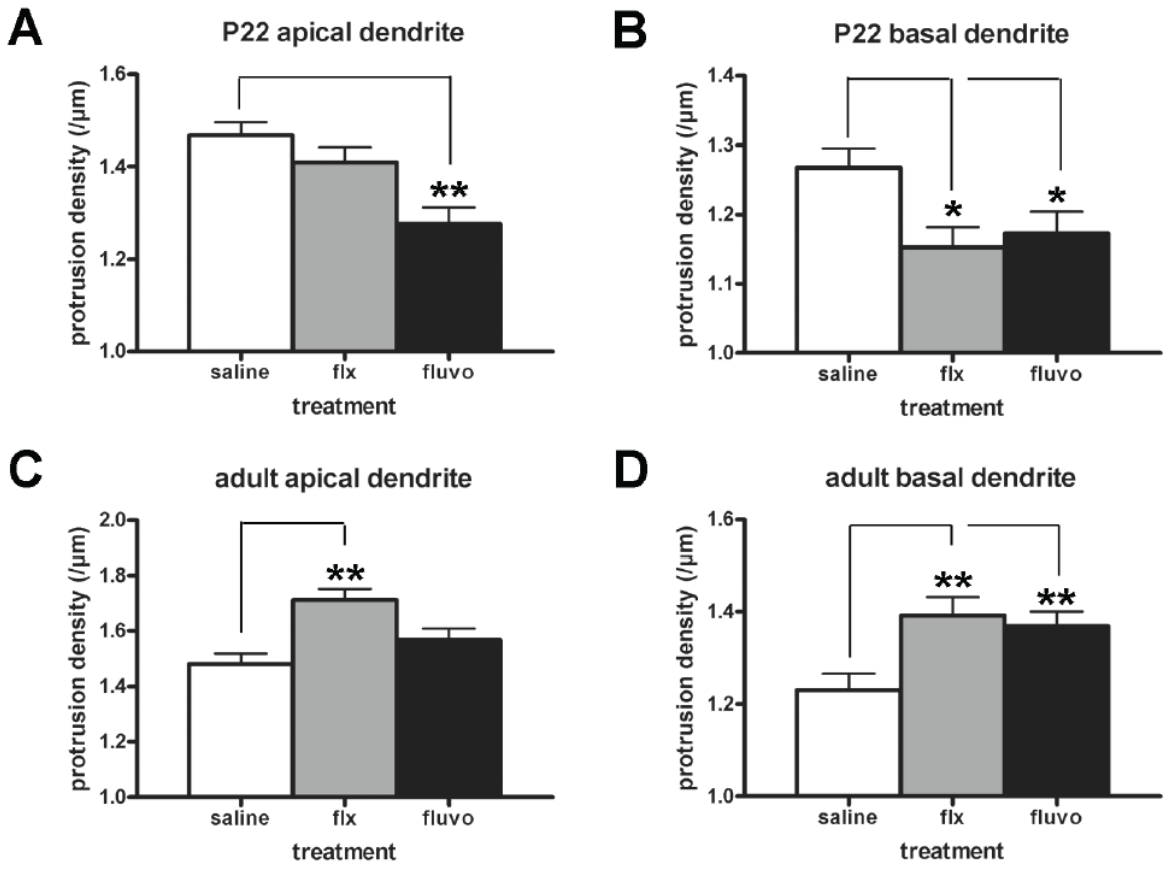

Figure 2 Spine density of CA1 pyramidal neurons after early life exposure to SSRIs. (A, B) Spine density of both apical and basal dendrites were examined at the age of P22. Both of the two groups given SSRIs showed a decreased spine density in basal dendrites (saline, *n* = 38; flx, *n* = 30; fluvo, *n* = 37) compared with saline group, while only fluvoxamine treated group showed a significant decrease of spine density of apical dendrites (saline, *n* = 33; flx, *n* = 27; fluvo, *n* = 33) in CA1 pyramidal neurons. (C, D) Spine density were also examined at the age of P90. Compared with the control group (n=26), the spine density of basal dendrites after fluoxetine (n=30) and fluvoxamine (n=22) exposure and the spine density of apical dendrites after fluoxetine (n=21) exposure were higher in mice at the age of P90. n = number of dendrites. Data are expressed by mean ± SEM and analyzed by LSD test. (**, p<0.05*; ***, p < 0.01*, compared with saline group.)

laboratory animals.

Results

Early life exposure to SSRIs affected spine density both in young and in adult mice

As serotonin plays an important role in brain development, we thus hypothesized that the early life disruption of serotonin homeostasis by SSRIs could affect the development of neuronal morphology. The time window from P0 to P4 was considered a critical period during which the increased level of serotonin could disrupt the formation of barrel cortex [11]. Therefore, we chose to use drugs after P4 to avoid disorganization of brain structure. Two SSRIs, fluoxetine (flx, 10mg/kg/day) and fluvoxamine (fluvo, 10mg/kg/day) at concentrations producing therapeutically relevant blood levels [12] were applied to neonatal mice from P4 to P21. Mice pups were separated from maternal breeding no more than 10 min per day.

We found that 18-day applications of fluoxetine and fluvoxamine significantly resulted in reduced spine density of basal dendrites at P22 but only fluvoxamine caused a reduction of spine density of apical dendrites. There was no significant difference of spine density of aptical

dendrites between control group and fluoxetine group (**Figure 2A** and **2B**). To test whether the influence of SSRIs on dendritic morphology is long-lasting, part of the mice were raised until P90. Interestingly, compared with the control group, the spine density of basal dendrites after fluoxetine and fluvoxamine exposure and the spine density of apical dendrites after fluoxetine exposure were increased in mice at the age of P90, 10 weeks after SSRI withdrawal (**Figure 2C** and **2D**). The chronic exposure to SSRI showed no obvious effect on neuronal variability (data not shown). Furthermore, there is no significant difference of body weight among three groups at P4 (flx vs. saline; fluvo vs. saline). However, the fluoxetine-treated group showed a slight decrease of body weight at the end of drug administration ($P<0.01$), while the fluvoxamine group showed no significant difference (data shown).

Long-term fluoxetine exposure impaired locomotor activity in adult mice

To investigate whether the morphological abnormalities induced by early life exposure to SSRIs were associated with behavioral deficits in adulthood, we performed a novel open field test. Mice were put individually in the novel open field chambers, and their locomotor activities were recorded for a total time of 60 min. Results showed that long-term fluoxetine treatments resulted in a reduction of total distance traveled during the 60 min (flx vs. saline, p = 0.009; **Figure 3A**). Furthermore, we found that fluvoxamine showed a similar effect on locomotor activity as fluoxetine (fluvo vs. saline, p = 0.0496; **Figure 3A**). These results indicated impaired locomotor activities. Meanwhile, fluoxetine group showed a reduction in moving time, while fluvoxamine group did not (flx vs. saline, p = 0.023; fluvo vs. saline, p = 0.115; **Figure 3B**). We also found that there were no obvious variation of center time (flx vs. saline, p = 0.679; fluvo vs. saline, p = 0.878) between control group and SSRIs treated groups (**Figure 3C**). These data implicated that the disruption of serotonin system in early life could lead to long-term consequences on locomotor activities. This result confirmed and extended the results of a previous report [12].

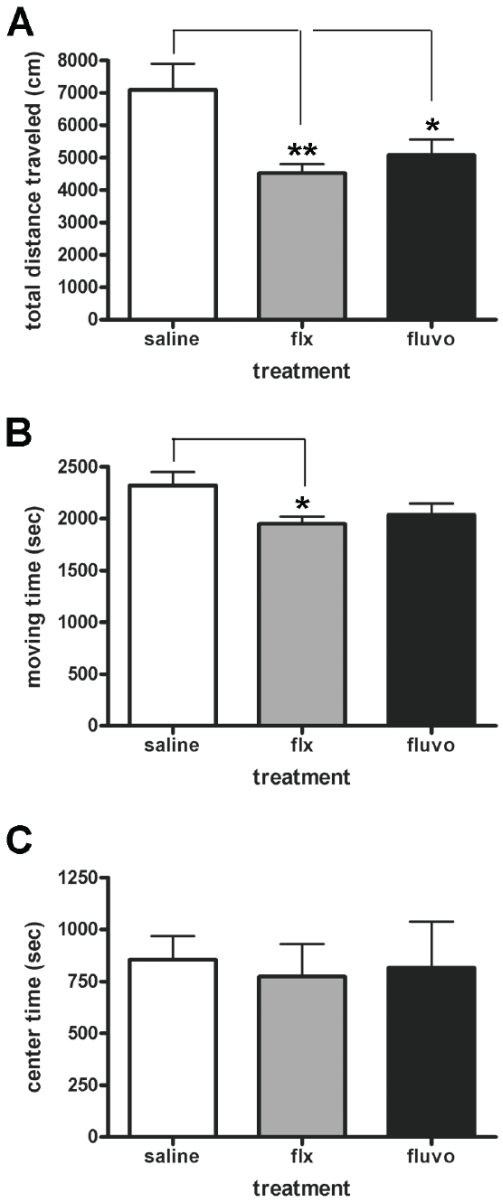

Figure 3. Open field test. (A) Impaired locomotor activities in adult mice after early life exposure to SSRIs. Both of the two groups given SSRIs exhibited a significant decrease of traveling distance during 60 min in the open field. (B) Reduced moving time in fluoxetine group. Total moving time in 60 min was recorded and only fluoxetine group showed a significant decrease of moving time compared with saline group. (C) Normal center time. All three groups showed the similar amount of time spent in center time, which indicated that there is no major difference of exploratory activity between saline- and drug-treated mice. Each plot represents data collected from 8 mice per group (saline, flx or fluvo). Data are expressed by mean ± SEM and analyzed by Student's *t* test, **, $p < 0.05$, **, $p < 0.01$*.

Discussion

There are three major findings in the present

study. First, the spine density of pyramidal neurons in the CA1 region of hippocampus were affected by 18-day administration of SSRIs in neonatal mice. Second, we found increased dendritic spine density in adult mice at P90, 10 weeks after SSRIs withdrawal. Third, we confirmed that early life exposure to SSRIs could alter adult locomotor activity in mice. These findings demonstrated that neonatal exposure to fluoxetine and fluvoxamine could have long-lasting effects on the development of dendritic spines and animal behaviors.

Interestingly, the effect of SSRIs on neuronal morphogenesis is more obvious on basal dendrite than on apical dendrite, which may implicate the differential mechanisms for dendritic development regulated by serotonin system. However, after a longer period withdrawal, the treatment of SSRIs in early life showed an opposite effect on neuronal morphology. The spine density restored to a level higher than the control group in adult mice. The mechanism underlying this increase is not clear yet. By binding to serotonin reuptake transporters, SSRIs can induce elevated serotonin concentration in synaptic cleft. This is widely considered to be the cellular response to SSRI medication for depression treatment. The high level of serotonin in synaptic cleft could continuously trigger a series of cellular signaling pathways in postsynaptic neurons. But the receptors mediated SSRI-induced effects of neuronal morphology are still unclear. It has been reported that actin dynamics and cytoskeleton remodeling may play important roles in neuronal morphogenesis [19]. SSRI treatment may affect the spine density of CA1 neurons via the synaptic remodeling and reorganization through activation of serotonin receptor-mediated signaling pathways.

Our study demonstrated an association between disruption of serotonin system in early life and the changed neuronal structure both in a short-term and a long-term withdrawal time in mice mimicking maternal use of SSRIs in clinical. In addition to morphological study, we also confirmed that SSRI treatment decreased locomotor activity by open field test, which is also a measurement of anxiety. These behavioral observations are consistent with a previous study performed on serotonin transporter knock out mice [12]. Taken together, our results demonstrated that early life exposure to SSRIs was capable of influencing the morphological plasticity of excitatory synapses and raised the caution for clinical use of SSRIs.

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