Melatonin Augments the Effects of Fluoxetine on Depression-Like Behavior and Hippocampal BDNF–TrkB Signaling

Kun Li¹ · Si Shen² · Yu-Tian Ji² · Xu-Yun Li³ · Li-San Zhang⁴ · Xiao-Dong Wang¹

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Abstract Depression is a debilitating psychiatric disorder with a huge socioeconomic burden, and its treatment relies on antidepressants including selective serotonin reuptake inhibitors (SSRIs). Recently, the melatonergic system that is closely associated with the serotonergic system has been implicated in the pathophysiology and treatment of depression. However, it remains unknown whether combined treatment with SSRI and melatonin has synergistic antidepressant effects. In this study, we applied a sub-chronic restraint stress paradigm, and evaluated the potential antidepressant effects of combined fluoxetine and melatonin in adult male mice. Sub-chronic restraint stress (6 h/day for 10 days) induced depression-like behavior as shown by deteriorated fur state, increased latency to groom in the splash test, and increased immobility time in the forced-swim test. Repeated administration of either fluoxetine or melatonin at 10 mg/kg during stress exposure failed to prevent depression-like phenotypes. However, combined treatment with fluoxetine and melatonin at the

selected dose attenuated stress-induced behavioral abnormalities. Moreover, we found that the antidepressant effects of combined treatment were associated with the normalization of brain-derived neurotrophic factor (BDNF)–tropomyosin receptor kinase B (TrkB) signaling in the hippocampus, but not in the prefrontal cortex. Our findings suggest that combined fluoxetine and melatonin treatment exerts synergistic antidepressant effects possibly by restoring hippocampal BDNF–TrkB signaling.

Keywords Melatonin · Fluoxetine · Depression · BDNF · TrkB

Introduction

Depression is a psychiatric disorder closely associated with stress exposure [1], and causes an enormous economic burden [2]. The typical symptoms of depression include anhedonia, long-lasting depressed mood, recurrent thoughts, and attempted suicide [3]. Although the currently available antidepressants are successful in alleviating the symptoms of depression [3, 4], our knowledge of the neurobiological underpinnings of depression is still limited.

5-Hydroxytryptamine (5-HT, serotonin) is a monoamine neurotransmitter crucial for mood regulation [5]. A large body of evidence has shown the critical role of 5-HT in the pathophysiology and treatment of depression [6]. Notably, selective serotonin reuptake inhibitors (SSRIs) are the most commonly-used antidepressants [7]. SSRIs such as fluoxetine exert antidepressant effects by inhibiting 5-HT turnover and increasing synaptic 5-HT levels [8]. In stress models of depression, fluoxetine has been shown to prevent depression-like behavior [9].

Kun Li and Si Shen have contributed equally to this work.

✉ Xiao-Dong Wang
xiaodongwang@zju.edu.cn

- ¹ Department of Neurobiology, Key Laboratory of Medical Neurobiology of the Ministry of Health of China, Zhejiang Province Key Laboratory of Neurobiology, Zhejiang University School of Medicine, Hangzhou 310058, China
- ² Zhejiang University School of Medicine, Hangzhou 310058, China
- ³ Experimental Teaching Center of Basic Medicine, Zhejiang University School of Medicine, Hangzhou 310058, China
- ⁴ Department of Neurology, Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou 310016, China

Melatonin is a circadian rhythm-related hormone synthesized from 5-HT and secreted primarily from the pineal gland during the dark period [10]. Disturbed circadian rhythms have been found in depressive patients, who frequently report insomnia and early-morning awakening. These findings imply a close link between the melatonergic system and depressive symptoms [11]. Recently, a disrupted level of melatonin has been implicated as a risk factor for depression [12], and its receptors MT1 and MT2 are potential therapeutic targets for depression [11]. Indeed, melatonin has been shown to exert antidepressant effects in preclinical studies [12].

Combined treatment with medications having different pharmacological properties may enhance the therapeutic effects of antidepressants [13]. In human studies, although melatonin alone does not alleviate depressive symptoms, it has antidepressant effects in combination with other antidepressants [12, 14]. Because both fluoxetine and melatonin target the 5-HT system and show antidepressant effects, it is possible that their combined administration may have synergistic antidepressant effects.

Brain-derived neurotrophic factor (BDNF) and its receptor tropomyosin receptor kinase B (TrkB) signaling is crucial for the development, plasticity, and remodeling of neuronal circuits [15]. Depression is closely associated with abnormal BDNF–TrkB signaling [16, 17] together with increased levels of circulating glucocorticoids [18, 19] that can be reversed by antidepressants. Disrupted BDNF–TrkB signaling in the hippocampus and the prefrontal cortex has been implicated in the pathophysiology of depression [15, 20–22]. In addition, glucocorticoids are released upon stress challenge and regulate metabolic, immune, and neuronal responses [19]. A dysregulated glucocorticoid level is a risk factor for depression [23]. Therefore, it is possible that fluoxetine and melatonin modulate BDNF–TrkB signaling and glucocorticoid levels to exert synergistic antidepressant effects.

To test these possibilities, we examined the effects of fluoxetine or melatonin alone and in combination, on sub-chronic stress-induced depression-like behavior, cognitive phenotypes, anxiety-related behavior, and molecular alterations in mice.

Materials and Methods

Animals

Male C57BL/6N mice (8 weeks old) were purchased from Shanghai SLAC Laboratory. The mice were maintained in a room at 21 ± 1 °C with 50%–60% relative humidity under a 12-h light/dark cycle (lights on at 08:00) with free access to food and water. All experimental procedures were

approved by the Animal Care and Use Committee of the Institute of Neuroscience, Zhejiang University.

Experimental Design

The experimental design is illustrated in Fig. 1. At 1 week before experiments, the mice were individually housed. A battery of behavioral tests was performed after drug treatment and sub-chronic restraint stress. At 30 min after the forced swim test, mice were anesthetized by isoflurane inhalation and rapidly decapitated for molecular experiments.

Sub-chronic Restraint Stress and Drug Treatment

Mice were randomly assigned to the following groups ($n = 7$ per group): control + vehicle (CTL), sub-chronic restraint stress + vehicle (SRS), sub-chronic restraint stress + fluoxetine (FLX), sub-chronic restraint stress + melatonin (MEL), and sub-chronic restraint stress + fluoxetine + melatonin (FLX + MEL). All mice received a daily intraperitoneal injection of vehicle (normal saline containing 5% ethanol) or drug between 09:00 and 10:00. Melatonin (10 mg/kg; M5250, Sigma-Aldrich, Louis, MO) was dissolved in 5% ethanol and diluted with saline. Fluoxetine (10 mg/kg; F132, Sigma-Aldrich) was dissolved in saline containing 5% ethanol. The doses of melatonin (10 mg/kg) and fluoxetine (10 mg/kg) were selected based on previous reports [24, 25]. After injection, the mouse was placed in a restraint tube (a 50 mL centrifugal tube with punched holes) for 6 h, while the control mouse remained undisturbed in its home cage. Drug treatment and sub-chronic restraint stress lasted for 10 consecutive days. Body weight and fur state were monitored daily. Fur state was classified according to a 4-point scale, where 1 stands for a perfect and clean fur, while 4 represents a disheveled and scruffy fur. Ratings of 2 and 3 demonstrate intermediate fur states [26].

Open Field Test

Locomotor activity and anxiety-like behavior were evaluated by the open field test [27]. The apparatus ($50 \times 50 \times 50$ cm³) was made of gray polyvinyl chloride. The illumination of the central zone (25×25 cm²) was 30 lux. The total distance traveled, time in the central zone, number of entries to the central zone, and number of rearings were recorded by ANY-maze 4.98 (Stoelting, Wood Dale, IL) for 5 min.

Fig. 1 Schematic of the experimental design.

Y-Maze Test

The short-term spatial working memory was tested by recording spontaneous alternation behavior in the Y-maze [28]. The apparatus was made of gray polyvinyl chloride with three symmetrical arms ($30 \times 10 \times 15 \text{ cm}^3$, 30 lux). The arms were marked by a triangle, bar, or plus sign as intra-maze spatial cues. Mice were placed in the center of the Y-maze and allowed to explore all arms freely for 5 min. Three consecutive choices of all three arms were counted as an alternation. The percentage of spontaneous alternation was determined by dividing the total number of alternations by the total number of choices minus 2.

Splash Test

The splash test assesses anhedonia-like behavior [29]. A 10% sucrose solution was evenly sprayed onto the dorsal coat, and the mice were individually placed in an acrylic apparatus ($11 \times 21 \times 9 \text{ cm}^3$). The latency to groom and the total amount of grooming were manually recorded for 5 min as indices of self-care and motivational behaviors.

Forced Swim Test

Depression-like behavior was assessed by the forced-swim test as described previously [30]. Mice were individually placed in a glass cylinder (height, 25 cm; diameter, 12 cm) containing 15 cm-deep water ($21 \pm 1^\circ \text{C}$) for 6 min. The duration of immobility and struggle during the last 5 min was recorded.

Corticosterone Assay

Blood samples were collected and centrifuged at 3,000 rpm for 15 min at 4°C . Plasma corticosterone concentrations were measured using a corticosterone ELISA kit (Cayman Chemical, Ann Arbor, MI) as described previously [31].

Western Blot

Western blot was performed as described previously [32]. Prefrontal cortex and hippocampal samples were dissected, snap-frozen, and stored at -80°C until use. Samples were

homogenized in ice-cold lysis buffer and centrifuged at 10,000 rpm for 20 min at 4°C . Protein concentrations were measured using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Protein samples (20 μg) were electrophoresed in SDS-PAGE gel (15% acrylamide) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked with Tris-buffered saline-Tween (TBST) [150 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 7.5), and 0.1% Tween] containing 5% non-fat milk for 2 h and were incubated with primary antibodies overnight at 4°C . The primary antibodies used were as follows: rabbit anti-BDNF (1:2000; ab108319, Abcam, Cambridge), rabbit anti-TrkB (1:1000; 4603, Cell Signaling, Danvers, MA), rabbit anti-phosphorylated-TrkB (p-TrkB; 1:1000; ab109684, Abcam) and mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:10000, E021010-01; EarthOx, Millbrae, CA). Next, the membranes were incubated with peroxidase-conjugated goat anti-rabbit (1:2000; ZB-2301; Zhongshan Golden Bridge Biotechnology, Beijing) or goat anti-mouse (1:2000; ZB-2305; Zhongshan Golden Bridge Biotechnology) secondary antibody for 3 h at room temperature. Bands were visualized using an enhanced chemiluminescence system and quantified by densitometry (Quantity One 4.2, Bio-Rad, Hercules, CA). All results were normalized to the value of the control group.

Statistical Analysis

Statistical analysis was performed using SPSS 20.0 (SPSS, Chicago, IL). Body weight and fur score were analyzed by one-way repeated measures analysis of variance (ANOVA) followed by the LSD *post hoc* test when appropriate. The remaining data were analyzed by one-way ANOVA followed by the LSD *post hoc* test when applicable. The level of statistical significance was set at $P < 0.05$. Data are expressed as mean $\pm$ SEM.

Results

Effects of Sub-chronic Stress and Drug Treatment on Body Weight, Fur State, and Plasma Corticosterone Levels

The body weight and fur state were monitored daily during sub-chronic restraint stress and drug treatment. No difference in body weight was found among groups ($F_{4, 30} = 0.962$, $P = 0.443$; Fig. 2A), whereas a main effect of condition on fur score was detected ($F_{4, 30} = 14.403$, $P < 0.001$). Compared with the CTL group, the SRS group displayed a significantly worse fur state, which was not improved by fluoxetine or melatonin treatment (all $P < 0.001$ versus CTL; Fig. 2B). However, combined fluoxetine and melatonin treatment attenuated the stress effect on fur state ($P < 0.01$ versus SRS), suggesting a synergistic effect of the combination.

At 30 min after the forced-swim test, blood samples were collected and plasma corticosterone was measured to evaluate the effects of stress and drug treatment on neuroendocrine activation. CTL and SRS mice had similar corticosterone levels (Fig. 2C). Fluoxetine or melatonin administration alone or in combination reduced plasma corticosterone levels ($P < 0.001$ versus CTL), indicating that drug treatment reduced the activation of the hypothalamic-pituitary-adrenal axis.

Sub-chronic Stress and Drug Treatment Had No Effect on Locomotion, Anxiety-Related Behavior, and Spatial Working Memory

After the 10-day restraint stress, we assessed the anxiety-related behavior and spatial working memory using the open field and Y-maze tests, respectively. In the open field test, there were no differences among all groups in the total distance traveled ($F_{4, 30} = 0.284$, $P = 0.886$), time in the central zone ($F_{4, 30} = 1.514$, $P = 0.223$), entries to the central zone ($F_{4, 30} = 1.723$, $P = 0.171$), and number of rearings ($F_{4, 30} = 1.514$, $P = 0.223$; Fig. 3), indicating that sub-chronic restraint stress and drug treatment had no effect on locomotion, exploration, and anxiety-related behavior. In the Y-maze spontaneous alternation task, all mice had comparable spatial working memory performance as shown by similar spontaneous alternation rates ($F_{4, 30} = 1.712$, $P = 0.173$; Fig. 4). In addition, the total number of arm entries remained unchanged in the stressed or drug treatment groups ($F_{4, 30} = 1.522$, $P = 0.221$), further indicating unaltered anxiety levels.

Combined Fluoxetine and Melatonin Treatment Reduced Depression-Like Behavior

Next, we assessed the effects of stress as well as melatonin and/or fluoxetine treatment on depression-like behavior. In

Fig. 2 Effects of sub-chronic restraint stress and drug treatment on body weight, fur state, and plasma corticosterone levels after an acute stress challenge. **A** All groups of mice had comparable body weight. **B** Progression of fur quality during the stress and drug treatment procedures. Combined fluoxetine and melatonin treatment improved the fur state in stressed mice. **C** Either fluoxetine or melatonin administration alone or combined treatment reduced the plasma corticosterone levels at 30 min after the forced swim test. *** $P < 0.001$ versus CTL. ## $P < 0.01$, ### $P < 0.001$ versus SRS. $n = 7$ mice per group.

Fig. 3 Sub-chronic restraint stress and drug treatment did not affect locomotor activity and anxiety-related behavior in the open field test. **A–D** All groups of mice were similar in the total distance traveled, time in the center zone, entries to the center zone, and number of rearings. $n = 7$ mice per group.

Fig. 4 Sub-chronic restraint stress and drug treatment did not affect spatial working memory in the Y-maze spontaneous alternation task. **A–B** All groups had comparable spontaneous alternation rates and number of arm entries. $n = 7$ mice per group.

the splash test, a significant main effect of condition on the latency to groom and number of grooming bouts was revealed (latency to groom: $F_{4, 30} = 8.729$, $P < 0.001$; number of grooming bouts: $F_{4, 30} = 14.313$, $P < 0.001$; Fig. 5A, B). Compared with the CTL group, mice in the SRS group had an increased latency to groom and a decreased number of grooming bouts (both $P < 0.001$ versus CTL; Fig. 5A, B). Administration of fluoxetine or melatonin alone did not reverse the sub-chronic stress effects (all $P < 0.001$ versus CTL), whereas combined fluoxetine and melatonin treatment normalized the performance in the splash test (latency to groom, $P < 0.001$; number of grooming bouts, $P < 0.01$ versus SRS). In the forced-swim test, a similar effect on immobility and struggle time was found (immobility time, $F_{4, 30} = 8.391$, $P < 0.001$; struggle time, $F_{4, 30} = 14.504$, $P < 0.001$; Fig. 5C, D). Sub-chronic stress

significantly increased the immobility time and reduced the struggle time, and treatment with fluoxetine or melatonin alone did not attenuate these effects (all $P < 0.001$ versus CTL). In comparison, combined fluoxetine and melatonin treatment normalized the immobility and struggle time (immobility time, $P < 0.01$; struggle time, $P < 0.001$ versus SRS). These results support the hypothesis that combined fluoxetine and melatonin treatment at a relatively low dose exerts a synergistic antidepressant effect.

Combined Fluoxetine and Melatonin Treatment Normalized BDNF–TrkB Signaling in the Hippocampus, but Not in the Prefrontal Cortex

It has been shown that both fluoxetine and melatonin modulate BDNF–TrkB signaling [33, 34], which has been

Fig. 5 Combined fluoxetine and melatonin treatment reduced depression-like behavior. **A–D** Combined fluoxetine and melatonin treatment decreased the latency to groom and increased the number of grooming bouts in the splash test, and decreased immobility time but increased struggle time in the forced-swim test. *** $P < 0.001$ vs CTL; ## $P < 0.01$, ### $P < 0.001$ vs SRS; $n = 7$ mice per group.

implicated in the pathogenesis of depression. Therefore, we assessed whether the synergistic antidepressant effect of combined fluoxetine and melatonin treatment was associated with the normalization of BDNF–TrkB signaling in the hippocampus and prefrontal cortex. One-way ANOVA revealed a main effect of condition on hippocampal protein levels of BDNF ($F_{4, 30} = 4.986$, $P < 0.01$) and p-TrkB ($F_{4, 30} = 8.410$, $P < 0.001$; Fig. 6A, B), the activated form of TrkB. Compared with the CTL group, the SRS group had reduced BDNF levels ($P < 0.05$) and TrkB phosphorylation ($P < 0.001$) in the hippocampus. Although a low dose of fluoxetine or melatonin failed to attenuate the stress effects (all $P < 0.05$ for CTL versus FLX or MEL), combined treatment normalized the hippocampal BDNF levels ($P < 0.01$ versus SRS) and TrkB signaling ($P < 0.05$ versus SRS). In contrast, we found that combined treatment had no effect on the protein level of BDNF in the prefrontal cortex (all $P < 0.001$ for CTL versus FLX, MEL, or FLX + MEL). Sub-chronic restraint stress and drug treatment did not influence prefrontal TrkB signaling ($F_{4, 30} = 0.885$, $P = 0.485$). In addition, the protein levels of TrkB and GAPDH in both brain regions were comparable among groups (data not shown). These results together indicate that fluoxetine and melatonin exert their synergistic effects by modulating BDNF–TrkB signaling in the hippocampus, but not in the prefrontal cortex.

Discussion

In this study, we found that combined treatment with fluoxetine and melatonin, which were administered at doses with no detectable antidepressant effect, attenuated sub-chronic restraint stress-induced depression-like behavior. Furthermore, the synergistic effect of combined treatment may be modulated by the normalization of hippocampal BDNF–TrkB signaling.

Restraint stress is a well-established animal model of depression [35], with the duration of stress exposure varying among different groups. Using the 6 h $\times$ 10 days restraint procedure, we induced depression-like behavior as shown by deteriorated fur state, increased latency to groom, and decreased number of grooming bouts in the splash test, and increased immobility time and decreased struggle time in the forced swim test in vehicle-treated mice. These results showed the validity of our stress paradigm. However, unlike many studies that report a reduction of body weight gain during or after chronic stress [36], we did not find a significant reduction of body weight gain during the sub-chronic stress period, similar to the results of Macedo *et al.* [37]. In addition, anxiety-related behavior and working memory remained undisturbed in sub-chronically stressed mice. It is likely that prolonged stress exposure is needed to reduce body weight gain, increase anxiety-related behavior, and impair working

Fig. 6 Combined fluoxetine and melatonin treatment normalized BDNF–TrkB signaling in the hippocampus (A–B), but not in the prefrontal cortex (C–D). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs CTL; # $P < 0.05$, ## $P < 0.01$ vs SRS group; $n = 7$ mice per group.

memory, which may also depend on the gender, age, and strain of the animals [38–40].

Fluoxetine is a commonly prescribed SSRI and is generally safe and well-tolerated in clinical use [8]. The effects of fluoxetine are dose- and time-dependent [41, 42]. Fluoxetine at a higher dose for a shorter duration [43] or at a lower dose for a longer duration [44] has more robust antidepressant effects. However, side-effects are present when higher doses are used [42]. In the current study, we found that fluoxetine at a relatively low dose (10 mg/kg) for a short duration (10 days) had no antidepressant effect, in line with previous studies [41]. Meanwhile, we also found that treatment with melatonin (10 mg/kg) alone had no effect on stress-induced depression-like behavior, consistent with human studies [45]. Although melatonin may restore the circadian rhythm in depression patients, it is not always effective in treating depression [14]. However, several animal studies have reported that exogenous melatonin has antidepressant effects [12, 14], which is inconsistent with our results. It

is likely that the dose and/or the duration of fluoxetine and melatonin we used were not high or long enough to exert antidepressant effects. Nonetheless, we found that the combination of both drugs at doses that had no antidepressant effect when injected alone attenuated the deteriorated fur state, decreased the latency to groom, and increased the number of grooming episodes in the splash test, and decreased the immobility time and increased the struggle time in the forced-swim test, indicating that the two drugs interact to abolish the negative effects of sub-chronic restraint stress. In future studies, the underlying mechanisms of such synergistic antidepressant effects merit more detailed investigation.

It has been shown that both fluoxetine [46] and melatonin [47] modulate the level of circulating glucocorticoids. We found that plasma corticosterone levels at 30 min after acute severe stress (forced swim test) were inhibited by administering fluoxetine or melatonin alone. In addition, combined treatment also reduced the corticosterone levels after stress challenge. The mismatch between

depression-like behavior and plasma corticosterone levels indicates that the antidepressant effects of drug treatment are independent of glucocorticoid levels.

The hippocampus and prefrontal cortex are key regions affected in depression [15]. BDNF–TrkB signaling in the hippocampus and prefrontal cortex is essential for stress adaptation, and their levels are regulated by antidepressants [16]. Accumulating evidence has implicated that stress or depression reduces BDNF–TrkB signaling in the hippocampus and prefrontal cortex, accompanied by reduced BDNF levels in the blood [16, 20]. Conversely, antidepressant treatments including SSRIs increase BDNF–TrkB signaling [16]. Furthermore, infusion of BDNF into the hippocampus has an antidepressant-like effect in animal models of depression, whereas mice lacking BDNF show blunted responses to antidepressants [20]. In line with previous findings, our data showed that hippocampal BDNF–TrkB signaling was suppressed after sub-chronic restraint stress [48]. Moreover, we found that the suppression of hippocampal BDNF–TrkB signaling was not prevented by administering fluoxetine or melatonin alone, but rather by combined fluoxetine and melatonin treatment. However, sub-chronic restraint stress and drug treatment did not alter the p-TrkB/TrkB ratio in the prefrontal cortex, and combined treatment failed to normalize prefrontal BDNF levels, findings inconsistent with previous studies [25]. This discrepancy may be ascribed to different stress paradigms. Taken together, these data suggest that the synergistic antidepressant effects of combined treatment may be mediated, at least in part, by normalizing BDNF–TrkB signaling in the stressed hippocampus. Further studies are needed to determine the molecular and cellular mechanisms underlying the modulation of hippocampal BDNF–TrkB signaling by combined fluoxetine and melatonin treatment.

The present study has a few limitations. First, it remains unclear whether the synergistic antidepressant effects of fluoxetine and melatonin are limited to the experimental paradigm we used. More evidence concerning such combined drug application on other animal models of depression and behavioral phenotypes should be provided in future studies. Second, to dissect the role of hippocampal BDNF–TrkB signaling in the therapeutic effects of combined fluoxetine and melatonin treatment, pharmacological and molecular approaches that can manipulate TrkB activation (e.g., the TrkB antagonist ANA-12) should be used.

In summary, our findings suggest that fluoxetine combined with melatonin at a relatively low dose alleviates depression-related behavioral abnormalities possibly by restoring hippocampal BDNF–TrkB signaling. These data may be helpful for optimizing the therapeutic strategy and dissecting the molecular mechanisms of depression.

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