Long-term fluoxetine administration induces changes in microtubule plasticity mediated by CRMP2 in differentiated PC12 cells

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Abstract

Backgroud Depression is a neuropsychiatric disease with a high risk of relapse. Clinical guidelines advocate whole course of antidepressant treatment, but the relapse rate of MDD is still high. Methods We intended to study the changes in microtubule scaffold plasticity induced by long-term antidepressant administration in PC12 cell to explore the possible mechanism of depression relapse. Results The cell activity of NC group decreased after 3 days of culture. The results of immunofluorescence showed the extension of processes was obvious on the first day in Flu group and NC group, but neurite connections were inhibited on the third day in Flu group.CRMP2 and Tubulin were co-located by IF, and Co-IP confirmed the interaction between CRMP2 and Tubulin. RT-PCR showed the mRNA expression of CRMP2 and Tubulin increased on the first and second day in Flu group, but decreased on the third day. Similarly, Western Blotting showed the contents of CRMP2 and Tubulin protein in Flu group increased on the first day, but decreased on the third day. The expression of CRMP2 and Tubulin was affected by changes in the activity of CRMP2. Conclusions The long-term fluoxetine administration can affect the microtubule scaffold plasticity in PC12 cell, and CRMP2 is involved in this process. This may be one of the biological bases of the high risk for depression relapse induced by long-term antidepressant administration.

Background

Major depression disorder (MDD), which is characterized by depression, slow thinking and weak will, is a neuropsychiatric disease with a high risk of relapse. Therefore, both domestic and foreign clinical guidelines advocate whole course of antidepressant treatment to control depressive symptoms in the acute phase, to prevent the relapse of depressive symptoms in the consolidation period and the maintenance period. With in-depth studies of neuroscience and the rapid development of psychopharmacology, a large number of new antidepressants, such as selective serotonin reuptake inhibitor (SSRIs), have been used in the clinic, and remarkable clinical therapeutic results have been obtained. However, the relapse rate of MDD is still high, and more than 85% of patients will relapse after the first depressive attack is alleviated[1]. Some researchers believe that this may be due to deficits in the timely and systematic treatment of MDD patients. A large number of prior clinical studies[2] have confirmed the view that long-term antidepressant administration can effectively control depressive symptoms and prevent the relapse of MDD. However, new research questions this view. For example, in a 23-year follow-up study of MDD patients, Yiend et al.[3] found that 65% of patients with depression experienced relapse, although long-term standardized antidepressant administration was recommended according to clinical guidelines. In addition, some MDD patients that are given systematic and standardized treatment still inevitably relapse, and this has attracted the attention of a large number of researchers.

Neuroplasticity plays an important role in the pathological mechanism of depression[4], and it has also been a prevalent topic of research in recent years. Neuroplasticity, capacity of neurons and neural networks in the brain to change their connections and behavior in response to new information, sensory stimulation, development, damage, or dysfunction. Although neural networks also exhibit modularity and

carry out specific functions, they retain the capacity to deviate from their usual functions and to reorganize themselves.A large number of studies have found that there are neuroplastic deficits in patients with MDD, including in the hippocampus, the prefrontal lobe and other parts of the brain and that neurons and glial cells are decreased, resulting in the atrophy of related brain areas. Psychopharmacological studies of chronic unpredictable mild stress (CUMS) have also found that antidepressant therapy can increase the growth of granular cells in the hippocampus. This suggests that neurogenesis induced by antidepressants may mediate neuroplasticity.

Microtubule scaffold plasticity plays an important role in the pathogenesis of MDD[5]. Tubulin is a heterodimer composed of α-Tubulin and β-Tubulin. The αβ dimer polymerizes at the end of microtubules and has a certain dynamic nature that allows Tubulin to extend forward and produce a characteristic tubular fiber structure that confers cell polarity. Because of this characteristic, Tubulin produces isolated domains in cells, mediates the process of nerve differentiation, such as axonal growth, synaptic activity, and cell polarity[6], and plays a guiding role in intracellular material transport[7]. Proteomics and animal depression model studies have also shown that there are changes in the scaffold microtubule system in MDD animal models. As our previous study[7, 8] showed, in a CUMS animal model, the microtubule structure of the hippocampal cell scaffold changes; specifically, microtubule dynamics and Tubulin expression decrease, and these changes are accompanied by axonal and dendritic changes in neurons. These studies suggest that microtubule scaffold plasticity plays a role in the pathological process involved in the occurrence and development of MDD.

Collapsin response mediator protein 2 (CRMP2) is a protein closely related to neuroplasticity[9, 10]. It is highly expressed in the axons and dendrites of neurons and is closely related to neuroplasticity in depression. A proteomic study of cadaver brains showed that the content of CRMP2 in the hippocampus of patients with MDD is significantly decreased. When the CRMP2 gene is knocked out[11] in rats, they exhibit depressive behaviors accompanied by ventricular enlargement, neuronal reduction and other neuroplastic changes. At the same time, CRMP2 plays a key role in the prolongation of axons and dendrites and mediates the formation of synaptic connections. Our previous study[12] showed that the high expression of CRMP2 can promote the growth of axons and dendrites in hippocampal neurons. In addition, CRMP2 is related to the repair mechanism of neurons[13]. A change in the growth rate of Tubulin in growth cones, which can cause the injury, repair or regeneration of neurons and glial axons is accompanied by an increase in the number of axons and is mediated by CRMP2. These results suggest that CRMP2 may be involved in neuroplasticity in depression by affecting the scaffold microtubule system.

The PC12 cell line is a classical cell line that is widely used in neurobiology and neuropharmacology[14]. It originates from rat pheochromocytoma and can be transformed from a poorly differentiated type to a well-differentiated type in vitro by nerve growth factor. The cell line shows neuronal characteristics, such as a gradual increase in processes, the enlargement of cell bodies and the thickening of branches. These characteristics[15] suggest that PC12 cells are advantageous for the study of scaffold microtubule systems. In addition, the cell line is also widely used in neuropsychopharmacology[16] and depression

cell models[17]. PC12 cells can be used as a cell model to study changes in the microtubule scaffold plasticity under long-term antidepressant administration.

This study hypothesized that antidepressant therapy affects the microtubule scaffold plasticity and that this affect plays a role in antidepressant efficacy but may also have an impact on depression relapse. Therefore, we intended to study the changes in the microtubule scaffold plasticity induced by long-term antidepressant administration and to explore the possible mechanism of depression relapse. We analyzed the effect of the long-term fluoxetineadministration, a classical antidepressant, on Tubulin. The interaction between the CRMP2 and Tubulin proteins was detected by immunoprecipitation. Finally, CRMP2 agonists or antagonists were given to regulate CRMP2 activity, and the effect of CRMP2 on the microtubule scaffold plasticity was further observed.

Methods

1. Cell culture and treatment

The differentiated PC12 cells were provided by the Cell Resource Centre of the Chinese Academy of Life Sciences (Shanghai, China). The PC12 cells were cultured in DMEM supplemented with penicillin (100 U/mL), streptomycin (100 U/mL), and 10% fetal bovine serum (Gibco, USA) in a humidified atmosphere of 5% CO_2 at 37 °C. The cells were divided into 4 groups: the normal control group (NC), the fluoxetine (Aladdin, China) administration group (Flu), the SB216763 (CRMP2 antagonist; Selleck, USA) administration group (SB), and the Wortmannin (CRMP2 agonist; Selleck) administration group (WT). They were cultured continuously for 3 days, and samples from each group were collected on the 1st, 2nd and 3rd days for evaluation.

2. Measurement of cell activity by the CCK8 assay

Cell viability was investigated to select an appropriate concentration of fluoxetine, SB216763 and Wortmannin using the CCK8 (Dojindo, Japan) assay. Each experimental group was further divided into different dose groups: 10nM, 100nM, 1μM, and 10μM Flu□1μM, 10μM, 100μM, and 1000μM SB and 50nM, 500nM, 5μM, and 50μM WT. Cells were seeded into six wells for each group. All samples were cultured continuously for 3 days, and the samples were collected for evaluation on the 1st, 2nd and 3rd days after administration. Cell viability was detected according to the kit instructions to determine the appropriate dose, which was used in subsequent experiments. This experiment was repeated at least 3 times.

3. Immunofluorescence (IF)

Cells grown on glass plates were fixed with 4% paraformaldehyde. Next, the cells were incubated with an rabbit anti-mouse CRMP2 primary antibody(1:500,Abcam,UK) and a mouse anti-mouse Tubulin primary antibody(1:1000,Abcam) at 4°C overnight, followed by incubation with an Alexa Fluor 594-conjugated goat anti-rabbit IgG secondary antibody (1:250, Abcam) and an Alex Fluor 488-conjugated goat anti-

mouse IgG secondary antibody (1:200, Abcam). The slides were counterstained with DAPI to visualize the cell nuclei. Images were recorded using a confocal laser scanning microscope. This experiment was repeated at least 3 times.

4. Real-time quantitative PCR (RT-PCR)

Total cellular RNA was extracted from PC12 cells from each experimental group using TRIzol reagent (Beyotime Biotechnology, China) based on the manufacturer's instructions. RNA (2μg) was reverse transcribed using a PrimeScript RT Kit (Takara, Japan). Real-time PCR was performed using SYBR master mix (Takara) on a Bio-Rad Connect Real-Time PCR platform. The primer sequences are as follows Table 1 .

5. Western blot analyses

At the end of the administrations, the cells were washed twice with PBS and then lysed with lysis buffer containing 1% protease inhibitor cocktail. The cell lysates were collected and centrifuged at 12,000 rpm for 12 min at 4 °C, and the supernatants were collected. The total protein content was measured by a BCA kit (Sigma, USA). An equal amount of protein (20μg) from each sample was separated by electrophoresis on SDS-polyacrylamide gels of appropriate concentrations and transferred onto nitrocellulose membranes. Five percent nonfat milk was used to block nonspecific binding sites for 70 min. Next, the membranes were incubated with a rabbit anti-mouse CRMP2 primary antibody (1:20000, Abcam), a mouse anti-mouse Tubulin primary antibody (1:5000, Abcam) and a rabbit anti-mouse GAPDH primary antibody (1:1000,Abcam) overnight at 4 °C, and they were incubated with secondary antibodies (HRP-labeled Goat Anti-Rat IgG,1:5000 and HRP-labeled Goat Anti-Rabbit IgG,1:10000, Abcam) at room temperature for 70 min. After washing three times with TBST, the bands were detected with a chemiluminescence detection system (Bio-Red, USA). This experiment was repeated at least 3 times.

6. Co-immunoprecipitation (Co-IP)

For the immunoprecipitation of CRMP2/Tubulin, the cell lysates were separated by centrifugation, incubated with 1μg anti-CRMP2(1:20000)/ anti-Tubulin (1:5000) antibodies (Abcam) overnight at 4 °C, and precipitated using Protein A agarose beads (Roche, Mannheim, Germany). The beads were washed eight times, and the bound proteins were released into the buffer by heating the samples at 100 °C for 7 min. The next steps are consistent with Western blot analyses.

7. Statistical analysis

All values are presented as the means ± standard deviation (SD). Statistical analyses were performed using SPSS 23.0 software (SPSS, Chicago, USA). A nonparametric Student's t-test was used to determine statistical differences. $P < 0.05$ was considered significant.

Results

1. Determination of the appropriate concentrations of Fluoxetine, SB216763 and Wortmannin in PC12 cells

First, PC12 cells were cultured continuously for 3 days, and their cell activity was detected by the CCK8 assay to determine the appropriate length of continuous administration in the subsequent experiments. As shown in Fig. 1A, the NC group was cultured continuously for 3 days. Compared with the cell activity on the first day, the cell activity on the second day (93.5 ±3.5%) and the third day (71.4±4.4%) decreased over time, and there was a significant difference between the two days (P <0.000). In addition, on the 4th day of culture (results not shown), microscopic observation showed that the cells exhibited vacuoles, atrophied processes, and cell activity that decreased to approximately 20%. Other results[18] showed that the cell survival rate decreased to approximately 50%, which stimulated cell apoptosis without stimulating cell death. This indicates senescence and apoptosis during this period, which are not suitable for the experiment.

Different doses caused differences in cell activity. To investigate the appropriate doses for the different experimental groups, after the cells were inoculated and adhered to the plate, the following drugs and doses were administered for 1 day, 2 days, and 3 days: Flu(10nM, 100nM, 1μM, and 10μM), SB(1 μM, 10 μM, 100 μM, and 1000 μM) and WT (50 nM, 500 nM, 5 μM, and50 μM). The CCK8 assay was used to detect cell activity on the 1st, 2nd and 3rd days. The difference in cell activity in each group treated with different doses was analyzed. As shown in Figs. 1B, 1C and 1D, the cell activity of the Flu administration group was the highest after 10^{-6} mol/L administration, the SB administration group exhibited the highest activity after 10 μM administration, and the WT administration group exhibited the highest activity after 5 μM administration (all P<0.05).

2. Fluoxetine improved the microtubule plasticity and increased the expression of Tubulin in the early stage of long-term administration, but aggravated deficits in microtubule plasticity in the later stage

The processes in the Flu group extended obviously on the first day but were inhibited on the third day. The intracellular expression of the Tubulin protein is important for the formation of microtubules for the cell scaffold, and the fluorescence of the protein can directly reflect changes in cell processes and cell junctions. After inoculation, PC12 cells were allowed to adhere to the plate, and then the processes gradually prolonged and extended. The fluorescence results showed that both the Flu group and NC group showed the obvious extension of processes on the first day (Fig. 2A). On the 3rd day, the processes of the NC group connected with each other to form a network-like structure. However, the processes of the Flu group did not yet exhibit the connected network structure observed in the NC group (Fig. 2C).

Merged images of CRMP2 and Tubulin fluorescence showed overlap between the CRMP2 expression region and the Tubulin expression region. There was fluorescence colocalization between the two proteins. The fluorescence colocalization in the NC group and Flu group was obvious on the first and second days but on the third day, the colocalization in the Flu group was significantly less than that in the NC group.

The Flu group exhibited increased levels of CRMP2 and Tubulin proteins on the first day, but these levels were decreased significantly on the third day. Fig. 3 shows that CRMP2 in the Flu group (0.84±0.26, *P*=0.004) was significantly higher than that in the NC group (0.48±0.09) on the first day, but there was no significant difference between the Flu (0.67±0.23, *P*=0.266) and NC groups (0.86±0.28) on the second day. On the 3rd day, the protein content (0.23±0.11, *P*=0.026) was lower in the Flu group than in the NC group (0.56±0.31). The Tubulin and CRMP2 protein content in the Flu group was the same after 3 days of continuous administration. The content of Tubulin in the Flu group (1.34±0.23, *P*=0.041) was higher than that in the NC group (0.86±0.17) on the first day, but there was no significant difference in the Tubulin content (0.73±0.29, *P*=0.123) between the Flu group and NC group (1.40±0.33) on the second day. On the 3rd day, the content of Tubulin (0.21±0.09, *P*=0.015) was lower than in the Flu group that in the NC group (0.77±0.25).

The mRNA expression of CRMP2 and Tubulin increased on the first and second day of Flu administration but decreased on the third day. As shown in Figs. 4A-1, 4B-1 and 4C-1, the expression of mRNA of CRMP2 on day 1(1.31±0.08，*P*=0.030) and day 2(1.60±0.07，*P*=0.001) in the Flu administration group was significantly higher than that on day 1(1.01±0.11) and day 2(1.00±0.07) in the NC group. However, on the third day, the CRMP2 mRNA level in the Flu group (0.46±0.04, *P*=0.001) was lower than that in the NC group (1.05±0.07). Similarly, as shown in Figs. 4A-2, 4B-2, 4C-2, the mRNA of Tubulin in the Flu administration group was higher than that in the NC group on the first day (1.35±0.05, *P*=0.017) and the second day (1.63±0.05, *P*=0.001). However, on the third day, the Tubulin mRNA level in the Flu group (0.64±0.07, *P*=0.026) was also lower than that in the NC group.

3. Co-IP validates the interaction between the CRMP2 and Tubulin proteins

The immunofluorescence results showed that there was immunofluorescence colocalization of CRMP2 and Tubulin. To investigate whether there is a direct interaction between the two proteins, we performed immunoprecipitation in 1day of NC group. Fig. 5A shows IP for Tubulin, IB for CRMP2, and CRMP2 in the cell lysate bound with magnetic beads containing a Tubulin antibody. The results (anti-CRMP2) showed that there was CRMP2 in the conjugates. Fig. 5B shows IP for CRMP2, IB for Tubulin, and Tubulin in the cell lysate bound with magnetic beads containing a CRMP2 antibody. The results (anti-Tubulin) showed that Tubulin existed in the conjugates. After double verification, it was fully confirmed that there was a direct interaction between the CRMP2 and Tubulin proteins. The coexpression of the two proteins was further verified by immunofluorescence at the protein level.

4. Effect of CRMP2 activity on Tubulin plasticity in PC12 cells

After determining the direct interaction between the CRMP2 and Tubulin proteins, we hypothesized that CRMP2 is involved in mediating microtubule scaffold plasticity. To answer this question, we tried to regulate CRMP2 activity with CRMP2 agonists and inhibitors for 3 days to observe the changes in Tubulin in cell scaffolds. We used the CCK8 assay to detect cell activity to determine the appropriate dose of SB216763 (a CRMP2 antagonist) and Wortmannin (a CRMP2 agonist). The results are shown in Fig. 1.

The cell activity in the SB group was the highest after 10 μM administration and the cell activity in the WT administration group was the highest after 5 μM administration.

In the SB group, the processes were significantly inhibited on the 1st and 2nd days, but neurite elongation and cell junctions increased on the 3rd day. In the WT group, the processes continued to elongate, and the intercellular junctions were enhanced for 3 days. The fluorescence results (Fig. 2) showed that the development of PC12 cells was inhibited on the first and second days in the SB administration group. On the first day, the extension of the cell processes was slow. On the second day, the processes began to extend, but they were still shorter than those in the normal control group. On the 3rd day, the processes of the cells were extended, and the intercellular connections increased significantly, attaining the network-like structure of the NC group. In the WT administration group, the processes were extended, and the intercellular junctions were enhanced from the first day to the third day. In addition, the fluorescence results of the SB group showed that, except for a small amount of CRMP2 and Tubulin colocalization on the first day, it was difficult to find the colocalization on the other two days. However, the colocalization of CRMP2 and Tubulin in the WT group was clearly visible from day 1 to day 3.

The content of CRMP2 in the SB group was significantly decreased after 3 days of continuous administration, while the content of the CRMP2 protein was significantly increased in the WT group. As shown in Fig. 4, the CRMP2 protein content was significantly higher in the WT group than in the NC group on the 1st (0.24±0.08, P=0.035), 2nd (0.37±0.16, P=0.007) and 3rd (0.15±0.09, P=0.007) days in the SB group was lower than that in the NC group on the 1st, 2nd and 3rd days. In contrast, the protein content of CRMP2 on the 1st (1.16±0.17, P=0.007), 2nd (2.05±0.32, P=0.000) and 3rd (1.62±0.25, P=0.000) days. The content of the Tubulin protein in the SB group decreased on the first day and the second day, but there was no difference between the SB group and the NC group on the third day. The Tubulin content was significantly increased in the WT group after continuous administration for 3 days. As shown in Fig. 3, the content of Tubulin in the SB group was inhibited on the first day (0.36±0.12, P=0.031) and the second day (0.44±0.13, P=0.032) and was lower than that in the NC group on the first and second days. However, there was no significant difference in the content of Tubulin (0.55±0.11, P=0.305) between the SB group and the NC group on the 3rd day. The content of Tubulin in the WT group was higher than that in the NC group on the first day (2.82±0.60, P=0.000), the second day (5.40±1.21, P=0.000) and the third day (3.34±0.58, P=0.000).

The mRNA expression of CRMP2 was inhibited in the SB group for 3 days but increased in the WT group for 3 days. Figs. 4A-1, 4B-1 and 4C-1 show that the mRNA expression of CRMP2 in the SB group was significantly inhibited on day 1 (0.40±0.06, P=0.003), day 2 (0.46±0.08, P=0.002) and day 3 (0.15±0.04, P=0.000) compared with that in the NC group on day 1 (1.01±0.11), day 2 (1.00±0.07) and day 3 (1.05±0.07). In contrast, the mRNA of CRMP2 in the WT group on the first day (2.42±0.11), the second day (1.87±0.06) and the third day (1.55±0.10) was higher than that in the NC group on the first day, the second day and the third day (all P<0.01). Tubulin mRNA expression in the SB group was inhibited on the 1st and 2nd days after continuous administration for 3 days, but there was no difference between the SB group and the NC group on the 3rd day. In contrast, the mRNA expression increased in the WT group after

3 days of continuous administration. As shown in Figs. 4A-2, 4B-2 and 4C-2, compared with that on the first and second days in the NC group, the mRNA level of Tubulin in the SB administration group was also significantly inhibited on the first day (0.51±0.09, P=0.004) and the second day (0.36±0.06, P =0.001) but on the 3rd day (1.87±0.10, P=0.002), it was higher in the SB group than in the NC group. In contrast, the mRNA of Tubulin in the WT group was significantly higher on the first day (1.82±0.12), the second day (1.93±0.11) and the third day (2.68±0.19) than that in the NC group (all P< 0.01).

Discussion

To study the changes in microtubule scaffold plasticity induced by long-term antidepressant administration to explore the possible mechanism of depression relapse we first measured cell activity to evaluate different experimental periods. In the past, more attention was paid to the kinetics of psychotropic drug efficiency, which is concerned with the rapid detection of cell activity after acute administration and the acquisition of the median lethal dose (LD50). We used the psychopharmacology method, using the CCK8 assay to reflect cell activity and to determine the experimental period for the long-term administration of cells. It was found that the activity of PC12 cells decreased with time during culture. On the 4th day of culture, the cells exhibited a large amount of apoptosis. Other results[18] showed that the cell survival rate decreased to approximately 50%, which suggests cell apoptosis without cell death. This indicates that the senescence and apoptosis present in this period were not suitable for the experiment. Therefore, we chose 3 days as the time frame for long-term administration.

In this study, it was found that fluoxetine administration enhances the plasticity of Tubulin in cell scaffolds in the early stage and promotes the extension of cell processes and the connection of cells. This is consistent with the results of previous studies[19, 20], which found that fluoxetine, an antidepressant, increases neurogenesis, synaptogenesis and synaptic plasticity in the hippocampus, cortex and amygdala[21]. Some researchers[22] have summarized the main effects of fluoxetine on neuroplasticity. First, fluoxetine administration increases the proliferation of neural progenitor cells. Second, fluoxetine stimulates dendritic branches and promotes the maturation of immature granulosa cells. Third, fluoxetine enhances the survival rate of immature neurons. Fourth, immature neurons are functionally integrated into local neural circuits and produce long-term synaptic plasticity enhancement. A large number of psychopharmacological studies[23, 24] have shown that fluoxetine has delayed efficacy in the treatment of depression, which is related to the therapeutic mechanism of SSRIs[25]. That is, fluoxetine administration depends more on the long-term adaptation of the presynaptic/postsynaptic serotonin concentration balance[26]. Therefore, some researchers have gradually changed their focus from structural plasticity to functional plasticity[21, 27]. They have found that fluoxetine promotes synaptic maturation and participates in nonclassical synaptic dynamics, inducing the expression of proteins that regulate vesicle transport and transmitter release. Tubulin plays an important role in structural plasticity. Improvements in structural plasticity also effectively promote the process of functional plasticity. Some studies have found that the combination of other drugs that promote structural plasticity can improve their efficacies; for example, imipramine combined with SSRIs can

effectively promote the recovery of acute depression and reduce the risk of stable relapse[28]. Furthermore, a study[29] found that imipramine can increase the number of neurons and the prolongation of axonal dendrites and inhibit the proliferation of microglia, which may alleviate the susceptibility to depression relapse to a certain extent.

However, these studies are based on the premise that the neurons are not yet mature. In the NC group of our study, the cell activity remained at a high level on the first day of culture, and the processes and junctions of the cells were formed after adherence to the plate. Recent studies on developmental plasticity[30, 31] have also found that SSRI antidepressants mainly play a role in immature neurons to induce faster plasticity. This provides new insight into the mechanism and treatment of depression from the point of view of the mechanism of developmental plasticity. That is, considering the reversible changes in neural plasticity in MDD patients, the timely administration of antidepressants to maintain the stability of the levels of related transmitters and ensure the effective transmission of neural information can effectively improve the control of depression-like symptoms. In this process, neuroplasticity is repaired as necessary, and antidepressants can also induce and promote the recovery of neuroplasticity. In this study, it was found that fluoxetine enhanced the expression of Tubulin and the content of the Tubulin protein in the early stage of fluoxetine administration. CRMP2 mediates participation in the above processes. By increasing the activity of CRMP2, the expression of Tubulin was further enhanced, the microtubule plasticity in the cell scaffolds was promoted, and the extension of cell processes and the connection of cells were significantly enhanced.

However, at the later stage of fluoxetine administration, the microtubule scaffold plasticity was impaired, the mRNA and protein contents of Tubulin were significantly decreased, and the intercellular junctions were sparse. The same result was found in an animal model of recurrent depression[32]; that is, long-term fluoxetine administration resulted in neuronal atrophy and the inhibition of axonal dendrite prolongation. These results suggest that neuroplastic injury in the late stage of the long-term fluoxetineadministration may be one of the neural structural bases for the increased susceptibility to depression relapse. Many studies[33] have shown that SSRI administration can affect depression-like behavior, but most of these studies have not considered the living environment of the subjects. Previous animal model studies[34] have demonstrated that hippocampal neuron injury is associated with depression, and fluoxetine can alleviate or even reverse the decrease in hippocampal neurons induced by stress. However, recent data[35, 36] has shown that fluoxetine does not effectively enhance neurogenesis or even lead to neurogenesis. fluoxetine administration under conditions of stress results in a decrease in the proliferation of hippocampal neurons and a decrease in the volume of the CA1 region[37].

Stress is an important part of the development of depression[38]. Early-life stress events, such as child abuse, emotional neglect or absence, are widely accepted and recognized as a major risk factor for depression later in life[39]. Some studies[22] have also found that the therapeutic effect of fluoxetine is highly dependent on the quality of the living environment, and epigenetic changes[40] are involved in the occurrence and development of life stress and depression. Our study showed that the mRNA expression of CRMP2 and Tubulin was higher on day 2 in the fluoxetine administration group than in the normal

control, but the protein content of CRMP2 and Tubulin was not different between the Flu group and the NC group. However, on the 3rd day, the mRNA and protein expression of CRMP2 and Tubulin were lower in the Flu group than in the NC group. This suggests that there may be a regulatory feedback pathway for scaffold Tubulin. The results of the SB group further confirmed that SB inhibited the expression of CRMP2 for 3 days and caused the content of Tubulin to be lower than that in the normal control group on the first and second days. However, there was no difference in the protein content between the SB group and the normal control group on the 3rd day. At present, the specific regulatory feedback mechanism is not clear, and further research is needed. However, some studies[41] have suggested that the occurrence of this transcriptional regulation may be related to epigenetics[41, 42]. Fluoxetine induces the epigenetic modification of hippocampal neurogenesis and the modification of related protein gene expression[43] through methylation, acetylation, etc., which lead to changes in transcription, translation and the microtubule plasticity of scaffolds. The findings provide a new way to treat depression; that is, fluoxetine administration partly improves neuroplasticity, but its therapeutic effect is affected by the living environment. During the maintenance period of depression, we actively improve the living environment of patients, carry out necessary cognitive behavioral therapy, improve the social participation of patients, and improve the curative effect, which may play a role in reducing the risk of depression relapse.

Recently, it has been suggested that MDD is a precursor of or an early clinical manifestation of Alzheimer's disease (AD)[44]. Cumulative studies[45, 46] have shown that MDD is associated with AD and that there may be a common mechanism of neuroplasticity between the two diseases[47].Polygenic risk scores can predict the risk of MDD developing into AD[48]. Some researchers[49] have found a reduction in hippocampal volume in patients with severe depression, and a one-year follow-up study also found that the volume of the left and right hippocampus in patients without clinical remission treated with antidepressants was smaller than baseline volumes. A reduction in hippocampal volume is the most prominent feature of the early pathological changes of AD[50], and it is also one of the most reliable predictors of the transformation from MDD to AD[51]. It has been found[52] that the abnormal expression and modification of scaffold Tubulin in MDD leads to changes and the aggregation of proteins, such as tau protein, which eventually forms neurofibrillary tangles. This is one of the key signs of neurodegeneration in AD. Our previous study[53] found that AKT/GSK3β/CRMP2 mediates microtubule scaffold plasticity in depression and improves depression-like behavior in animals by regulating CRMP2 activity. It has been shown that[54] this pathway plays an important role in the aging process of nerves in AD, and the regulation of this pathway can cause earlier and more serious neuronal injury.

Depression causes changes in hippocampal formation[49, 55], mainly including a decrease in the number and length of dendrites. Environmental factors such as stress are also involved in this process. With the progression of depression, nerve injury gradually advances from a reversible injury to an irreversible nerve injury, and fluoxetine administration results in the aggravation of plasticity impairment. Previous studies[56] have also confirmed that elderly patients with depression have a high susceptibility to relapse, which explains the phenomenon of neuronal atrophy and axonal prolongation inhibition induced by long-term fluoxetine administration in an animal model of depression relapse[32]. This is in agreement with the results of this study that fluoxetine administration causes deficits in the microtubule scaffold

plasticity at the later stage. In the normal control group, cell activity decreased to 71.4±4.4% at the later stage of culture. This suggests that the cells were close to the level of cell senescence and that the microtubule scaffold plasticity were irreversibly damaged. In addition, we also found that CRMP2 is involved in the process of microtubule scaffold plasticity during fluoxetine administration. By increasing the activity of CRMP2, the cells maintained the cell junctions and extension for 3 days, suggesting that CRMP2 may be an important regulatory factor in maintaining the homeostasis of microtubules in senescent cell scaffolds. This provides a new possible strategy for treating depression relapse, which needs to be further studied.

There are some shortcomings in this study. First, immunoprecipitation only verified the interaction between CRMP2 and Tubulin in the normal control group and did not compare the binding rates of the two proteins. Second, it is necessary to establish a cell model of depression to further study the changes in microtubule scaffold plasticity after long-term fluoxetine administration. Finally, animal experiments are needed to further verify the changes in microtubule scaffold plasticity during long-term antidepressantsadministration.

Conclusion

Long-term fluoxetineadministration causes changes in the microtubule scaffold plasticity and effectively improves the plasticity in the early stage. There is a protein-protein interaction between CRMP2 and Tubulin, and CRMP2 mediates the changes in the microtubule scaffold plasticity induced by fluoxetine. The microtubule scaffold plasticity is damaged by fluoxetine in the later stage. This may be one of the biological bases of the high risk for depression relapse induced by long-term antidepressant administration.

Abbreviations

MDD: Major depression disorder; *SSRIs:* selective serotonin reuptake inhibitor; *CUMS:* chronic unpredictable mild stress; *CRMP2:* Collapsin response mediator protein 2; *LD50:* median lethal dose; *AD:* Alzheimer's disease; *NC:* the normal control group; *Flu:* the fluoxetine administration group; *SB:* the SB216763 (CRMP2 antagonist) administration group; *WT:* the Wortmannin (CRMP2 agonist) administration group; *IF:* Immunofluorescence; *RT-PCR*Real-time quantitative PCR*Co-IP*Co-immunoprecipitation.

Declarations

Ethics approval and consent to participateNot applicable

Consent for publicationNot applicable

Availability of data and materialThe datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests：The authors declare that they have no competing interests.

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Authors' contributions：WGH and WZT designed the study. WZT, WYY and XL conducted an experiment to acquire data. YC and WHL performed the statistical analysis and interpretation of data. WZT prepared the first draft. WGH contributed to the conceptualization of the study and reviewed several manuscript drafts. All authors contributed to and approved the final manuscript.

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Figures

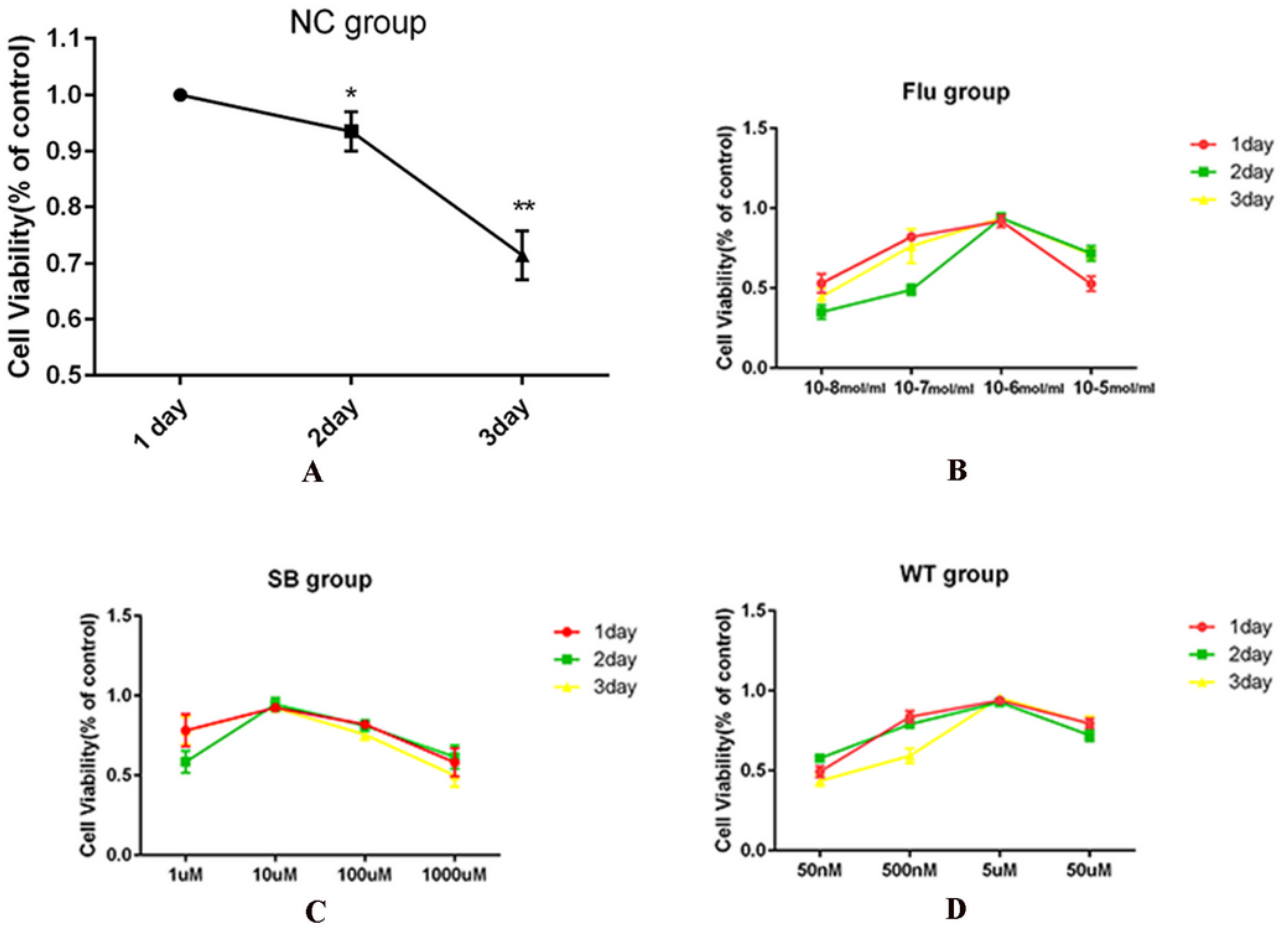

Figure 1

Determination of the appropriate concentrations of fluoxetine, SB216763 and Wortmannin in PC12 cells. (A) Changes in cell activity in the normal control group cultured continuously for 3 days. (B) Cell activity in the groups treated with different doses of Flu, including 10nM, 100nM, 1μM, and 10μM, on the 1st, 2nd and 3rd days. (C) Cell activity in the groups treated with different doses of SB, including 1 μM, 10 μM, 100 μM, and 1000 μM, on the 1st, 2nd and 3rd days. (D) Cell activity of in the groups treated with different doses of WT, including 50 nM, 500 nM, 5 μM, and 50 μM, on the 1st, 2nd and 3rd days. CCK8 assays were carried out to investigate the survival rates of the PC12 cells. These results are shown as the mean ± SD (n = 3).*P < 0.05 and **P < 0.01.

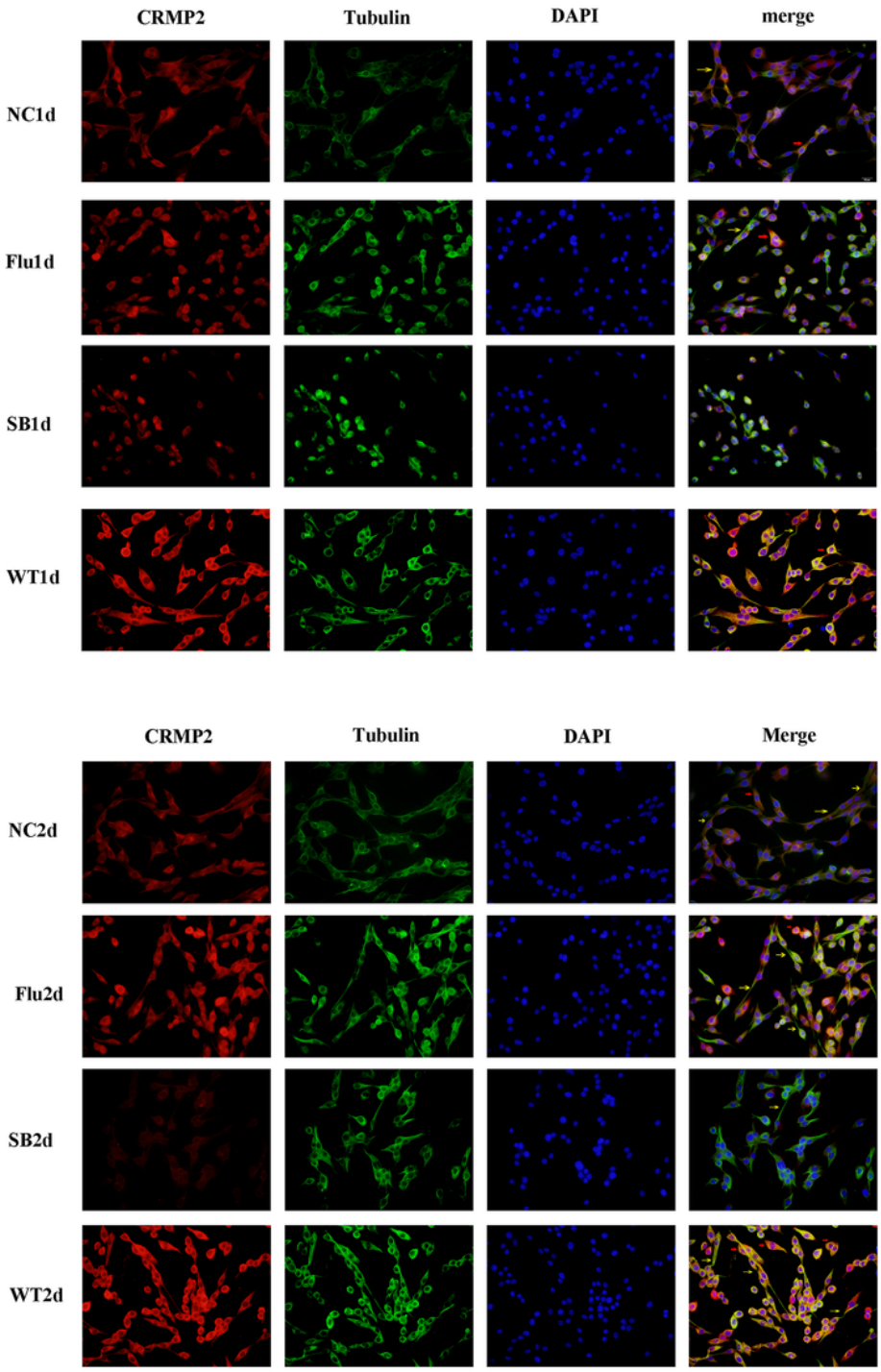

Figure 2

Fig. 2A) shows the immunofluorescence results (×200) of each administration group on the first day. The yellow arrow indicates the cell process connection, and the red arrow indicates the co-localization of CRMP2 and Tubulin. Fig. 2B) shows the immunofluorescence results (×200) of each administration group on the second day. The yellow arrow indicates the cell process connection, and the red arrow indicates the co-localization of CRMP2 and Tubulin.

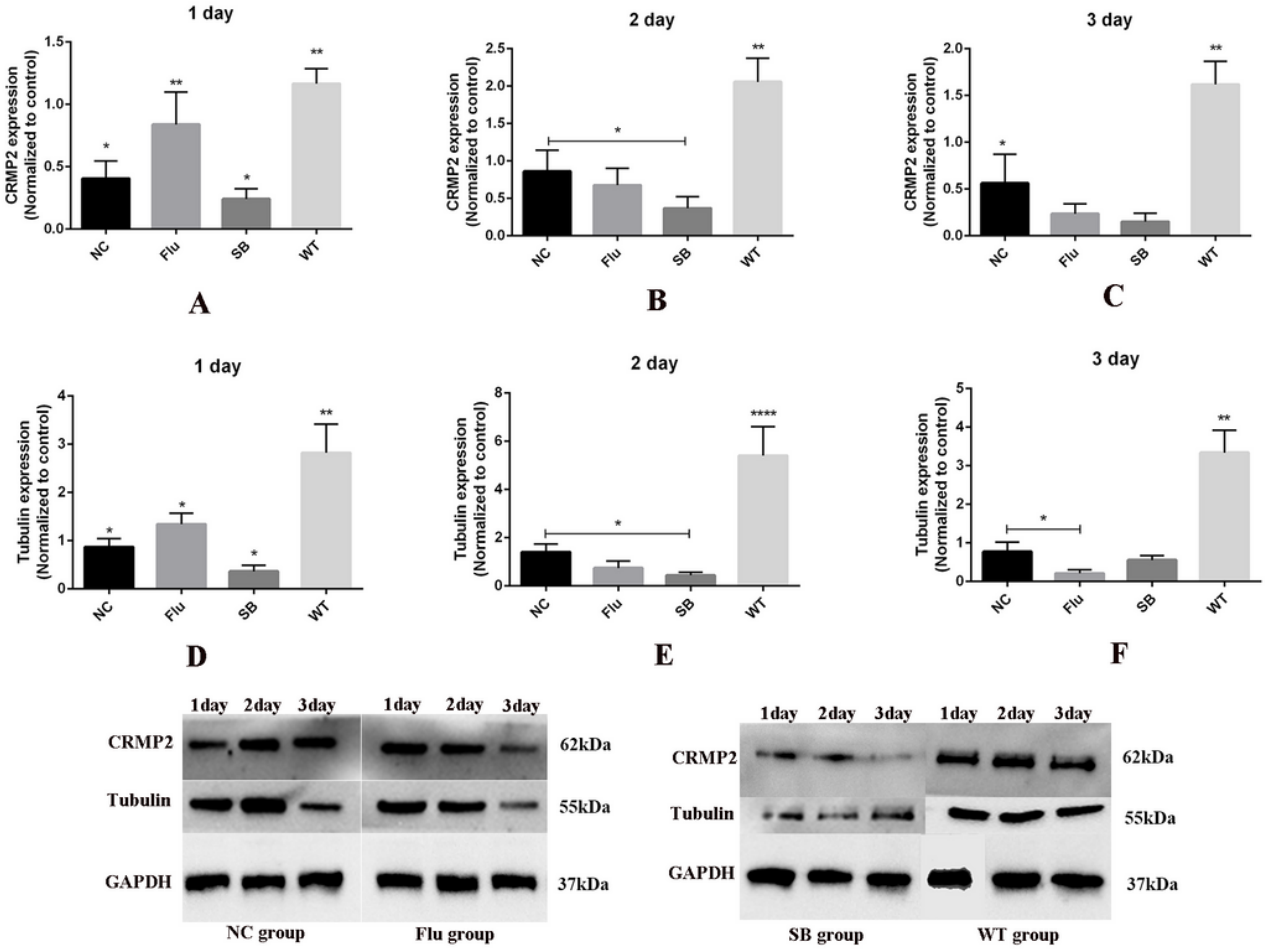

Figure 3

shows the protein content of CRMP2 and Tubulin in each experimental group. A), B) and C) show the protein content of CRMP2 on the 1st, 2nd and 3rd days, respectively. D), E) and F) show the protein content of Tubulin on the 1st, 2nd and 3rd days, respectively. These results are shown as the mean ± SD (n = 3). *P < 0.05, **P < 0.01 and ****P<0.001.

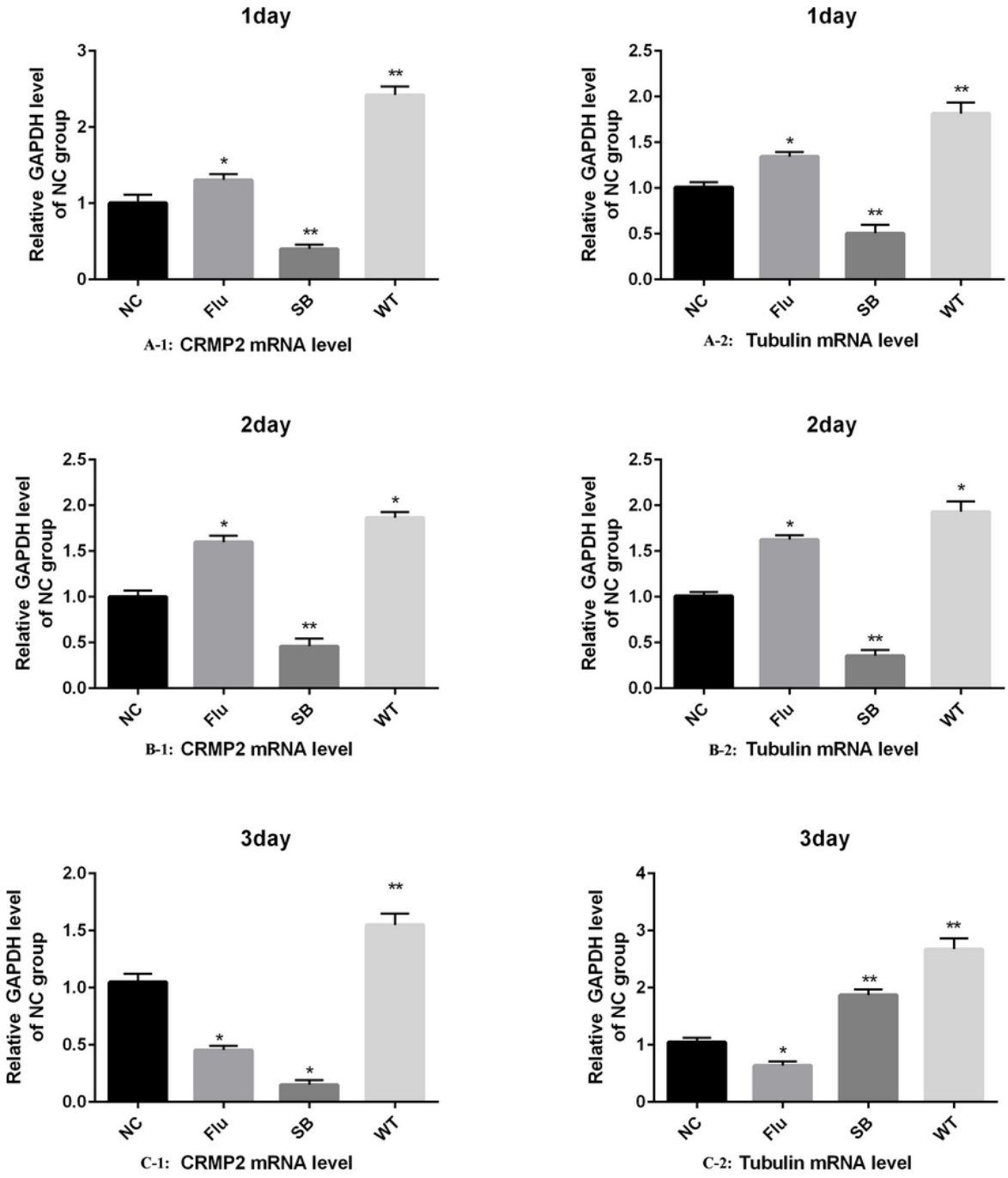

Figure 4

shows the mRNA expression of CRMP2 and Tubulin in each experimental group. A-1) and A-2) show the mRNA expression of CRMP2 and Tubulin, respectively, in each experimental group on the first day of administration. B-1) and B-2) show the mRNA expression of CRMP2 and Tubulin, respectively, in each experimental group on the second day of administration. C-1) and C-2) show the mRNA expression of

CRMP2 and Tubulin, respectively, in each experimental group on the third day of administration. These results are shown as the mean ± SD (n = 3). *P < 0.05 and **P < 0.01.

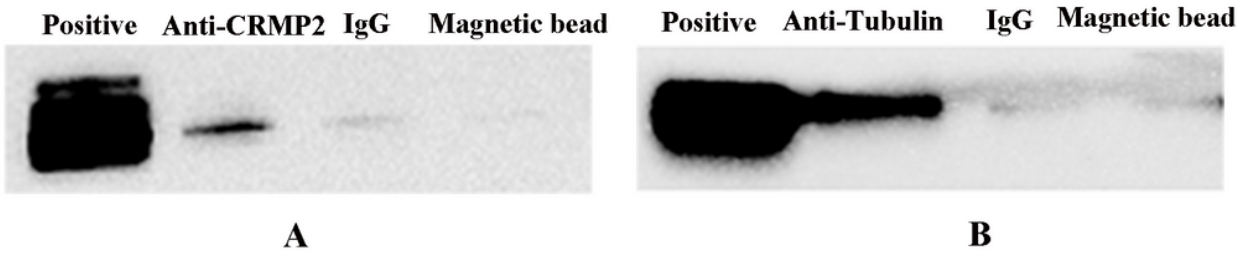

Figure 5

The results of CRMP2 and Tubulin immunoprecipitation in 1 day of NC group. A) The results of CRMP2 binding to the cell lysate with magnetic beads containing a Tubulin antibody. From the left to the right is the cell lysate, anti-CRMP2, the IgG negative control and the magnetic bead negative control. B) The results of Tubulin binding to the cell lysate with magnetic beads containing a CRMP2 antibody. From the left to the right is the cell lysate, anti-Tubulin, the IgG negative control and the magnetic bead negative control.

Supplementary Files

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- Table1.pdf