Fluoxetine treatment for more than 2 days decreases the cellular neuroplasticity and microtubule plasticity in differentiated PC12 cells

Zuotian Wu
Wuhan University Renmin Hospital  https://orcid.org/0000-0002-4293-7286

Gaohua Wang (✉ wgh6402@163.com)
Huiling Wang
Wuhan University Renmin Hospital

Ling Xiao
Wuhan University Renmin Hospital

Yanyan Wei
Wuhan University Renmin Hospital

Can Yang
Wuhan University Renmin Hospital

Research article

Keywords: Antidepressant, Fluoxetine, Plasticity, CRMP2, Depression

Posted Date: August 31st, 2019

DOI: https://doi.org/10.21203/rs.2.13724/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
Abstract

Background: Recent studies indicate that antidepressants treatment restores neuroplasticity. But some researchers claimed that antidepressants, including fluoxetine (FLU), may exacerbate neuroplasticity, which is contradictory and rarely studied. Since almost all of those studies treated with drugs for 1 to 2 days as treatment models of antidepressants, it is possible that FLU treatments for longer periods would have opposite effects on neuroplasticity. Results: In the present study, we examined the effects of FLU treatment up to 3 days on the cellular neuroplasticity and microtubule plasticity in PC12 cells. The cell viability of cells happened a small decease at 2 days (93.5±3.5%), followed by highly significant decreases at 3 days (71.4±4.4%). As report previously, cellular neuroplasticity was significantly upregulated with FLU treatment at 1 day, but that was inhibited FLU treatment at 3 days. Similarly, the expression of tubulin, which is microtubulin plasticity marker, was also upregulated with FLU treatment at 1 day. But it decreased significantly in cells treated with FLU at 3 days. Furthermore, we found tubulin interacted with CRMP2, which accelerated to cellular neuroplasticity, and the regulation of CRMP2 activity influenced microtubule plasticity. Conclusions: The results demonstrate that cellular neuroplasticity and microtubule plasticity were increased with FLU treatment at 1 day, but treatment with FLU for more than 2 days has opposite effect on them. The reduction in cellular neuroplasticity and microtubule plasticity with FLU treatment for more than 2 days might be involved in some aspects of the drugs’ therapeutic effects on depression.

Background

It has been accepted that some antidepressants [1] are suggested to have a neuroprotective effect. The selective serotonin reuptake inhibitor fluoxetine (FLU) reduces cell death in PC12 cells and hippocampus-derived cell line [2, 3]. However, it has been shown that FLU and other antidepressants exacerbate cell death in HT22 immortalized hippocampal neurons, PC12 cells, human neuroblastoma cell line and human colon carcinoma cells [4-6]. Therefore, antidepressants, including FLU, may exert a cyto-protective effect or exacerbate cell damage, which is contradictory and rarely studied.

Recent studies [7] indicate that antidepressants treatment restores neuroplasticity which is increasingly considered central to etiopathogenesis of depression [8]. Microtubule plasticity is an important part of neuroplasticity. The highly polarized architecture of the neuron underlies its ability to integrate and transmit information. Tubulin, which is microtubule plasticity marker, provides not only structural scaffolding for the neuron but also participates in active functional polarization [9]. Tubulin is non-covalent polymers composed of αβ-tubulin heterodimers, which are intrinsically polar polymers. The αβ dimer polymerizes at the end of microtubules and has a certain dynamic nature, which allows tubulin to extend forward and produce a characteristic tubular fiber structure that confers cell polarity [10]. As our previous studies [11, 12] showed, hippocampal microtubule plasticity decreased in the chronic unpredictable mild stress (CUMS) animal model, accompanied by axonal and dendritic inhibition in neurons. These studies suggest that microtubule plasticity plays a role in the pathological process involved in the antidepressant-induced plasticity. In addition, Collapsin response mediator protein 2
(CRMP2) is a protein closely related to neuroplasticity [13, 14], which is predominantly expressed in the nervous system during development and play important roles in axon formation from neurites and in growth cone guidance and collapse through their interactions with microtubules [15]. CRMP2 plays a key role in the prolongation of axons and dendrites and mediates the formation of synaptic connections. Our previous study [16] showed that the high expression of CRMP2 can promote the growth of axons and dendrites in hippocampal neurons. CRMP2 is also related to the repair mechanism of neurons [17].

PC 12 cells are widely used in psychopharmacological study [18, 19] and depression cell models [20], which originates from rat adrenal pheochromocytoma. Upon the nerve growth factor stimulation, PC12 cells are differentiated and display neurite growth [18, 21, 22], which is advantageous for the study of neuronal plasticity and express tubulin. Since almost all of those studies [3, 24-26] treated with drugs for 1 to 2 days as treatment models of antidepressants, we hypothesized that antidepressant treatment for longer periods would have opposite effects on neuroplasticity and microtubule plasticity. To test this hypothesis, we administered FLU from 1 day to 3 days to study the effects on the cellular neuroplasticity and microtubule plasticity in PC12 cells.

**Methods**

1. **PC12 cells culture and evaluation of cell viability**

The differentiated PC12 cells were provided by the Cell Resource Centre of the Chinese Academy of Life Sciences (Shanghai, China). Cells were differentiated by treating with 100 ng/ml nerve growth factor for 9 days [27]. The cells were cultured in DMEM medium with penicillin (100 U/ml), streptomycin (100 U/ml), and 10% fetal bovine serum (Gibco, USA) in 5% CO2 at 37 °C. They were cultured for 3 days, and were collected on 1 day, 2 days and 3 days for testing.

Cell viability was determined by cell counting kit-8 (CCK-8) assay (Dojindo, Japan). The cells were seeded into a 96-well plate to culture with 10% FBS-containing DMEM. All samples were cultured for 3 days, and were texted with treatment on 1 day, 2 days and 3 days. 10μl of CCK-8 reagents were added to each well to incubate for 2 h at 37 °C, which was measured the optical density absorbance at wavelength of 450 nm.

2. **Immunofluorescence (IF)**

Cells grown on glass plates were fixed with 4% paraformaldehyde. The cells were incubated with a 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 the Multifunctional automated inverted Fluorescence Microscopy (ZEISS, Germany).
3. Real-time quantitative PCR (RT-PCR)

Total RNA was isolated by trizol (invitrogen, USA) extraction according to the manufacturer’s instructions. RNA (2µg) was reverse transcribed to cDNA using a PrimeScript RT Kit (Takara, Japan). The reaction mixture was added to the RNA solution and incubated at 42°C for 1 hour, heated at 70°C for 5 minutes, and chilled at 48°C. Real-time PCR was performed using SYBR master mix (Takara) on a Bio-Rad Connect Real-Time PCR platform (Bio-Red, USA). The reaction was carried out in a DNA thermal cycler under the following conditions: 95°C for 30 s; 95°C for 5s and 60°C for 30 s, repeat 40 times; 95°C for 10 s, 60°C for 5 s. The values of CRMP2 and Tubulin PCR product were normalized against the amount of PCR product for GAPDH obtained for the same sample. The primer sequences are as follows:

4. Western blotting

The PC12 cells samples were prepared and analyzed by quantitative immunoblotting as previously described [28]. A rabbit monoclonal anti-CRMP2 antibody (1:20000, Abcam), a mouse monoclonal anti-tubulin antibody (1:5000, Abcam) and a monoclonal rabbit anti- GAPDH primary antibody (1:1000, Abcam) were used. The proteins (20µg) by SDS-PAGE with 10% polyacrylamide gels were transferred electrophoretically to polyvinylidene fluoride (PVDF) membranes according to the TGX Stain-FreeTM FastCastTM Acrylamide Kit (Bio-Red). The membranes were blocked with 5% nonfat dry milk in TBS+0.1%Tween-20 (TBST) for one hour and incubated with primary antibody in BondTM Primary Antibody Diluent overnight at 4°C. After three washes in TBST, the membranes 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 one hour. Proteins were detected by the BIO-RAD ChemiDoc Touch Image System (Bio-Red). The expression of CRMP2 protein (62 kDa) and tubulin protein (55 kDa) were normalized to GAPDH expression (37 kDa). Densitometric signals from Western blots were analyzed with BIO-RAD software.

5. Co-immunoprecipitation (Co-IP)

The cell lysates were separated by centrifugation, incubated with 1µg anti-CRMP2 (Abcam, 1:50)/ anti-Tubulin (Abcam, 1:50) antibodies overnight at 4 °C, and precipitated using Protein A agarose beads (Roche, Mannheim, Germany). After the magnetic beads were separated, the supernatant was collected for SDS-PAGE detection. The bound proteins were released into the buffer by heating the samples at 100 °C for 7 min. The proteins by SDS-PAGE with 10% polyacrylamide gels were transferred electrophoretically to PVDF. The membranes were incubated with primary antibody overnight at 4°C and secondary antibodies for one hour. Proteins were detected by the BIO-RAD ChemiDoc Touch Image System (Bio-Red).

6. Statistical analysis
Data were expressed as means ± standard deviation (SD), and all determinations were repeated three times. Statistical analyses were performed using SPSS 23.0 software (SPSS, Chicago, USA). The GraphPad Prism 7.0 software (GraphPad Software, Inc., San Diego, CA) was used to perform for drawing. One-way analysis of variance (ANOVA) and t-test were used to examine the differences between the different treatment groups. A difference was considered statistically significant at \( P < 0.05 \).

**Results**

1. **Effect of treatment on PC12 cell viability**

The differentiated PC12 cells were cultured for 3 days, and their cell viabilities were detected by the CCK8 assay. As showed in Fig.1A, compared with cell viability at 1 day, the cell viability of PC12 cells happened a small decease at 2 days (93.5±3.5%), followed by highly significant decreases at 3 days (71.4±4.4%). There was a significant difference in cell viability between the 2 days and 3 days (\( P < 0.01 \)). In addition, the cells showed cell damage and neuronal atrophy at 4 days of culture (results not shown), with cell viability reducing to nearly 20%, which are not suitable for the subsequent experiments.

To investigate the appropriate concentrations of FLU (Aladdin, China), SB216763 (SB, CRMP2 antagonist) and Wortmannin (WT, CRMP2 agonist; Selleck, USA), cells were divided into FLU group (0.01 µM, 0.1 µM, 1 µM, 10 µM) and SB group (1 µM, 10 µM, 100 µM, 1000 µM) and WT group (0.05 µM, 0.50 µM, 5 µM, 50 µM). As shown in Fig.1B, 1C and 1D, the cell viabilities of the FLU (1 µM), SB (10 µM) and WT (5 µM) treatment were the highest (all \( P < 0.05 \)), which were used in subsequent experiments.

2. **FLU treatment has opposite effects on the regulation of cellular neuroplasticity and microtubule plasticity at 1 day than at 3 days**

Cellular neuroplasticity in PC12 cells was determined by detection of tubulin, a spherical protein that is the basic structural unit of the cells, so that the IF results of tubulin directly reflects changes in cell morphology. FLU significantly improved cellular neuroplasticity at 1 day, but inhibited them at 3 days. The IF results found a more significant increase of cellular neuroplasticity, such as neurite outgrowth, neurite length and number of neuritis, in FLU group than NC group at 1 day (Fig. 2A shows). The cell morphology of NC group formed a network-like structure at 3 days, but FLU group did not exhibit the connected network structure observed in the normal control (NC) group (Fig. 2C shows).

We used western blotting and RT-PCR to further detect the expression of tubulin, which is microtubule plasticity marker. The results of western blotting (Fig.3 shows) showed the level of tubulin protein was increased with FLU treatment for 1 day (1.34±0.23, \( P = 0.041 \)) compared to that in NC group (0.86±0.17). But there was no significant difference in the tubulin content (0.73±0.29, \( P = 0.123 \)) between FLU group and NC group (1.40±0.33) at 2 days. The tubulin protein content with FLU treatment at 3 days (0.21±0.09, \( P = 0.015 \)) was lower than that in NC group (0.77±0.25). Similarly, the mRNA expression of tubulin was increased with FLU treatment for 1 day but decreased at 3 days. As shown in Figs. 4A-2, 4B-2, 4C-2, the mRNA expression of tubulin with FLU treatment for 1 day (1.35±0.05, \( P = 0.017 \)) and 2 days (1.63±0.05,
were higher than that in NC group at 1 day (1.01±0.06) and 2 days (1.01±0.04). However, the tubulin mRNA level with FLU treatment at 3 days (0.64±0.07, P=0.026) was lower than that in NC group (1.05±0.07).

This study also found that treatment with FLU had effects on the expression of CRMP2, a protein closely related to cellular neuroplasticity. FLU treatment increased levels of CRMP2 protein at 1 day, but this level was decreased significantly at 3 days. Fig.3 shows CRMP2 in FLU group (0.84±0.26, P=0.004) was significantly higher than that in NC group (0.48±0.09) at 1 day, but there was no significant difference between FLU group (0.67±0.23, P=0.266) and NC group (0.86±0.28) at 2 days. CRMP2 protein content with FLU treatment (0.23±0.11, P=0.026) for 3 days was lower than that in NC group (0.56±0.31).

Similarly, as showed in Fig. 4A-1, 4B-1 and 4C-1, the mRNA expression of CRMP2 with FLU treatment at 1 day (1.31±0.08, P=0.030) and 2 days (1.60±0.07, P=0.001) were significantly higher than that at 1 day (1.01±0.11) and 2 days (1.00±0.07) in NC group. However, CRMP2 mRNA level in FLU group (0.46±0.04, P=0.001) was lower than that in NC group (1.05±0.07) at 3 days.

3. Effect of CRMP2 activity on cellular neuroplasticity and microtubule plasticity in PC12 cells

The above dates indicate that treatment with FLU has opposite effects on the regulation of cellular neuroplasticity and microtubule plasticity at 1 day than at 3 days, and it also affects the expression of CRMP2. What is more, the IF results showed there was colocalization between CRMP2 and tubulin (Fig.2 shows). To further investigate this result, we used Co-IP validates a direct interaction between CRMP2 and tubulin (Fig.3J shows), which is consistent with other studies [29-31]. Some studies [32] show that brain-specific CRMP2 knockout (cKO) mice display molecular, cellular, structural and behavioural deficits. The cKO mice exhibit microtubule injury in other tissues, such as enlarged ventricles and ventricular. Loss of CRMP2 in the hippocampus leads to aberrant dendrite development and defective synapse formation in CA1 neurons. Furthermore, CRMP2 knockdown in newborn neurons results in stage-dependent defects in their development during adult hippocampal neurogenesis. On this basis, we tried to regulate CRMP2 activity with SB treatment and WT treatment in longer periods (up to 3 days) to study the cellular neuroplasticity and microtubule plasticity in PC12 cells.

We found that the expression of CRMP2 was significantly inhibited after giving SB treatment. CRMP2 protein content (Fig.3 shows) with SB treatment for 1 day (1.16±0.17, P=0.007), 2 days (2.05±0.32, P=0.000) and 3 days (1.62±0.25, P=0.000) were lower than NC group. Similarly, As Figs.4A-1, 4B-1 and 4C-1 showed the CRMP2 mRNA expression with SB treatment for 1 day (0.40±0.06, P=0.003), 2 days (0.46±0.08, P=0.002) and 3 days (0.15±0.04, P=0.000) were inhibited, compared with NC group. However, the expression of CRMP2 increased with WT treatment. The CRMP2 protein content (Fig.3 shows) with WT treatment for 1 day (0.24±0.08, P=0.035), 2 days (0.37±0.16, P=0.007) and 3 days (0.15±0.09, P=0.007) were more than NC group. Similarly, CRMP2 mRNA expression (Figs.4A-1, 4B-1 and 4C-1 show) with WT treatment for 1 day (2.42±0.11, P<0.01), 2 days (1.87±0.06, P<0.01) and 3 days (1.55±0.10, P<0.01) were also more than NC group.
The above results showed that SB and WT effectively regulated the expression of CRMP2. Next, the tubulin results of IF (Fig. 2 shows) found cellular neuroplasticity was significantly inhibited with SB treatment for 1 day and 2 days, but increased at 3 days, which formed a network-like structure like the cell morphology of NC group. However, the cellular neuroplasticity was enhanced with WT treatment from 1 day to 3 days. In addition, it was difficult to find the colocalization between CRMP2 and tubulin with SB treatment at 2 days and 3 days, except for a small amount of co-expression at 1 day. However, the colocalization between CRMP2 and tubulin was clearly visible in WT group from 1 day to 3 days.

As shown in Fig. 3, the tubulin protein content with SB treatment for 1 day (0.36±0.12, P=0.031) and 2 days (0.44±0.13, P=0.032) was lower than that in NC group. However, there was no significant difference in tubulin content between the SB group (0.55±0.11, P=0.305) and the NC group at 3 days. As shown in Figs. 4A-2, 4B-2 and 4C-2, compared with NC group, tubulin mRNA expression with SB treatment for 1 day (0.51±0.09, P=0.004) and 2 days (0.36±0.06, P=0.001) was inhibited, followed by highly significant increases at 3 days (1.87±0.10, P=0.002). In contrast, the tubulin mRNA expression in the WT group was significantly higher at 1 day (1.82±0.12, P<0.01), 2 days (1.93±0.11, P<0.01) and 3 days (2.68±0.19, P<0.01) than that in the NC group. Similarly, the level of tubulin in WT group was higher at 1 day (2.82±0.60, P=0.000), 2 days (5.40±1.21, P=0.000) and 3 days (3.34±0.58, P=0.000) than those in NC group.

Discussion

The present findings showed that treatment with FLU significantly upregulated the cellular neuroplasticity and microtubule plasticity at 1 day, resulting in increase of CRMP2 and tubulin expression. However, cellular neuroplasticity and microtubule plasticity were inhibited with FLU treatment for up to 3 days. These findings suggest that treatment with FLU for longer periods (more than 2 days) has an opposite effects on the regulation of cellular neuroplasticity and microtubule plasticity at 1 day than at 3 days.

Effects of treatment with FLU on cellular neuroplasticity and microtubule plasticity

The previous studies [3, 24-26] treated with FLU for time periods ranging from 1 day to 2 days, while we medicated with FLU for longer periods up to 3 days. This prolonged period found treatment with FLU has an opposite effects on the regulation of cellular neuroplasticity and microtubule plasticity at 1 day than at 3 days. Currently, the mechanism underlying this phenomenon with FLU treatment remains unclear. There are a few possibilities, as follows.

The growth state of cells or cell viability played an important role in this phenomenon. Almost all of previous studies [3, 24-26] regard treated with drugs from 1 day to 2 days as treatment models of antidepressants, during which the cells are in the growth phase or stable phase. The cells maintain high cell viability, which is consistent with the results of this study. FLU treatment for 1 day promoted cellular neuroplasticity and microtubule plasticity, which was consistent with the results of previous studies [33, 34], which found that FLU treatment up to 2 days increases neurogenesis, synaptogenesis and synaptic plasticity in the hippocampus, cortex and amygdale [35]. Some researchers [36, 37] have summarized the main effects of FLU on neuroplasticity. First, FLU treatment increases the proliferation of neural
progenitor cells [38]. Second, FLU stimulates dendritic branches and promotes the maturation of immature granulosa cells. Third, FLU enhances the survival rate of immature neurons [39]. Fourth, immature neurons are functionally integrated into local neural circuits and produce long-term synaptic plasticity enhancement [8].

However, the cellular neuroplasticity and microtubule plasticity were impaired with FLU treatment for 3 days. Other studies [40] showed the cell viability decreased to 50%, which suggests cell apoptosis. The cell viability decreased sharply at 3 days, suggesting that the cells were about to enter the apoptosis stage or neuronal injury. The same result was found in other study of antidepressant treatment [41], which found FLU treatment resulted in neuronal atrophy and the inhibition of axonal dendrite prolongation. Other studies [42] have shown that FLU treatment affects depression-like behavior, but most of these studies have not considered the neuronal injury. Previous animal model studies [43] have demonstrated that hippocampal neuron injury is associated with depression, and FLU can alleviate the decrease in hippocampal neurons. But, recent data [44, 45] has shown that FLU treatment results in a decrease in the proliferation of hippocampal neurons and a decrease in the volume of the CA1 region [46] under conditions of neuronal injury.

In this study, we also found that FLU treatment enhanced the expression of CRMP2 at 1 day. When we up-regulate the activity of CRMP2, the cellular neuroplasticity and microtubule plasticity were significantly promoted. Our previous study [47] shows CRMP2 is related to the repair and regeneration of adult brain neurons [17], which regulates the growth of axons and dendrites by affecting the aggregation or depolymerization of microtubule dimers in the neuronal axon growth cone and regulating the dynamics of microtubules [48]. It participates in the neuroplasticity process [30]. We found that CRMP2 interacts with tubulin, and other studies [30] showed their interaction at the positive end of microtubule, which promotes the binding of tubulin dimer and improves the efficiency of microtubule synthesis. Therefore, CRMP2 can extend the growth of microtubule-supported terminal growth cone of axon and form new synapses. The phosphorylation level of CRMP2 regulates the binding of the protein to microtubule dimer, and when it exists in the form of high phosphorylation, it loses the ability to bind to tubulin and reduces the growth of microtubules [49]. It is suggested that CRMP2 is involved in synaptic plasticity by mediating microtubule dynamics, which may affect the transmission of neurotransmitters between nerve cells or neural loops. Therefore, in many studies, CRMP2 has been considered as a novel microtubule-associated protein in scaffolds [50].

There is another interesting discovery in this research. Our study showed that the tubulin and CRMP2 mRNA expression with FLU treatment for 2 days were higher than NC group, but there were no in the level of tubulin and CRMP2 protein difference between FLU group and NC group. The expression of tubulin and CRMP2 with FLU treatment for 3 days was both lower than NC group. This suggests that there may be a regulatory feedback pathway or another regulatory pathway for tubulin. The result of SB group further confirmed this finding. The expression of CRMP2 has been suppressed with SB treatment from 1 day to 3 days. The tubulin expression was decreased with SB treatment from 1 day to 2 days, but there was no difference in tubulin protein content between SB group and NC group at 3 days. The regulatory
pathway mechanism is rare and not clear at present so that further research is needed. We think that it may be a selective cellular injury process, which purpose is to ensure the most critical structure and function of cell, when cell damage exceeds its steady-state equilibrium.

The possibility of mechanisms other than those described above cannot be excluded, and further study will be necessary to determine the mechanisms underlying an opposite effects on the regulation of microtubule plasticity with FLU treatment at 1 day than 3 days.

**Implications of FLU treatment for depression**

The FLU treatment to depression is determined the degree of neuronal injury. The majority of researchers have accepted this view [8] that depression is not a simple neurofunctional disease, but a mental disorder with structural damage to the nervous system. When the neuronal injury is mild, the FLU treatment could improve the cellular neuroplasticity and microtubule plasticity to improve antidepressant effect. Many studies [36, 51, 52] have confirmed this view. However, when the neuronal injury is severe, FLU alone treatment would aggravate cellular neuroplasticity and microtubule plasticity, which may be one of the biological bases of depression recurrence.

Depression is a prevalent neuropsychiatric disorder with a high risk of recurrence, affecting around 16% of the population worldwide [53]. Despite the moderate capacity to achieve remission, over 85% of remitted patients suffer recurrent episodes of depression, within 15 years after an initial event [54, 55]. There are extremely complex reasons for the recurrence of depression, and compliance of patients is the main problem. The evidence [56] shows that whole course antidepressant treatment, with overcoming compliance problems, effectively controls depressive symptoms and prevent the recurrence of depression. Therefore, the clinical guidelines advocate whole course antidepressant treatment to control depressive symptoms. But other study [57] found that the recurrence rate was 64% in a 23-year follow-up study of depression patients, with a standardized whole course antidepressant treatment according to clinical guidelines. Fluctuation of depression itself is also other influencing factors, such as seasonal fluctuations, menstrual cycle, age, psychosocial factors and so on. The biological mechanisms of depression recurrence underlying antidepressant treatment may be the important factor in this context.

Neuroplasticity are increasingly considered central to the etiopathogenesis of and recovery from depression. Some depression recurrence model studies [58] found treatment with FLU was effective to promote sustained reversion of a depressive-like phenotype, which is consistent with our results about increase in cellular neuroplasticity and microtubule plasticity with FLU treatment for 1 day. However, previous exposure to a depressive-like episode impacts on the behavioral and neuroanatomical changes triggered by subsequent re-exposure to similar experimental conditions. As a result, this aggravates nerve injury so that stress re-exposure in fluoxetine-treated animals resulted in an overproduction of adult-born neurons along with neuronal atrophy of granule neurons, accounting for an increased susceptibility to recurrent behavioral changes typical of depression. Our finding about FLU treatment for 3 days confirms this view. Depression recurrence model found the proper control of adult hippocampal neuroplasticity triggered by antidepressants is essential to counteract recurrent depressive-like episodes. Some studies
have found that FLU treatment combined with other drugs, which promote structural plasticity, for example imipramine [3], can effectively promote the recovery of acute depression and reduce the risk of stable relapse [59]. Imipramine re-established hippocampal neurogenesis and neuronal dendritic arborization contributing to resilience to recurrent depressive-like behavior [60]. Therefore, this study showed the effect of CRMP2 activity on cellular neuroplasticity and microtubule plasticity. This may be an alternative way to improve the FLU antidepressant effect.

Conclusions

We have provided the evidence for the effect of FLU treatment on cellular neuroplasticity and microtubule plasticity, which has opposite effects at 1 day than at 3 days. The reduction in cellular neuroplasticity and microtubule plasticity with FLU treatment for more than 2 days might be involved in some of the therapeutic effects on depression and side-effects of FLU.

Abbreviations

FLU: fluoxetine; CUMS: chronic unpredictable mild stress; CRMP2: Collapsin response mediator protein 2; IF: immunofluorescence; RT-PCR: Real-time quantitative PCR; Co-IP: Co-immunoprecipitation; SB: SB216763; WT: Wortmannin; NC: normal control; cKO: CRMP2 knockout.

Declarations

Ethics approval and consent to participate: Not applicable
Consent for publication: Not applicable
Availability of data and material: The 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.
Funding: This study was supported by the National Natural Science Foundation of China (No. 81571325, No.81871072 and NO.81401117) and the Medical Science Advancement Program of Wuhan University (NO.TFLC2018001).

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.

Acknowledgements: Not Applicable

References

1. Ishima T, Fujita Y, Hashimoto K: Interaction of new antidepressants with sigma-1 receptor chaperones and their potentiation of neurite outgrowth in PC12 cells. European journal of
2. Chiou SH, Chen SJ, Peng CH, Chang YL, Ku HH, Hsu WM, Ho LL, Lee CH: Fluoxetine up-regulates expression of cellular FLICE-inhibitory protein and inhibits LPS-induced apoptosis in hippocampus-derived neural stem cell. Biochemical and biophysical research communications 2006, 343(2):391-400.

3. Kolla N, Wei Z, Richardson JS, Li XM: Amitriptyline and fluoxetine protect PC12 cells from cell death induced by hydrogen peroxide. Journal of psychiatry & neuroscience : JPN 2005, 30(3):196-201.

4. Arimochi H, Morita K: Characterization of cytotoxic actions of tricyclic antidepressants on human HT29 colon carcinoma cells. European journal of pharmacology 2006, 541(1-2):17-23.

5. Bartholomä P, Erlandsson N, Kaufmann K, Rössler OG, Baumann B, Wirth T, Giehl KM, Thiel G: Neuronal cell death induced by antidepressants: lack of correlation with Egr-1, NF-kappa B and extracellular signal-regulated protein kinase activation. Biochemical pharmacology 2002, 63(8):1507-1516.

6. Levkovitz Y, Gil-Ad I, Zeldich E, Dayag M, Weizman A: Differential induction of apoptosis by antidepressants in glioma and neuroblastoma cell lines: evidence for p-c-Jun, cytochrome c, and caspase-3 involvement. Journal of molecular neuroscience : MN 2005, 27(1):29-42.

7. Maya Vetencourt JF, Sale A, Viegi A, Baroncelli L, De Pasquale R, O’Leary OF, Castrén E, Maffei L: The antidepressant fluoxetine restores plasticity in the adult visual cortex. Science (New York, NY) 2008, 320(5874):385-388.

8. Castrén E: Neuronal network plasticity and recovery from depression. JAMA psychiatry 2013, 70(9):983-989.

9. Gundersen GG, Kreitzer G, Cook T, Liao G: Microtubules as determinants of cellular polarity. The Biological bulletin 1998, 194(3):358-360.

10. Sauer M, Stannat W: Reliability of signal transmission in stochastic nerve axon equations. Journal of computational neuroscience 2016, 40(1):103-111.

11. Abad-Rodríguez J, Díez-Revuelta N: Axon glycoprotein routing in nerve polarity, function, and repair. Trends in biochemical sciences 2015, 40(7):385-396.

12. Yang C, Wang G, Wang H, Liu Z, Wang X: Cytoskeletal alterations in rat hippocampus following chronic unpredictable mild stress and re-exposure to acute and chronic unpredictable mild stress. Behavioural brain research 2009, 205(2):518-524.

13. Bisgaard CF, Bak S, Christensen T, Jensen ON, Enghild JJ, Wiborg O: Vesicular signalling and immune modulation as hedonic fingerprints: proteomic profiling in the chronic mild stress depression model. Journal of psychopharmacology (Oxford, England) 2012, 26(12):1569-1583.

14. Ge L, Zhu MM, Yang JY, Wang F, Zhang R, Zhang JH, Shen J, Tian HF, Wu CF: Differential proteomic analysis of the anti-depressive effects of oleamide in a rat chronic mild stress model of depression. Pharmacology, biochemistry, and behavior 2015, 131:77-86.

15. Liu BP, Strittmatter SM: Semaphorin-mediated axonal guidance via Rho-related G proteins. Current opinion in cell biology 2001, 13(5):619-626.
16. Zhu LQ, Zheng HY, Peng CX, Liu D, Li HL, Wang Q, Wang JZ: **Protein phosphatase 2A facilitates axonogenesis by dephosphorylating CRMP2.** *The Journal of neuroscience: the official journal of the Society for Neuroscience* 2010, **30**(10):3839-3848.

17. Liz MA, Mar FM, Santos TE, Pimentel HI, Marques AM, Morgado MM, Vieira S, Sousa VF, Pemble H, Wittmann T et al: **Neuronal deletion of GSK3β increases microtubule speed in the growth cone and enhances axon regeneration via CRMP-2 and independently of MAP1B and CLASP2.** *BMC biology* 2014, **12**:47.

18. Greene LA, Tischler AS: **Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor.** *Proceedings of the National Academy of Sciences of the United States of America* 1976, **73**(7):2424-2428.

19. Shi DD, Huang YH, Lai CSW, Dong CM, Ho LC, Li XY, Wu EX, Li Q, Wang XM, Chen YJ et al: **Ginsenoside Rg1 Prevents Chemotherapy-Induced Cognitive Impairment: Associations with Microglia-Mediated Cytokines, Neuroinflammation, and Neuroplasticity.** *Molecular neurobiology* 2019.

20. Zhao X, Zeng Z, Gaur U, Fang J, Peng T, Li S, Zheng W: **Metformin protects PC12 cells and hippocampal neurons from H O -induced oxidative damage through activation of AMPK pathway.** *Journal of cellular physiology* 2019.

21. Das KP, Freudenrich TM, Mundy WR: **Assessment of PC12 cell differentiation and neurite growth: a comparison of morphological and neurochemical measures.** *Neurotoxicology and teratology,* **26**(3):397-406.

22. Kadota T, Yamaai T, Saito Y, Akita Y, Kawashima S, Moroi K, Inagaki N, Kadota K: **Expression of dopamine transporter at the tips of growing neurites of PC12 cells.** *The journal of histochemistry and cytochemistry: official journal of the Histochemistry Society* 1996, **44**(9):989-996.

23. Xue J, Wu T, Li J, Zhu C, Xia Y: **Promoting the Outgrowth of Neurites on Electrospun Microfibers by Functionalization with Electrospayed Microparticles of Fatty Acids.** *Angewandte Chemie (International ed in English)* 2019, **58**(12):3948-3951.

24. Li YF, Zhang YZ, Liu YQ, Wang HL, Cao JB, Guan TT, Luo ZP: **Inhibition of N-methyl-D-aspartate receptor function appears to be one of the common actions for antidepressants.** *Journal of psychopharmacology (Oxford, England)* 2006, **20**(5):629-635.

25. Yang Y, Ang W, Long H, Chang Y, Li Z, Zhou L, Yang T, Deng Y, Luo Y: **Scaffold Hopping Toward Agomelatine: Novel 3, 4-Dihydroisoquinoline Compounds as Potential Antidepressant Agents.** *Scientific reports* 2016, **6**:34711.

26. Mandela P, Ordway GA: **KCl stimulation increases norepinephrine transporter function in PC12 cells.** *Journal of neurochemistry* 2006, **98**(5):1521-1530.

27. Tatton WG, Chalmers-Redman RM, Ju WJ, Mammen M, Carlile GW, Pong AW, Tatton NA: **Propargylamines induce antiapoptotic new protein synthesis in serum- and nerve growth factor (NGF)-withdrawn, NGF-differentiated PC-12 cells.** *The Journal of pharmacology and experimental therapeutics* 2002, **301**(2):753-764.
28. Rosenbrock H, Koros E, Bloching A, Podhorna J, Borsini F: Effect of chronic intermittent restraint stress on hippocampal expression of marker proteins for synaptic plasticity and progenitor cell proliferation in rats. *Brain research* 2005, 1040(1-2):55-63.

29. Zheng Y, Sethi R, Mangala LS, Taylor C, Goldsmith J, Wang M, Masuda K, Karamajejadranbar M, Mannion D, Miranda F *et al.*: Tuning microtubule dynamics to enhance cancer therapy by modulating FER-mediated CRMP2 phosphorylation. *Nature communications* 2018, 9(1):476.

30. Fang W, Gao G, Zhao H, Xia Y, Guo X, Li N, Li Y, Yang Y, Chen L, Wang Q *et al.*: Role of the Akt/GSK-3β/CRMP-2 pathway in axon degeneration of dopaminergic neurons resulting from MPP+ toxicity. *Brain research* 2015, 1602:9-19.

31. Wilson SM, Moutal A, Melemedjian OK, Wang Y, Ju W, François-Moutal L, Khanna M, Khanna R: The functionalized amino acid (S)-Lacosamide subverts CRMP2-mediated tubulin polymerization to prevent constitutive and activity-dependent increase in neurite outgrowth. *Frontiers in cellular neuroscience* 2014, 8:196.

32. Zhang H, Kang E, Wang Y, Yang C, Yu H, Wang Q, Chen Z, Zhang C, Christian KM, Song H *et al.*: Brain-specific Crmp2 deletion leads to neuronal development deficits and behavioural impairments in mice. *Nature communications* 2016, 7.

33. Wang JW, David DJ, Monckton JE, Battaglia F, Hen R: Chronic fluoxetine stimulates maturation and synaptic plasticity of adult-born hippocampal granule cells. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2008, 28(6):1374-1384.

34. Shuto T, Kuroiwa M, Sotogaku N, Kawahara Y, Oh YS, Jang JH, Shin CH, Ohnishi YN, Hanada Y, Miyakawa T *et al.*: Obligatory roles of dopamine D1 receptors in the dentate gyrus in antidepressant actions of a selective serotonin reuptake inhibitor, fluoxetine. *Molecular psychiatry* 2018.

35. Popova D, Castrén E, Taira T: Chronic fluoxetine administration enhances synaptic plasticity and increases functional dynamics in hippocampal CA3-CA1 synapses. *Neuropharmacology* 2017, 126:250-256.

36. Alboni S, van Dijk RM, Poggini S, Milior G, Perrotta M, Drent T, Brunello N, Wolfer DP, Limatola C, Amrein I *et al.*: Fluoxetine effects on molecular, cellular and behavioral endophenotypes of depression are driven by the living environment. *Molecular psychiatry* 2017, 22(4):552-561.

37. Castrén E, Hen R: Neuronal plasticity and antidepressant actions. *Trends in neurosciences* 2013, 36(5):259-267.

38. Banasr M, Hery M, Printemps R, Daszuta A: Serotonin-induced increases in adult cell proliferation and neurogenesis are mediated through different and common 5-HT receptor subtypes in the dentate gyrus and the subventricular zone. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* 2004, 29(3):450-460.

39. Ohira K, Miyakawa T: Chronic treatment with fluoxetine for more than 6 weeks decreases neurogenesis in the subventricular zone of adult mice. *Molecular brain* 2011, 4:10.

40. Peng R, Dai W, Li Y: Neuroprotective effect of a physiological ratio of testosterone and estradiol on corticosterone-induced apoptosis in PC12 cells via Traf6/TAK1 pathway. *Toxicology in vitro : an*
41. Han YS, Lee CS: Antidepressants reveal differential effect against 1-methyl-4-phenylpyridinium toxicity in differentiated PC12 cells. European journal of pharmacology 2009, 604(1-3):36-44.

42. Tsankova NM, Berton O, Renthal W, Kumar A, Neve RL, Nestler EJ: Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action. Nature neuroscience 2006, 9(4):519-525.

43. Small SA, Schobel SA, Buxton RB, Witter MP, Barnes CA: A pathophysiological framework of hippocampal dysfunction in ageing and disease. Nature reviews Neuroscience 2011, 12(10):585-601.

44. David DJ, Samuels BA, Rainer Q, Wang JW, Marsteller D, Mendez I, Drew M, Craig DA, Guiard BP, Guilloux JP et al: Neurogenesis-dependent and -independent effects of fluoxetine in an animal model of anxiety/depression. Neuron 2009, 62(4):479-493.

45. Wu MV, Shamy JL, Bedi G, Choi CW, Wall MM, Arango V, Boldrini M, Foltin RW, Hen R: Impact of social status and antidepressant treatment on neurogenesis in the baboon hippocampus. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 2014, 39(8):1861-1871.

46. Bremner JD, Narayan M, Anderson ER, Staib LH, Miller HL, Charney DS: Hippocampal volume reduction in major depression. The American journal of psychiatry 2000, 157(1):115-118.

47. Wu Z, Wang G, Wei Y, Xiao L, Wang H: PI3K/AKT/GSK3β/CRMP-2-mediated neuroplasticity in depression induced by stress. Neuroreport 2018, 29(15):1256-1263.

48. Gu Y, Ihara Y: Evidence that collapsin response mediator protein-2 is involved in the dynamics of microtubules. The Journal of biological chemistry 2000, 275(24):17917-17920.

49. Fukata Y, Kimura T, Kaibuchi K: Axon specification in hippocampal neurons. Neuroscience research 2002, 43(4):305-315.

50. Piubelli C, Carboni L, Becchi S, Mathé AA, Domenici E: Regulation of cytoskeleton machinery, neurogenesis and energy metabolism pathways in a rat gene-environment model of depression revealed by proteomic analysis. Neuroscience 2011, 176:349-380.

51. Sun Y, Sun X, Qu H, Zhao S, Xiao T, Zhao C: Neuroplasticity and behavioral effects of fluoxetine after experimental stroke. Restorative neurology and neuroscience 2017, 35(5):457-468.

52. Perez-Caballero L, Torres-Sanchez S, Bravo L, Mico JA, Berrocoso E: Fluoxetine: a case history of its discovery and preclinical development. Expert opinion on drug discovery 2014, 9(5):567-578.

53. Hollon SD, Shelton RC, Wisniewski S, Warden D, Biggs MM, Friedman ES, Husain M, Kupfer DJ, Nierenberg AA, Petersen TJ et al: Presenting characteristics of depressed outpatients as a function of recurrence: preliminary findings from the STAR*D clinical trial. Journal of psychiatric research 2006, 40(1):59-69.

54. Mueller TI, Leon AC, Keller MB, Solomon DA, Endicott J, Coryell W, Warshaw M, Maser JD: Recurrence after recovery from major depressive disorder during 15 years of observational follow-up. The American journal of psychiatry 1999, 156(7):1000-1006.
55. Baldessarini RJ, Lau WK, Sim J, Sum MY, Sim K: **Duration of initial antidepressant treatment and subsequent relapse of major depression.** *Journal of clinical psychopharmacology* 2015, **35**(1):75-76.

56. Keller MB, Kocsis JH, Thase ME, Gelenberg AJ, Rush AJ, Koran L, Schatzberg A, Russell J, Hirschfeld R, Klein D *et al:* **Maintenance phase efficacy of sertraline for chronic depression: a randomized controlled trial.** *Jama* 1998, **280**(19):1665-1672.

57. Yiend J, Paykel E, Merritt R, Lester K, Doll H, Burns T: **Long term outcome of primary care depression.** *Journal of affective disorders* 2009, **118**(1-3):79-86.

58. Alves ND, Correia JS, Patrício P, Mateus-Pinheiro A, Machado-Santos AR, Loureiro-Campos E, Morais M, Bessa JM, Sousa N, Pinto L: **Adult hippocampal neuroplasticity triggers susceptibility to recurrent depression.** *Translational psychiatry* 2017, **7**(3):e1058.

59. Sanmukhani J, Anovadiya A, Tripathi CB: **Evaluation of antidepressant like activity of curcumin and its combination with fluoxetine and imipramine: an acute and chronic study.** *Acta poloniae pharmaceutica,* **68**(5):769-775.

60. Tan M, Cha C, Ye Y, Zhang J, Li S, Wu F, Gong S, Guo G: **CRMP4 and CRMP2 Interact to Coordinate Cytoskeleton Dynamics, Regulating Growth Cone Development and Axon Elongation.** *Neural plasticity* 2015, **2015**:947423.

### Tables

**Table 1**

| Gene Name | Sequence 5’-3’ | Length | Product length |
|-----------|----------------|--------|----------------|
| CRMP2     | Forward        | 21     | 216            |
|           | Reverse        | 20     |                |
| Tubulin   | Forward        | 20     | 142            |
|           | Reverse        | 21     |                |
| GAPDH     | Forward        | 21     | 281            |
|           | Reverse        | 21     |                |

### Figures

---
Figure 1

Effect of treatment on PC12 cell viability. A) The cell viability of NC group from 1 day to 3 days. B) The cell viability with FLU treatment in different doses from 1 day to 3 days: 0.01μM, 0.1μM, 1μM, and 10μM. C) The cell viability with SB treatment in different doses from 1 day to 3 days: 1µM, 10µM, 100µM, and 1000µM. D) The cell viability with WT treatment in different doses from 1 day to 3 days: 0.05nM, 0.5nM, 5µM, and 50µM. The cell viability was investigated by CCK8 assays. These results are shown as the mean ± SD (n = 3).*P < 0.05 and **P < 0.01.
Figure 2

A), B), C) show the IF results (×200) of each treatment group for 1 day, 2 days and 3 days. The yellow arrow indicates the cellular neuroplasticity, and the red arrow indicates the co-localization of CRMP2 and tubulin.

Figure 3

The results of western blotting and Co-IP. A), B), C) show the CRMP2 protein content of each treatment group for 1 day, 2 days and 3 days. D), E), F) show the tubulin protein content of each treatment group for 1 day, 2 days and 3 days. J) shows a direct interaction between CRMP2 and tubulin. These results are shown as the mean ± SD (n = 3). *P < 0.05, **P < 0.01 and ****P<0.001.
Figure 4

The results of RT-PCR. A-1), B-1), C-1) show the CRMP2 mRNA expression of each treatment group for 1 day, 2 days and 3 days. A-2), B-2), C-2) show the tubulin mRNA expression of each treatment group for 1 day, 2 days and 3 days. These results are shown as the mean ± SD (n = 3). *P < 0.05 and **P < 0.01.