PIBB 生物化学与生物物理进展
Progress in Biochemistry and Biophysics
2023, 50(6): 1391~1402
www.pibb.ac.cn

The Quercetin-modulated Activity-dependent Inhibition of Endocytosis at a Central Synapse*

LI Shun[1,5)**], GAO Yi-Ming[2,3)**], XU Yue[3,4)], HU Jia-Qi[3,5)], TANG Wen-Xu[3)], SUN Xiao-Quan[3)], XUE Lei[2,3,4)***], WANG Wan-Chun[6)***]

([1)]*Jiangxi University of Traditional Chinese Medicine, Nanchang* 330004, China;
[2)]*State Key Laboratory of Medical Neurobiology and MOE Frontiers Center for Brain Science, Fudan University, Shanghai* 200433, China;
[3)]*Department of Physiology and Neurobiology, School of Life Sciences, Fudan University, Shanghai* 200438, China;
[4)]*Research Institute of Intelligent Complex Systems, Fudan University, Shanghai* 200433, China;
[5)]*Center for Rehabilitation Medicine, Department of Pain Management, Zhejiang Provincial People's Hospital, Affiliated People's Hospital, Hangzhou Medical College, Hangzhou* 310014, China;
[6)]*Department of Traditional Chinese Medicine Surgery, Affiliated Hospital of Jiangxi University of Chinese Medicine, Nanchang* 330006, China)

Abstract **Objective** Quercetin, a flavonol compound widely distributed in fruits, vegetables, and medicinal plants, has been suggested to act as a neuroprotective agent. In the present study, we investigated the presynaptic effect of quercetin on synaptic transmission and plasticity. **Methods** Using whole-cell patch clamp and capacitance measurement technique, we recorded miniature excitatory postsynaptic currents (mEPSC), presynaptic calcium influx, vesicle release and recycling, and the replenishment of readily releasable pool (RRP). Additionally, we stimulated the axon with 5-200 Hz and recorded short-term depression (STD) in the postsynaptic neuron. **Results** We found that 100 μmol/L quercetin in the extracellular solution did not affect the mEPSC amplitude and frequency, indicating a presynaptic mechanism modulating synaptic transmission. At the presynaptic nerve terminals, 100 μmol/L quercetin did not induce notable changes in calcium influx or vesicle release, but significantly inhibited clathrin-dependent slow endocytosis following exocytosis. The inhibition of endocytosis led to a slowdown of vesicle mobilization during stimulation, a reduction in readily releasable pool replenishment after stimulation, and enhancement of short-term depression during high-frequency repetitive stimulation in the postsynaptic principal neurons. **Conclusion** These results provide new insights into quercetin-modulated neuronal signaling and suggest a protective effect that prevents excessive excitatory synaptic transmission in brain circuits.

Key words quercetin, synaptic transmission, vesicle exocytosis, vesicle endocytosis, capacitance measurement
DOI: 10.16476/j.pibb.2023.0191

Synapses are the essential building blocks of the central nervous system (CNS) and responsible for transmitting information between neurons[1]. The highly efficient process of presynaptic vesicle recycling plays a crucial role in maintaining precise synaptic transmission and plasma membrane homeostasis[2]. When the action potential (AP) arrives at the nerve terminal and activates the voltage-dependent calcium channels, the calcium influx triggers the neurotransmitter-containing vesicles to fuse with the presynaptic membrane, leading to exocytosis[3]. In the mammalian CNS, neurons can fire in the range of 10 to several hundred Hertz.

* This work was supported by grants from the National Key Research & Development Program of China (2022YFC3602700, 2022YFC3602702), the Science and Technology Innovation 2030-Brain Science and Brain-Inspired Intelligence Project (2021ZD0201301), The National Natural Science Foundation of China (81960874, 31971159), the Innovation Program of Shanghai Municipal Education Commission (2019-01-07-00-07-E00041), the Key Project of Natural Science Foundation of Jiangxi Province (20202ACB206010), Shanghai Municipal Science and Technology Major Project (2018SHZDZX01), ZJ Lab and Shanghai Center for Brain Science and Brain-Inspired Technology.
** These authors contributed equally to this work.
*** Corresponding author.
WANG Wan-Chun. Tel: 86-791-86361514,
E-mail: 1210569577@qq.com
XUE Lei. Tel: 86-791-86361514, E-mail: lxue@fudan.edu.cn
Received: May 16, 2023 Accepted: May 23, 2023

However, typical presynaptic terminals only contain several hundred synaptic vesicles per active zone (AZ) [4]. Therefore, efficient endocytosis following exocytosis is critical to prevent exhaustion of vesicles and to help maintain the efficiency of synaptic transmission[5].

Quercetin is a flavonol compound present in traditional Chinese medicine but also widely found in various fruits, vegetables, and medicinal plants[6]. Recently, cumulative evidence has shown that quercetin can penetrate the blood-brain barrier and has multiple physiological effects, such as in anti-oxidative stress, the anti-inflammatory response, and promotion of nerve regeneration[7-9]. Quercetin may also have therapeutic effects on ischemia and hypoxia-induced brain injury and Alzheimer's disease, suggesting great potential in the treatment of neurodegenerative diseases[10-11].

Previous studies have reported that quercetin can modulate neuronal excitability. Ren *et al*. [12] showed that quercetin can reduce the firing rate of pyramidal neurons in the prefrontal cortex. Toyota *et al*. [13] observed similar phenomena in which acute administration of quercetin inhibited the firing rate in trigeminal ganglion cells. In rat retinal ganglion cells, quercetin can reduce the frequency of miniature excitatory postsynaptic currents (mEPSCs) and increase the frequency of miniature inhibitory postsynaptic currents (mIPSCs) in both ON and OFF type cells to suppress the excitotoxicity caused by glaucoma[14]. In addition, quercetin has been shown to enhance the paired-pulse ratio (PPR) and long-term potentiation (LTP) in the rat hippocampus, relieving chronic lead exposure-induced impairment of synaptic plasticity[15]. On the other hand, the quercetin-modulated presynaptic mechanisms remain largely unclear. Lu *et al*.[16] showed that quercetin inhibits N-type and P/Q-type calcium channels and reduces excessive glutamate release in the synaptic boutons of the cerebral cortex, exerting a neuroprotective effect. Another study reported an opposite conclusion in the rat hippocampal CA1 neurons that quercetin promoted presynaptic glutamate release by reducing the postsynaptic PPR, leading to a dose-dependent increase in the excitatory postsynaptic current (EPSC) amplitude[17]. The uncertainty surrounding quercetin-modulated presynaptic mechanisms requires direct electrophysiological recordings at the presynaptic nerve terminals.

In the present study, we examined how quercetin modulates synaptic transmission at a giant glutamatergic synapse, the calyx of Held, located at the medial nucleus of the trapezoid body (MNTB) in the brainstem[18]. The large presynaptic nerve terminal allows for direct recordings of presynaptic calcium current (*I*Ca) and calcium-triggered vesicle exo-endocytosis *via* capacitance measurements at a high temporal resolution [19-20]. Our findings show that quercetin inhibits vesicle endocytosis in a calcium-independent manner, which leads to slowdown of vesicle mobilization and the readily releasable pool (RRP) replenishment. Furthermore, quercetin enhances short-term depression (STD) in a frequency-dependent manner and dramatically reduces the EPSC amplitude upon high-frequency stimulation. Our results provide new insights into quercetin-modulated neuronal signaling and short-term plasticity *via* inhibition of presynaptic vesicle endocytosis, which leads the reduced synaptic transmission and enhanced STD.

1 Materials and methods

1.1 Slice preparation

Sprague-Dawley rats (8−10 d old, p8−p10) of either sex were decapitated and tissue blocks containing the MNTB placed in a low-Ca^{2+} artificial cerebrospinal fluid (ACSF) solution (125 mmol/L NaCl, 25 mmol/L $NaHCO_3$, 3 mmol/L myo-inositol, 2 mmol/L Na-pyruvate, 2.5 mmol/L KCl, 1.25 mmol/L NaH_2PO_4, 0.4 mmol/L ascorbic acid, 25 mmol/L glucose, 3 mmol/L $MgCl_2$, and 0.05 mmol/L $CaCl_2$). Brain slices (~200 μm thick) were sectioned using a vibratome (VT 1200s, Leica, Germany) and incubated in normal ACSF with 2 mmol/L $CaCl_2$ at 37° C for 30−40 min before experiments. Quercetin was diluted in dimethylsulfoxide (DMSO) at a concentration of 10 mmol/L and stored frozen (−20°C). For use, the stock solution was vortexed to ensure complete mixing of components and diluted into the extracellular solution to a final working concentration of 10−250 μmol/L, with a DMSO concentration of 0.1% maintained in all solutions. All electrophysiological recordings were made at room temperature (22−24°C).

All of the methods were carried out in accordance with approved guidelines, and all animal experimental protocols were approved by the Animal Care and Use Committee of Fudan University and

Jiangxi University of Chinese Medicine.

1.2 Electrophysiology

The voltage-clamp recordings of AMPA receptor-mediated mEPSCs and EPSCs were obtained using an EPC-10 amplifier (HEKA, Lambrecht, Germany). The pipette (2−3 MΩ) solution contained 125 mmol/L K-gluconate, 20 mmol/L KCl, 4 mmol/L Mg-ATP, 10 mmol/L Na_2-phosphocreatine, 0.3 mmol/L GTP, 10 mmol/L HEPES, and 0.5 mmol/L EGTA (pH 7.2, adjusted with KOH). The extracellular solution was similar to the ACSF except with 2 mmol/L $CaCl_2$. The series resistance (<10 MΩ) was compensated by 95% (lag 10 μs) throughout the experiment. The mEPSCs were recorded at a holding membrane potential of −80 mV with 0.5 μmol/L tetrodotoxin (TTX). Bicuculline (10 μmol/L), D-APV (10 μmol/L), and strychnine (10 μmol/L) were included in the extracellular solution to inhibit the GABAA receptor, NMDA receptor, and glycine receptor-mediated currents. For EPSC recordings, a bipolar electrode was placed near the midline of the MNTB. Stimulation pulses (0.1 ms, 2−20 V with AM2100, A-M systems, USA) at 5−200 Hz were delivered to evoke APs at the presynaptic nerve terminal, which induced an AMPA receptor-mediated EPSC at the principal neuron of the MNTB.

The presynaptic membrane capacitance measurements were obtained using an EPC-10 amplifier with lock-in software. The presynaptic pipette (3.5−5 MΩ) solution contained 125 mmol/L Cs-gluconate, 20 mmol/L CsCl, 4 mmol/L Mg-ATP, 10 mmol/L Na_2-phosphocreatine, 0.3 mmol/L GTP, 10 mmol/L HEPES, and 0.05 mmol/L BAPTA (pH 7.2, adjusted with CsOH). The series resistance (<10 MΩ) was compensated by 65% (lag 10 μs). Slow endocytosis was induced by a 20-ms depolarization pulse from −80 mV to +10 mV ($depol_{20ms}$), and rapid endocytosis was induced by 10 $depol_{20ms}$ at 10 Hz ($depol_{20ms\times10}$). To measure the vesicle mobilization during stimulation, 10 pulses of $depol_{20ms}$ at 1 Hz were applied.

1.3 Data analysis

The Mini Analysis Program (version 6.07, Synaptosoft, USA) was used to analyze the mEPSCs. Capacitance jumps were measured 250 ms after depolarization to avoid artifacts. The initial rate of endocytosis ($Rate_{endo}$) was measured within 2 s after depolarization with $depol_{20ms}$ or $depol_{20ms\times10}$. The residual capacitance 15 s after $depol_{20ms}$ (ΔCm_{15s}) or 30 s after $depol_{20ms\times10}$ (ΔCm_{30s}) was measured to represent endocytosis recovery. All data were expressed as mean±*SE*. *P* values were determined by Student's *t*-test, one-way ANOVA with Bonferroni's multiple comparisons test, or Kruskal-Wallis test, and $P<0.05$ was considered significant.

2 Results

2.1 Quercetin does not affect the sensitivity of postsynaptic AMPA receptors

To investigate how quercetin affects synaptic transmission in the CNS, we examined the mEPSCs of the principal neurons at the rat calyx of Held synapses in the brain stem. The postsynaptic neurons were held at a resting potential of −80 mV in the whole-cell configuration with 0.5 μmol/L TTX in the extracellular solution. Bicuculline (10 μmol/L), D-APV (10 μmol/L), and strychnine (10 μmol/L) were also included. In control p8−p10 rats, the mean mEPSC amplitude and frequency were (30.7±2.6) pA and (0.8±0.2) Hz (722 events, $n=5$; Figure 1a−c). The mean 10%−90% rise time and 20%−80% decay time were (0.34±0.02) ms and (1.58±0.05) ms, respectively (Figure 1d). These results were similar to our previous studies at calyx-type synapses[21-22]. In the quercetin-treated groups, we applied 10 μmol/L quercetin to the extracellular solution at least 30 min before recordings were made. The mEPSC amplitude and frequency were not significantly different from the control group (amplitude: (33.2±2.3) pA, $P=0.84$; frequency: (0.8±0.3) Hz, $P=0.99$; 884 events, $n=6$; Figure 1a−c). We also found no significant differences in the 10%−90% rise time and 20%−80% decay time (rise time: (0.36±0.03) ms, $P=0.53$; decay time: (1.65±0.10) ms, $P=0.24$; $n=6$; Figure 1d). Previous studies have tested quercetin concentrations up to 200−300 μmol/L[16, 23]. Therefore, we also investigated 100 and 250 μmol/L extracellular quercetin. However, neither mEPSC amplitude and frequency nor 10%−90% rise time and 20%−80% decay time showed significant differences among the control and two high-concentration quercetin-treated groups (Figure 1c−d). We also plotted the cumulative probability curve of the mEPSCs in the control and quercetin-treated groups and found no significant differences among all four groups (Kruskal-Wallis test, $P=0.31$; Figure 1e). Therefore, we conclude that quercetin

does not affect the spontaneous mEPSCs, suggesting that quercetin does not alter the sensitivity of the postsynaptic AMPA receptors of principal neurons at calyces.

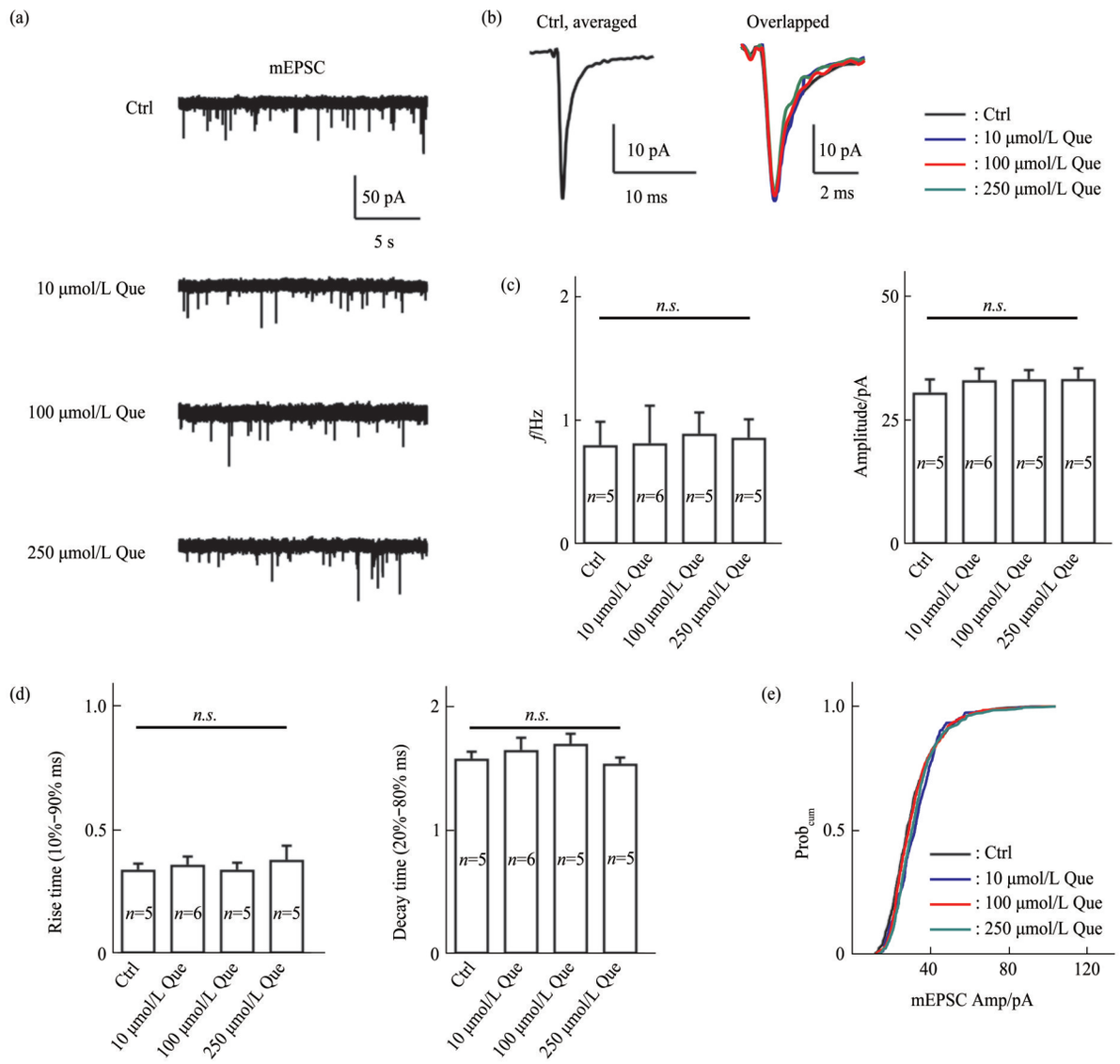

Fig. 1 Quercetin does not affect the sensitivity of postsynaptic AMPA receptors

(a) Representative mEPSC traces in the control and three quercetin-treated groups. Different concentrations of quercetin (10, 100, and 250 μmol/L) were applied to the extracellular solution at least 30 min before recordings were made. (b) Left, averaged mEPSC traces in the control group (722 events, *n*=5, black). Right, averaged mEPSC traces from the control and three quercetin-treated groups (10 μmol/L, 884 events, *n*=6, red; 100 μmol/L, 804 events, *n*=5, blue; 250 μmol/L, 760 events, *n*=5, green) were overlapped. (c) Statistics for the mEPSC frequency and amplitude in the control (*n*=5) and three quercetin-treated (*n*=5−6) groups. *P* values were calculated using a one-way ANOVA with Bonferroni's multiple comparisons test. *n.s.*, not significant. (d) Statistics for the 10%−90% rise time and 20%−80% decay time of mEPSCs in the control (*n*=5) and three quercetin-treated (*n*=5−6) groups. *P* values were calculated using a one-way ANOVA with Bonferroni's multiple comparisons test. *n.s.*, not significant. (e) The mEPSC amplitude $Prob_{cum}$ in the control (722 events, *n*=5, black) and three quercetin-treated groups from (b). *P* values were calculated using a Kruskal-Wallis test. Ctrl: control; Que: quercetin.

2.2 Quercetin inhibits presynaptic vesicle endocytosis at the calyx of Held synapses

Having shown that quercetin has no effect on the postsynaptic mEPSCs, we further investigated whether quercetin affects synaptic transmission from the presynaptic site. At the calyx-type synapse, the giant presynaptic nerve terminal provides a unique opportunity to study the presynaptic key parameters

modulating synaptic transmission, including the calcium influx, vesicle exocytosis, and endocytosis[20].

At the presynaptic nerve terminal, APs depolarize the presynaptic membrane and induce calcium influx to trigger neurotransmitter release[2]. Endocytosis, which retrieves the membrane released by its immediately preceding exocytosis, is also of vital importance in maintaining the efficiency of synaptic transmission[24]. Here, we measured the *I*Ca and vesicle exo-endocytosis at the presynaptic nerve terminal of the calyx-type synapse with direct capacitance recordings. As we have already shown that quercetin does not affect the mEPSCs in a concentration range of 10−250 μmol/L (Figure 1), we chose 100 μmol/L extracellular quercetin, a relatively high concentration, for all of the studies in the presynaptic nerve terminal.

Our previous studies showed that the kinetics of vesicle exo-endocytosis critically depend on stimulation intensities[25-26]. Specifically, $depol_{20ms}$ can deplete the RRP and induce clathrin-dependent, dynamin-dependent slow endocytosis[2], whereas $depol_{20ms\times10}$ can induce exocytosis to a greater extent and a rapid dynamin-dependent, but clathrin-independent, form of endocytosis[27]. In control rats, $depol_{20ms}$ induced a *I*Ca and capacitance jump reflecting exocytosis (Δ*C*m) of (2.1±0.1) nA and (511±27) fF (*n*=6; Figure 2a−c), respectively, which were similar to our recent study[20]. The immediately subsequent capacitance decay could be fitted monoexponentially with a time constant (τ) of (16.5±0.1) s (*n*=6), showing slow endocytosis. The $Rate_{endo}$ measured 1−2 s after $depol_{20ms}$ was (42±4) fF/s (*n*=6; Figure 2d). In the quercetin-treated group, $depol_{20ms}$ induced an *I*Ca and exocytosis of (2.0±0.2) nA and (573±37) fF (*n*=6), respectively, which were not significantly different from controls (*I*Ca: *P*=0.75; Δ*C*m: *P*=0.24; Figure 2a−c). However, endocytosis was inhibited in the quercetin-treated group. $Rate_{endo}$ was slightly but not significantly reduced ((37±5) fF/s, *P*=0.56; *n*=6), and the capacitance decay did not return to baseline within 30 s, which is difficult to fit monoexponentially (Figure 2a, e). The remaining capacitance jump 15 s after $depol_{20ms}$ was (56±4)% (ΔCm_{15s}%; *n*=6; Figure 2e) of the maximum Δ*C*m induced by $depol_{20ms}$, which was significantly higher than in controls ((40±2)%, *n*=6; *P*=0.01), indicating that quercetin inhibits slow endocytosis.

Next, we applied intensive stimulation of $depol_{20ms\times10}$ to induce the clathrin-independent but dynamin-dependent rapid endocytosis at calyces. In controls, $depol_{20ms\times10}$ induced a much larger calcium influx (measured by calcium charge, Q*I*Ca; (316±19) pC; *n*=9), leading to a higher capacitance jump ((1 461±97) fF, *n*=9; Figure 2f−h). The capacitance decay reflecting endocytosis could be fit bi-exponentially with rapid and slow time constants of (2.0±0.1) s and (20.6±0.1) s, respectively. The $Rate_{endo}$ representing rapid endocytosis was (238±24) fF/s (*n*=9). In the presence of 100 μmol/L quercetin in the extracellular solution, $depol_{20ms\times10}$ induced a similar calcium influx and capacitance jump as in controls (Q*I*Ca: (326±27) pC, *P*=0.78; Δ*C*m: (1 526±101) fF, *P*=0.66; *n*=9; Figure 2f−h). The rapid endocytosis was also slightly but not significantly reduced ($Rate_{endo}$: (222±13) fF/s, *P*=0.61; *n*=9; Figure 2i). However, the residual capacitance jump measured 30 s after $depol_{20ms\times10}$ (ΔCm_{30s}%) was significantly higher than in controls (Control: (16±3)%; Quercetin: (32±3)%; *P*=0.002; *n*=9; Figure 2j), indicating inhibition of slow endocytosis. A previous study demonstrated that calcium initiates all forms of endocytosis[28]. In our study, we found that quercetin inhibited slow endocytosis while not affecting the *I*Ca and exocytosis. Therefore, our results may indicate that quercetin inhibits presynaptic vesicle endocytosis, especially slow endocytosis, in a calcium-independent manner.

2.3 Quercetin slows vesicle mobilization and RRP replenishment

Recruiting vesicles to the RRP after exocytosis is critical for RRP replenishment. Inhibition of endocytosis may slow replenishment down. To test this possibility, we applied 10 pulses of $depol_{20ms}$ at 1 Hz at the presynaptic nerve terminal of calyces and examined the RRP recovery during the stimulation. The 1 s between pulses, which was much longer than for 10 Hz stimulation, could help us measure the endocytosis rate more accurately during stimulation. The first $depol_{20ms}$ depleted the RRP[29], and the capacitance jump evoked by the 2nd to the 10th depolarizing pulse would reflect vesicle mobilization to the RRP. In the presence of 100 μmol/L quercetin in the extracellular solution, we found that the RRP replenishment rate was significantly reduced starting from the 2nd $depol_{20ms}$ (Figure 3a, b). A previous study showed that calcium/calmodulin sped up both the

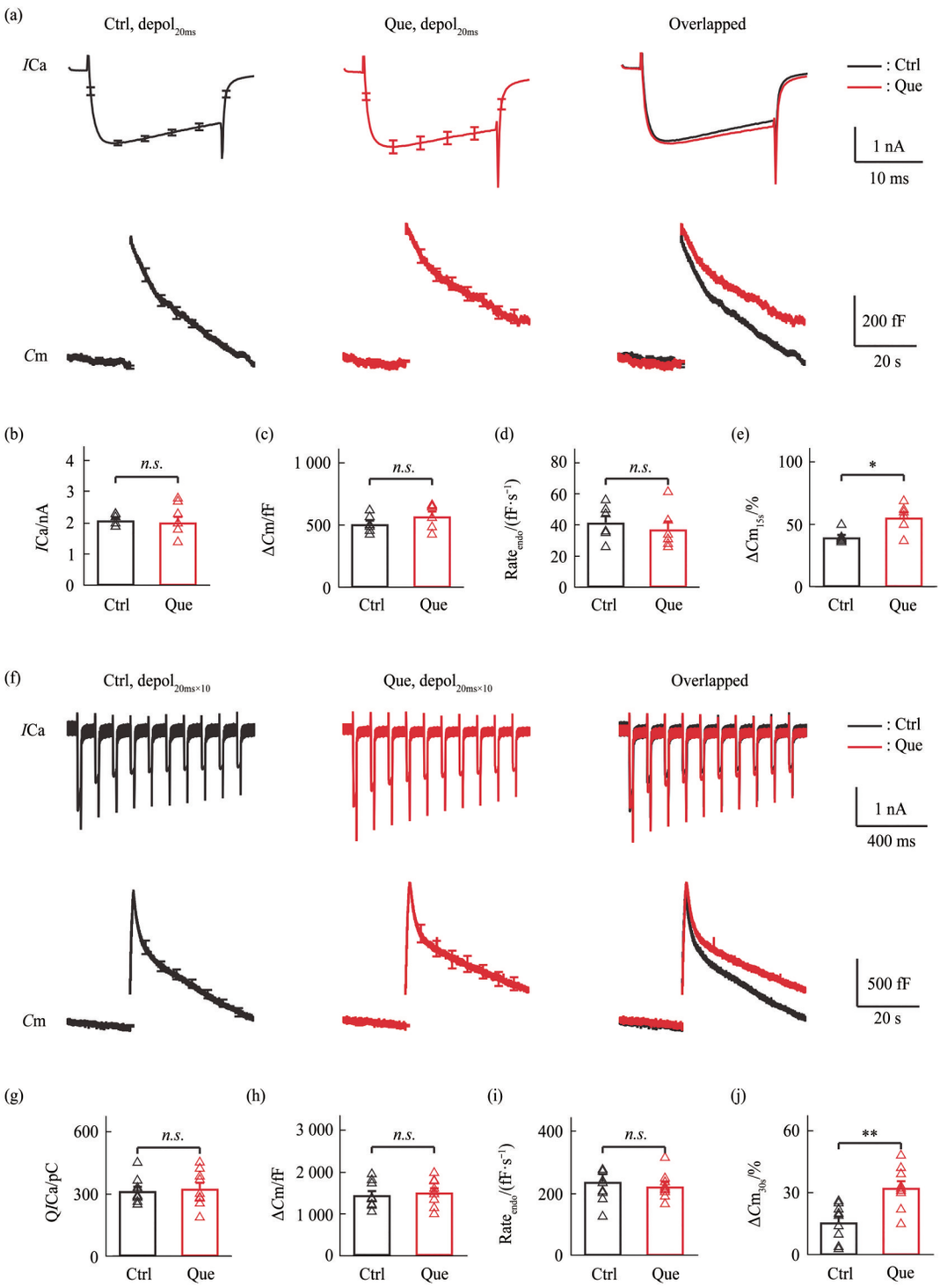

Fig. 2　Quercetin inhibits presynaptic vesicle endocytosis at the calyx−type synapses

(a) Left, average presynaptic calcium current (*I*Ca, top) and membrane capacitance (*C*m, bottom) induced by depol$_{20ms}$ in the control group (0.1% DMSO in the extracellular solution, black). Middle, similar to Left but for the 100 μmol/L quercetin-treated synapses (red). Right, overlap of the average *I*Ca and *C*m from Left and Middle. (b−e) Statistics for *I*Ca, capacitance jump (Δ*C*m), Rate$_{endo}$, and Δ*C*m$_{15s}$% induced by depol$_{20ms}$ from the control and 100 μmol/L quercetin-treated groups (Ctrl, *n*=6, black; Quercetin, *n*=6, red). *P* values were calculated using an unpaired Student's *t*-test. **P*<0.05; *n.s.*, not significant. (f) Left, average presynaptic *I*Ca (top) and *C*m (bottom) induced by depol$_{20ms×10}$ in the control group (0.1% DMSO in the extracellular solution, black). Middle, similar to Left but for the 100 μmol/L quercetin-treated synapses (red). Right, overlap of the average *I*Ca and *C*m from Left and Middle. (g−j) Statistics for Q*I*Ca, Δ*C*m, Rate$_{endo}$, and Δ*C*m$_{30s}$% induced by depol$_{20ms×10}$ from the control and 100 μmol/L quercetin-treated groups (Ctrl, *n*=9, black; Quercetin, *n*=9, red). *P* values were calculated using an unpaired Student's *t*-test. ***P*<0.01; *n.s.*, not significant. Ctrl: control; Que: quercetin.

endocytosis rate and vesicle recruitment to the RRP[28, 30]. However, our results demonstrated that the reduced capacitance jump in the presence of quercetin was not caused by changes in calcium influx, suggesting that quercetin may directly inhibit RRP replenishment by inhibiting endocytosis.

To further consolidate our conclusion, we measured the RRP replenishment at various times (Δt= 0.1−30 s) after $depol_{20ms}$ and plotted the RRP replenishment curve. In controls, the RRP replenishment could be fitted double exponentially with a rapid time constant of 0.2 s and slow time constant of 7.1 s, consistent with previous reports (Figure 3c, upper; Figure 3d)[25, 30]. In the presence of 100 μmol/L quercetin in the extracellular solution, the RRP recovery was much slower than in controls, with a rapid time constant of 0.2 s and a slow time constant of 13.1 s (Figure 3c, lower; Figure 3d). The slowing of RRP replenishment further confirmed that quercetin reduces RRP replenishment by inhibiting endocytosis.

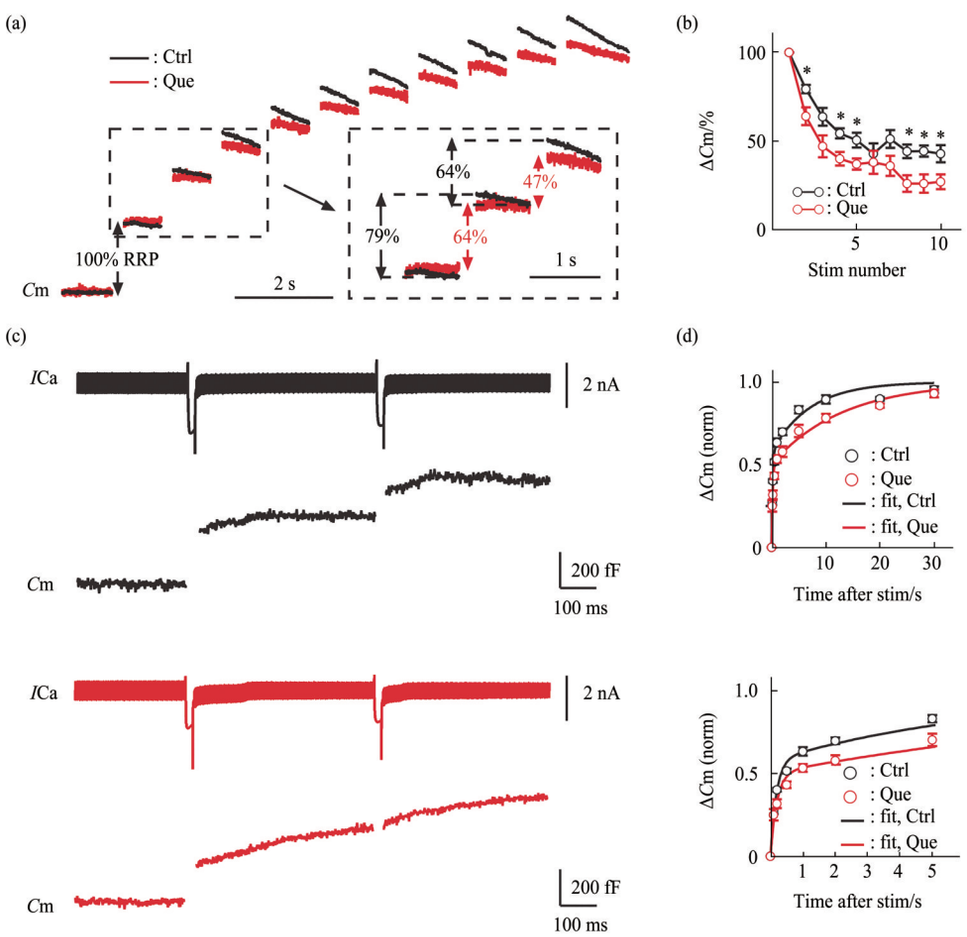

Fig. 3　Quercetin slows down vesicle mobilization and RRP replenishment

(a) Average *C*m induced by 10 pulses of 20-ms depolarization at 1 Hz in the control (0.1% DMSO in the extracellular solution, black) and 100 μmol/L quercetin-treated groups (red). The Δ*C*m induced by the first 20-ms depolarization was normalized. Inset shows the capacitance jumps induced by the 2nd and 3rd 20-ms depolarization. (b) The Δ*C*m induced by each 20-ms depolarization pulse during 10 such pulses at 1 Hz in the control (0.1% DMSO in the extracellular solution, black) and 100 μmol/L quercetin-treated groups (red). Data were normalized to the Δ*C*m induced by the first 20-ms depolarization (Ctrl, n=5; Quercetin, n=6). P values were calculated using an unpaired Student's t-test. *P<0.05. (c) Top, representative presynaptic *I*Ca and *C*m induced by a 20-ms depolarization applied at 500 ms after a conditioning 20-ms depolarization in the control group (0.1% DMSO in the extracellular solution, black). Bottom, similar to Top but for the 100 μmol/L quercetin-treated group (red). (d) Δ*C*m induced by a 20-ms depolarization applied at various intervals after a conditioning 20-ms depolarization (Ctrl, n=4−5, black; Quercetin, n=5−7, red). Data were normalized to the Δ*C*m induced by the conditioning pulse and fit with a double exponential curve (Ctrl, A_1=0.6, τ_1=0.2 s, A_2=0.4, τ_2=7.1 s; Quercetin, A_1=0.5, τ_1=0.2 s, A_2=0.5, τ_2=13.1 s). Top and bottom panels are the same data shown in different time scales. Ctrl: control; Que: quercetin.

2.4　Quercetin enhances short−term depression

The quercetin-induced inhibition of endocytosis and replenishment of RRP may lead to reduced postsynaptic responses. We investigated this possibility with fiber stimulation at the midline of the trapezoid body to evoke APs at the calyx-type synapses and recorded the EPSCs from the postsynaptic principal neuron[31-32]. At calyces, basal neuronal firing can be increased to 600−800 Hz upon stimulation[18, 33]. Thus, we evoked 40 APs at a range of physiological stimulation frequencies at calyces, 5 to 200 Hz [34]. During the stimulation, the EPSC amplitude rapidly declined to a steady-state level (Figure 4a–d). We calculated the steady-state depression ratio ($EPSC_{ss}/EPSC_1$) by averaging the EPSCs from the 31st to the 40th EPSC. As shown in Figure 4e, the steady-state depression ratio decreased as the stimulation frequency increased. In the presence of 100 μmol/L quercetin in the extracellular solution, the depression ratio was not significantly different from that of the control group at a low stimulation frequency of 5 Hz (Figure 4a, e). However, at higher stimulation frequencies (50–200 Hz), quercetin significantly enhanced synaptic depression (50 Hz: ctrl (4.5±1.1)% (n=7) and quercetin (1.4±0.5)% (n=6), P=0.03; 100 Hz: ctrl (3.4±0.5)% (n=6) and quercetin (1.4±0.5)% (n=6), P=0.02; 200 Hz: ctrl (3.0±0.6)% (n=6) and quercetin (1.0±0.4)% (n=4), P=0.049; Figure 4e). These results demonstrate that inhibition of vesicle endocytosis and RRP replenishment led to reduced EPSCs during high-frequency stimulation, with the steady-state depression being more pronounced at higher stimulation frequencies. In addition, we also calculated the PPR from the first two EPSC events of STD recordings both in the control and quercetin-

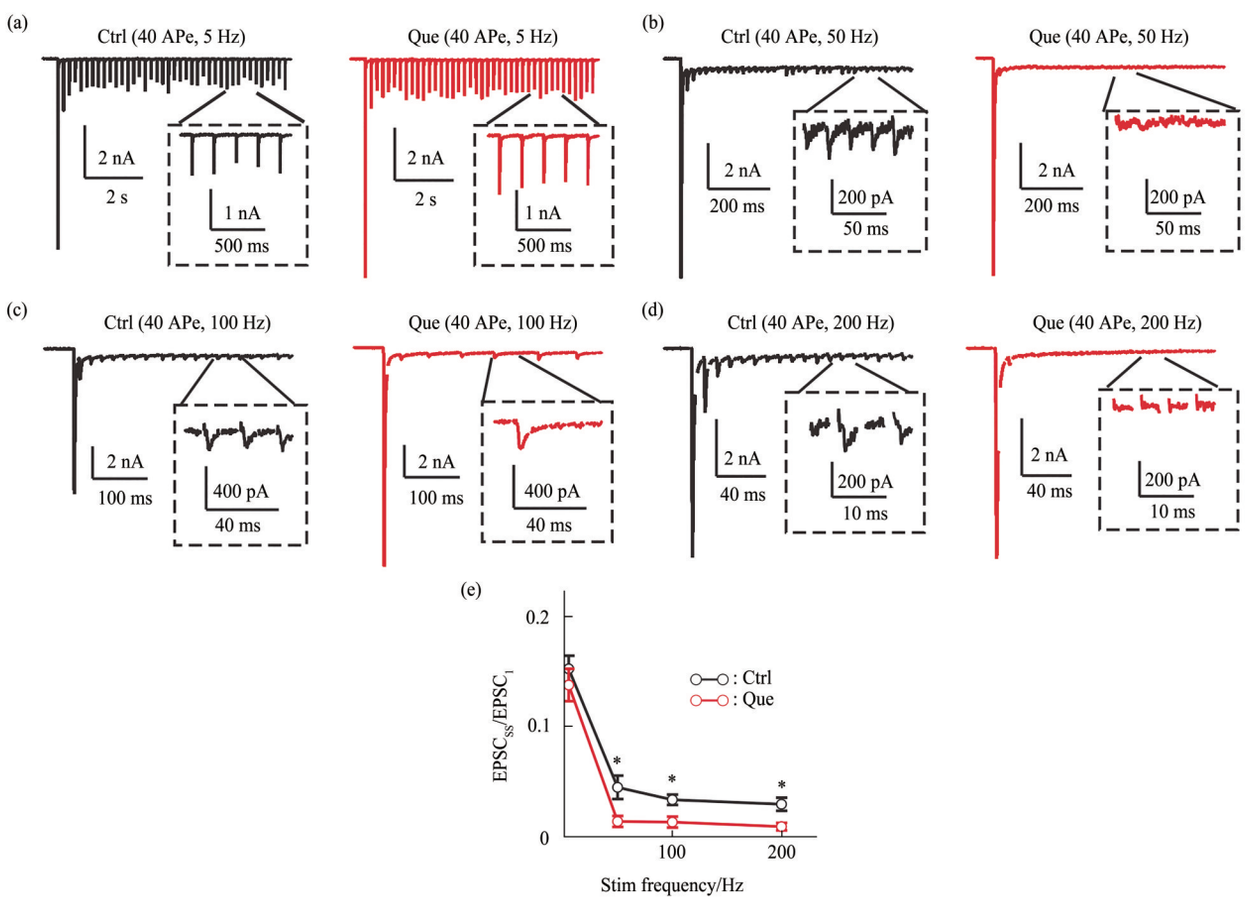

Fig. 4　Quercetin enhances short−term depression under intense stimulation

(a) Left, representative traces of EPSCs evoked by 40 APs at 5 Hz in the control (black) group. Right, similar to Left but for the 100 μmol/L quercetin-treated group (red). Insets show the steady-state EPSCs at larger scale. (b−d) Similar to (a) but with 50, 100, and 200 Hz stimulation. (e) Frequency dependence of short-term depression evaluated as the plot of the ratio of $EPSC_{ss}$ (averaged from the 31st to the 40th EPSC) to the first EPSC ($EPSC_1$) in the stimulation train (Ctrl, n=6, black; Quercetin: n=6, red). P values were calculated using an unpaired paired Student's t-test. *P<0.05. Ctrl: control; Que: quercetin.

treated groups. We found PPR changed after quercetin treatment, further confirming that quercetin was involved in the presynaptic modulation of synaptic transmission.

3 Discussion

Efficient synaptic transmission in the CNS critically relies on presynaptic vesicle exo- and endocytosis. In the present study, we report that quercetin, a flavonol compound commonly found in fruits, vegetables, and medicinal plants, can inhibit vesicle endocytosis at rat calyx-type synapses located in the brainstem. Inhibition of endocytosis leads to slowed vesicle mobilization during stimulation, a reduction in RRP replenishment after stimulation, and enhancement of STD during high-frequency repetitive stimulation, a form of short-term plasticity in the postsynaptic principal neurons.

Accumulated studies have demonstrated that quercetin can modulate neuronal excitability *via* different underlying mechanisms in the CNS. In acutely isolated rat hippocampal neurons, quercetin has been reported to reduce voltage-dependent sodium currents and delay depolarization to exert neuroprotective effects[35]. Recently, TWIK-related potassium channel-1 (TREK-1) was shown to be a new target of quercetin action, which can control neuronal excitability by keeping the membrane potential below the depolarization threshold[36]. Ren *et al*. [12] showed that quercetin increased TREK-1 current by activating PKC and reduced the excitability of pyramidal neurons in the prefrontal cortex, thereby alleviating manic behavior in mice. In addition, quercetin has been observed to modulate other channel activities in the CNS, including calcium channels[16], BK channels (large-conductance Ca^{2+}-regulated potassium channels) [37], and TRPV1 channels[38]. The quercetin-modulated neuronal activities may further lead to modulation of the efficiency of synaptic transmission and plasticity in different brain regions. However, many studies of quercetin-modulated synaptic transmission are still controversial[16-17] due to a lack of direct evidence from the presynaptic side. The possible mechanisms for presynaptic function mainly come from the mEPSC/mIPSC frequency recordings or PPR evaluations.

APs depolarize the presynaptic membrane, inducing calcium influx, which triggers neurotransmitter release [39-40]. Endocytosis is crucial to maintaining the efficiency of synaptic transmission by retrieving the membrane released by its immediately preceding exocytosis[41-42]. Our previous studies have shown that the kinetics of vesicle exo-endocytosis critically depend on stimulation intensities [26, 28]. Two major forms of vesicle endocytosis were investigated in the present study. $Depol_{20ms}$ can deplete the RRP and induce clathrin-dependent, dynamin-dependent slow endocytosis [2, 43-44], whereas $depol_{20ms\times10}$ can induce a rapid dynamin-dependent, but clathrin-independent, form of endocytosis [27-28, 43, 45]. We found that administration of quercetin in the extracellular solution did not affect the *I*Ca and exocytosis, but slightly reduced the rapid endocytosis and significantly inhibited the slow endocytosis (Figure 2). The slowdown of endocytosis further inhibited vesicle mobilization and RRP replenishment, which may provide a protective effect that prevents excessive excitatory synaptic transmission.

We found that quercetin enhanced the synaptic depression during high-frequency repetitive stimulation because of slowed endocytosis (Figure 4). A previous study reported that, in synapsin I/II double knock-out (DKO) mice, the kinetics of vesicle exo-endocytosis are not altered. However, the STD was dramatically enhanced in DKO mice during high-frequency stimulation[31]. Synapsin proteins may help boost the release probability during repetitive stimulation. Whether quercetin can also inhibit the synapsin function and contribute to the enhanced STD remains unclear.

Our previous study showed that calmodulin is the calcium sensor of endocytosis, and calcium/calmodulin facilitates RRP replenishment, which is critical in maintaining release during repetitive firing[28]. However, in the present study, we found that quercetin inhibits vesicle endocytosis and RRP replenishment while not affecting the calcium influx and exocytosis. This observation is somewhat surprising because it is not consistent with the calcium/calmodulin-dependent mechanism. Interestingly, a previous study also reported that the RRP replenishment time course was slowed by more intense stimulation, which presumably should induce more calcium influx and activate calmodulin to a higher level[25]. We also confirmed this phenomenon

with similar protocols[30]. In addition, calcineurin, which is activated by calcium/calmodulin, has been shown to control the speed of both rapid and slow endocytosis by dephosphorylating endocytic proteins[46]. Whether calcineurin is also involved in the calcium/calmodulin-independent mechanism needs to be further clarified. Thus, it would be of great interest to explore a possible mechanism other than the calcium/calmodulin-dependent mechanism in future studies, which may help elucidate the generation of postsynaptic short-term plasticity upon intense stimulation.

4 Conclusion

In conclusion, we investigated the quercetin-modulated presynaptic mechanisms at a glutamatergic central synapse. By uncovering the precise kinetics in synaptic transmission and plasticity, our research may provide new insights for clinical application of quercetin.

References

[1] Saheki Y, De Camilli P. Synaptic vesicle endocytosis. Cold Spring Harb Perspect Biol, 2012, **4**(9): a005645

[2] Wu L G, Hamid E, Shin W, *et al*. Exocytosis and endocytosis: modes, functions, and coupling mechanisms. Annu Rev Physiol, 2014, **76**: 301-331

[3] Sakaba T. Kinetics of transmitter release at the calyx of Held synapse. Proc Jpn Acad Ser B Phys Biol Sci, 2018, **94**(3): 139-152

[4] Wu L G, Ryan T A, Lagnado L. Modes of vesicle retrieval at ribbon synapses, calyx-type synapses, and small central synapses. J Neurosci, 2007, **27**(44): 11793-11802

[5] Xue L, Mei Y A. Synaptic vesicle recycling at the calyx of Held. Acta Pharmacol Sin, 2011, **32**(3): 280-287

[6] Boots A W, Haenen G R, Bast A. Health effects of quercetin: from antioxidant to nutraceutical. Eur J Pharmacol, 2008, **585**(2-3): 325-337

[7] Ishisaka A, Ichikawa S, Sakakibara H, *et al*. Accumulation of orally administered quercetin in brain tissue and its antioxidative effects in rats. Free Radic Biol Med, 2011, **51**(7): 1329-1336

[8] Godoy J A, Lindsay C B, Quintanilla R A, *et al*. Quercetin exerts differential neuroprotective effects against H(2)O(2) and Aβ aggregates in hippocampal neurons: the role of mitochondria. Mol Neurobiol, 2017, **54**(9): 7116-7128

[9] Han X, Xu T, Fang Q, *et al*. Quercetin hinders microglial activation to alleviate neurotoxicity *via* the interplay between NLRP3 inflammasome and mitophagy. Redox Biol, 2021, **44**: 102010

[10] Faria A, Pestana D, Teixeira D, *et al*. Flavonoid transport across RBE4 cells: a blood-brain barrier model. Cell Mol Biol Lett, 2010, **15**(2): 234-241

[11] Saw C L, Guo Y, Yang A Y, *et al*. The berry constituents quercetin, kaempferol, and pterostilbene synergistically attenuate reactive oxygen species: involvement of the Nrf2-ARE signaling pathway. Food Chem Toxicol, 2014, **72**: 303-311

[12] Ren K, Liu H, Guo B, *et al*. Quercetin relieves D-amphetamine-induced manic-like behaviour through activating TREK-1 potassium channels in mice. Br J Pharmacol, 2021, **178**(18): 3682-3695

[13] Toyota R, Ito H, Sashide Y, *et al*. Suppression of the excitability of rat nociceptive primary sensory neurons following local administration of the phytochemical, quercetin. J Pain, 2023, **24**(3): 540-549

[14] Zhou X, Li G, Yang B, *et al*. Quercetin enhances inhibitory synaptic inputs and reduces excitatory synaptic inputs to OFF- and ON-type retinal ganglion cells in a chronic glaucoma rat model. Front Neurosci, 2019, **13**: 672

[15] Hu P, Wang M, Chen W H, *et al*. Quercetin relieves chronic lead exposure-induced impairment of synaptic plasticity in rat dentate gyrus *in vivo*. Naunyn Schmiedebergs Arch Pharmacol, 2008, **378**(1): 43-51

[16] Lu C W, Lin T Y, Wang S J. Quercetin inhibits depolarization-evoked glutamate release in nerve terminals from rat cerebral cortex. Neurotoxicology, 2013, **39**: 1-9

[17] Liu Y, Ding S, Luan Y, *et al*. Ginkgo biloba extracts inhibit post-ischemic LTP through attenuating EPSCs in rat hippocampus. Metab Brain Dis, 2021, **36**(8): 2299-2311

[18] Von Gersdorff H, Borst J G. Short-term plasticity at the calyx of Held. Nat Rev Neurosci, 2002, **3**(1): 53-64

[19] Liu Q Z, Hao M, Zhou Z Y, *et al*. Propofol reduces synaptic strength by inhibiting sodium and calcium channels at nerve terminals. Protein Cell, 2019, **10**(9): 688-693

[20] Wu Y, Liu Q, Guo B, *et al*. BDNF activates postsynaptic TrkB receptors to induce endocannabinoid release and inhibit presynaptic calcium influx at a calyx-type synapse. J Neurosci, 2020, **40**(42): 8070-8087

[21] Wu X S, Xue L, Mohan R, *et al*. The origin of quantal size variation: vesicular glutamate concentration plays a significant role. J Neurosci, 2007, **27**(11): 3046-3056

[22] He L, Xue L, Xu J, *et al*. Compound vesicle fusion increases quantal size and potentiates synaptic transmission. Nature, 2009, **459**(7243): 93-97

[23] Shin T J, Choi S H, Lee B H, *et al*. Effects of quercetin on human α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor-mediated ion currents. Biol Pharm Bull, 2010, **33**(9): 1615-1619

[24] Gan Q, Watanabe S. Synaptic vesicle endocytosis in different model systems. Front Cell Neurosci, 2018, **12**: 171

[25] Wu X S, Wu L G. Rapid endocytosis does not recycle vesicles within the readily releasable pool. J Neurosci, 2009, **29**(35): 11038-11042

[26] Sun Z C, Ge J L, Guo B, *et al*. Extremely low frequency electromagnetic fields facilitate vesicle endocytosis by increasing presynaptic calcium channel expression at a central synapse. Sci

Rep, 2016, **6**: 21774

[27] Wu W, Xu J, Wu X S, *et al*. Activity-dependent acceleration of endocytosis at a central synapse. J Neurosci, 2005, **25**(50): 11676-11683

[28] Wu X S, Mcneil B D, Xu J, *et al*. Ca(2+) and calmodulin initiate all forms of endocytosis during depolarization at a nerve terminal. Nat Neurosci, 2009, **12**(8): 1003-1010

[29] Sun J Y, Wu L G. Fast kinetics of exocytosis revealed by simultaneous measurements of presynaptic capacitance and postsynaptic currents at a central synapse. Neuron, 2001, **30**(1): 171-182

[30] Guo J, Ge J L, Hao M, *et al*. A three-pool model dissecting readily releasable pool replenishment at the calyx of held. Sci Rep, 2015, **5**: 9517

[31] Sun J, Bronk P, Liu X, *et al*. Synapsins regulate use-dependent synaptic plasticity in the calyx of Held by a Ca2+/calmodulin-dependent pathway. Proc Natl Acad Sci USA, 2006, **103**(8): 2880-2885

[32] Xue L, Wu L G. Post-tetanic potentiation is caused by two signalling mechanisms affecting quantal size and quantal content. J Physiol, 2010, **588**(Pt 24): 4987-4994

[33] Hermann J, Pecka M, Von Gersdorff H, *et al*. Synaptic transmission at the calyx of Held under *in vivo* like activity levels. J Neurophysiol, 2007, **98**(2): 807-820

[34] Sommer I, Lingenhöhl K, Friauf E. Principal cells of the rat medial nucleus of the trapezoid body: an intracellular *in vivo* study of their physiology and morphology. Exp Brain Res, 1993, **95**(2): 223-239

[35] Yao Y, Han D D, Zhang T, *et al*. Quercetin improves cognitive deficits in rats with chronic cerebral ischemia and inhibits voltage-dependent sodium channels in hippocampal CA1 pyramidal neurons. Phytother Res, 2010, **24**(1): 136-140

[36] Djillani A, Mazella J, Heurteaux C, *et al*. Role of TREK-1 in health and disease, focus on the central nervous system. Front Pharmacol, 2019, **10**: 379

[37] Kampa R P, Sek A, Szewczyk A, *et al*. Cytoprotective effects of the flavonoid quercetin by activating mitochondrial BK(Ca) channels in endothelial cells. Biomed Pharmacother, 2021, **142**: 112039

[38] Yang C C, Hung Y L, Li H J, *et al*. Quercetin inhibits histamine-induced calcium influx in human keratinocyte *via* histamine H4 receptors. Int Immunopharmacol, 2021, **96**: 107620

[39] Sudhof T C. The presynaptic active zone. Neuron, 2012, **75**(1): 11-25

[40] Sudhof T C. The synaptic vesicle cycle. Annu Rev Neurosci, 2004, **27**: 509-547

[41] Xue L, Mcneil B D, Wu X S, *et al*. A membrane pool retrieved *via* endocytosis overshoot at nerve terminals: a study of its retrieval mechanism and role. J Neurosci, 2012, **32**(10): 3398-3404

[42] Smith C, Neher E. Multiple forms of endocytosis in bovine adrenal chromaffin cells. J Cell Biol, 1997, **139**(4): 885-894

[43] Xu J, Mcneil B, Wu W, *et al*. GTP-independent rapid and slow endocytosis at a central synapse. Nat Neurosci, 2008, **11**(1): 45-53

[44] Xue L, Zhang Z, Mcneil B D, *et al*. Voltage-dependent calcium channels at the plasma membrane, but not vesicular channels, couple exocytosis to endocytosis. Cell Rep, 2012, **1**(6): 632-638

[45] Hosoi N, Holt M, Sakaba T. Calcium dependence of exo- and endocytotic coupling at a glutamatergic synapse. Neuron, 2009, **63**(2): 216-229

[46] Sun T, Wu X S, Xu J, *et al*. The role of calcium/calmodulin-activated calcineurin in rapid and slow endocytosis at central synapses. J Neurosci, 2010, **30**(35): 11838-11847

槲皮素抑制中枢神经突触前兴奋性依赖的胞吞动力学*

李　顺[1,5)**]　高一鸣[2,3)**]　徐　玥[3,4)]　胡佳琦[3,5)]　汤雯絜[3)]　孙小荃[3)]
薛　磊[2,3,4)***]　王万春[6)***]

([1)] 江西中医药大学，南昌 330004；
[2)] 复旦大学医学神经生物学国家重点实验室及脑科学教育部前沿研究中心，上海 200433；
[3)] 复旦大学生命科学学院生理与神经生物系，上海 200438；
[4)] 复旦大学智能复杂系统研究所，上海 200433；
[5)] 浙江省人民医院康复医学中心，疼痛科，杭州医学院附属人民医院，杭州 310014；
[6)] 江西中医药大学附属医院中医外科，南昌 330006）

摘要　目的　槲皮素是一种广泛分布于药用植物中的黄酮类化合物，传统被认为具有神经保护作用。本研究利用位于大鼠脑干花萼状突触的突触前神经末梢进行膜片钳记录，研究槲皮素调控突触传递和可塑性的突触前机制。**方法**　利用全细胞膜片钳结合膜电容记录，在突触后记录微小兴奋性突触后电流（mEPSC），在突触前神经末梢记录钙内流和神经囊泡的释放、回收以及可立即释放库（RRP）的恢复动力学。并且利用纤维刺激在轴突给予5~200 Hz的刺激，诱发突触后EPSC，记录突触后短时程抑制（STD）。**结果**　100 μmol/L槲皮素不影响突触后mEPSC的振幅、频率以及AMPA受体的动力学特征。在突触前神经末梢，槲皮素不改变钙内流或囊泡的释放，但显著抑制胞吐后网格蛋白依赖的慢速胞吞。抑制胞吞会导致突触前囊泡动员的减慢，降低RRP的补充速率，并且增强高频刺激下的短时程可塑性STD。**结论**　本研究为槲皮素调控中枢神经突触传递提供全新的突触前神经机制，槲皮素有助于抑制中枢神经过度兴奋，进而发挥神经保护作用。

关键词　槲皮素，突触传递，囊泡胞吐，囊泡胞吞，电容测量
中图分类号　Q4，Q6　　**DOI**：10.16476/j.pibb.2023.0191

* 国家重点研发计划（2022YFC3602700，2022YFC3602702），科技创新2030-脑科学与脑启发智能项目（2021ZD0201301），国家自然科学基金（81960874，31971159），上海市教委创新计划（2019-01-07-00-07-E00041），江西省自然科学基金（20202ACB206010），上海市重大科技专项（2018SHZDZX01），张江实验室和上海脑科学与类脑研究中心资助项目。
** 并列第一作者。
*** 通讯联系人。
王万春　Tel：0791-86361514，E-mail：1210569577@qq.com
薛磊　Tel：0791-86361514，E-mail：lxue@fudan.edu.cn
收稿日期：2023-05-16，接受日期：2023-05-24