# Electronic Supplementary Information

## Ginsenoside $Rg_1$ Modulates Vesicular Dopamine Storage and Release during Exocytosis Revealed with Single-Vesicle Electrochemistry

Junlan Zhou,[†ab] Jing Zhang,[†ab] Lijiao Cao,[ab] Yuying Liu,[abc] Luyao Liu,[ab]

Chunlan Liu,[*ab] Xianchan Li[*abc]

[a] Key Laboratory of Mass Spectrometry Imaging and Metabolomics (Minzu University of China), National Ethnic Affairs Commission, Beijing 100081, China

[b] Center for Imaging and Systems Biology, College of Life and Environmental Sciences, Minzu University of China, Beijing 100081, China

[c] State Key Laboratory of Natural and Biomimetic Drugs and Department of Pharmaceutical Analysis, School of Pharmaceutical Sciences, Peking University, Beijing 100191, China

*** Corresponding Author**

E-mail: Xianchan Li, xcli@hsc.pku.edu.cn; Chunlan Liu, liuchunlan@muc.edu.cn.

## Contents:

## Material and methods

*Chemicals and solutions*

Chemical reagents, of analytical grade, were purchased from Sinopharm Group Chemical Reagents Co., Ltd. (Shanghai, China) and used as obtained. The HEPES physiological saline consists of 150 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, and 5 mM glucose. The high $K^+$ stimulation solution contains 85 mM NaCl, 70 mM KCl, 1.2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, and 5 mM glucose. The phosphate buffer saline (PBS) contains 2.7 mM KCl, 136.9 mM NaCl, 1.8 mM $KH_2PO_4$, and 12 mM $Na_2HPO_4$. The Dulbecco's phosphate-buffered saline (DPBS) consists of 2.7 mM KCl, 1.5 mM $KH_2PO_4$, 137.9 mM NaCl, 8.1 mM $Na_2HPO_4 \cdot 7H_2O$. All solutions were prepared with 18 MΩ·cm water from the Purelab Classic purification system (ELGA, Sweden), and the solution pH was adjusted to 7.4 with concentrated NaOH (3.0 M).

*Microelectrodes fabrication*

The disk microelectrodes and the nano-tip conical electrodes were prepared using the following methods. A 7 μm diameter carbon fiber was inserted into a borosilicate glass capillary (1.5 mm O.D., 0.84 mm I.D., VitalSense Scientific Instrument Co., Ltd, China). The borosilicate glass capillaries were subsequently pulled into two separate electrodes by a microelectrode puller (PC-100, Narishige Inc., Japan). For disk microelectrodes, the carbon fibers extending from the glass tip were cut entirely using a surgical scalpel under a microscope and sealed with epoxy. The glued electrodes were cured at 100 °C in an oven overnight and then beveled at a 45° angle (EG-401, Narishige Inc., Japan). For nano-tip conical electrodes, the carbon fibers extending from the glass tip were cut to 100-200 μm. Then the fiber was flame-etched with a butane flame to form a needle-like shape followed by epoxy glue and dry at 100 °C overnight.

Both kind of microelectrodes were tested by cyclic voltammetry (-0.4 to 0.6 V vs. Ag / AgCl, 100 mV / s) in 100 μM dopamine prepared in PBS (pH 7.4). Only electrodes showing good reaction kinetics and stable steady-state currents were used for electrochemical experiments (seen in Fig. S1).

*Cell culture and treatment*

PC12 cells were purchased from Peking Union Medical College Hospital. The cells were cultured in RPMI-1640 media (Hyclone) supplemented with 10% horse serum (Gibco), 5% fetal bovine serum (Gibco), 100 units/ml penicillin, and 100 μg/ml streptomycin penicillin (Sigma) in a 5% $CO_2$, 100% humidity atmosphere at 37 ℃. The cells were grown on Petri dishes (Corning) and passaged every 7-9 days. The cell medium was replaced every 2-3 days across the lifespan of all cultures.[1]

For ginsenoside $Rg_1$ experiments, PC12 cells were incubated in RPMI-1640 media containing 25 μM ginsenoside $Rg_1$ for 3 h in a humidified incubator. Before single vesicle electrochemical

experiments, the media was removed from the dish and the cells were rinsed three times with HEPES physiological saline.

*Transmission electron microscopy*

Transmission electron microscopy (TEM, HT7700, Hitachi Ltd., Japan) was used to visualize vesicles in PC12 cells. Briefly, PC12 cell suspension centrifugates at 3000 rpm for 5 min to get the cell pellets. After washing 3 times with PBS, cells were immobilized with 2.5% glutaraldehyde at 4 ℃ for 24 h and 1% osmium tetroxide at room temperature for 2 h, subsequently. Dehydration was done by rising concentrations of ethanol (70%, 85%, 95% and 99.5%) and later with 100% acetone and embedded in Agar 100 resin. 70 nm thick sections were cut with an ultramicrotome (UC7, Leica, German) and poststained with 2% uranyl acetate and lead citrate double stain solution for 15 min before TEM imaging.

The TEM images were analyzed with Image-Pro Plus (version 6.0, Media Cybernetics, Inc., America) to measure the diameters of vesicles and dense cores, defined as the distance between the bilateral farthest points of vesicles or dense cores. All results are compared using the Mann-Whitney rank-sum test.

*Calcium influx measurement*

After washing 3 times with DPBS buffer, PC12 cells were incubated with 5 μM fluorogenic calcium-sensitive dye Cal 520 (ab171868, abcam) for 1 hour in a cell incubator and for another 30 min at room temperature. $Rg_1$-treated cells were then incubated with 25 μM $Rg_1$ for 3 hours while control cells were incubated with RPMI-1640. During the experiment, both two groups bathed in the HEPES physiological saline at 37℃.

The calcium measurement was done with a fluorescent microplate reader (cytation 5, Bio Tek) by setting excitation and emission wavelengths as 469 nm and 526 nm, respectively. After collecting 10 s baseline, PC12 cells were stimulated with 70 mM $K^+$ solution to induce calcium influx. The fluorescence intensity, representing the intracellular calcium concentration, is processed in Gen5 software. Background subtraction and analysis were performed with GraphPad Prism.

*Single-cell experiments*

All single-cell experiments were performed under an inverted microscope (IX73, Olympus) in a Faraday cage at 37 ℃. The working electrode was held at +700 mV versus an Ag/AgCl reference electrode via a patch-clamp amplifier (EPC 10 USB double, HEKA). For SCA, the disk microelectrode was put above the cell along with a glass micropipette containing high $K^+$ stimulating solution positioned 30 μm from the cell. The stimulation was performed through a microinjection system (Pump 11 Elite, Harvard).

*Data processing and statistics*

The amperometric signals were analyzed with IgorPro 6.22 software originated from David Sulzer's group. The filter used was 1 kHz (binomial sm.). Five times of noise standard deviation

was used as the threshold for peak analysis. Only the traces with more than 10 peaks had been used for analysis to minimize the discrepancy of the medians. Data between the different groups were statistically compared with a two-tailed Wilcoxon-Mann-Whitney test.

The kinetic parameters, including the peak current $I_{max}$, $t_{1/2}$ (the time when peak width at 50 % of $I_{max}$), $t_{rise}$ (the time when peak rises from 25 % to 75 % of $I_{max}$ on the ascending part of the spike), and $t_{fall}$ (the time when peak drops from 75% to 25% of $I_{max}$ on the descending part of the spike). The bulge before the main peak is called the pre-spike foot. $I_{foot}$ is defined as its foot current ($I_{foot}$ > 1pA), while $t_{foot}$ is defined as its duration time. The number of molecules released ($N_{molecules}$ and $N_{foot}$) can be calculated by Faraday's law (N = Q/nF), where Q is the area under the peak, n is the number of electrons per molecule transferred in the oxidation reaction (2 for dopamine), and F is the Faraday constant, 96 485 $C \cdot mol^{-1}$.[2-4]

The exponential decays were fitted with double exponential in IgorPro 6.22 and the parameters ($T_1$ and $T_2$) were obtained. The normalized distance, *d*, was calculated as previously reported.[3]

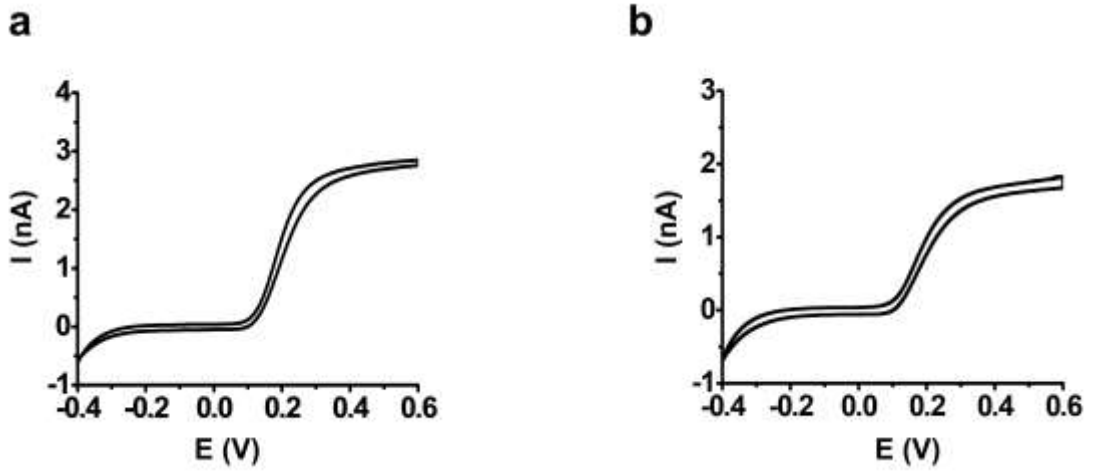


**Fig. S1** The cyclic voltammetry of disk (a) and nanotip conical (b) microelectrodes in 100 μM dopamine prepared in PBS (pH 7.4). Reference electrode, Ag / AgCl.

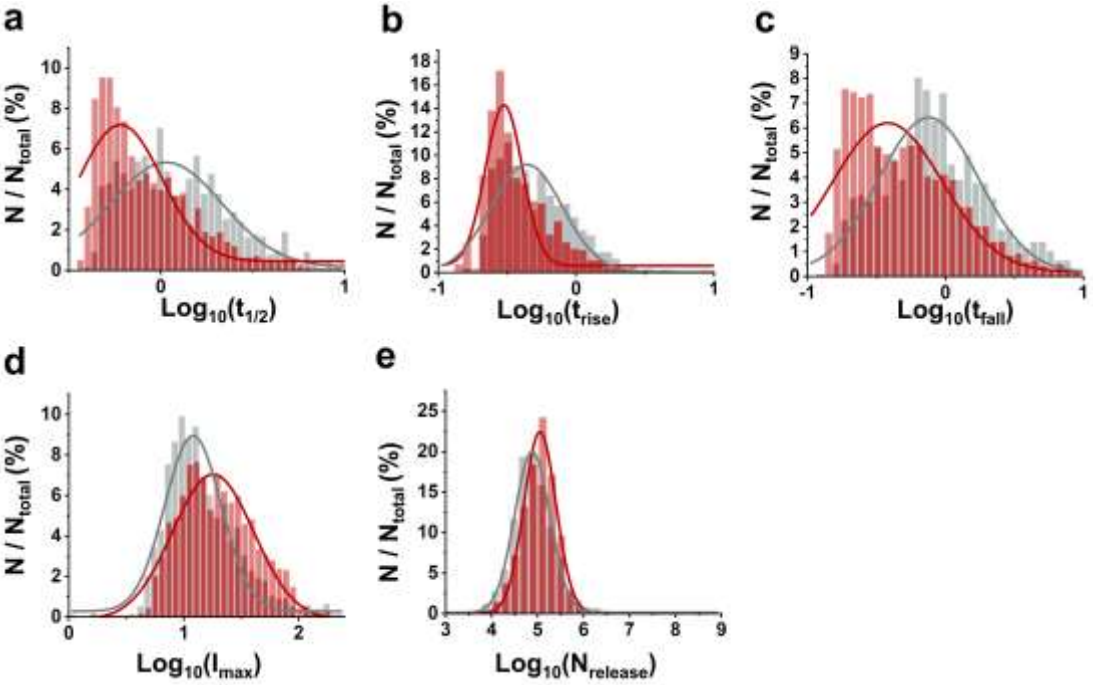


**Fig. S2** Normalized frequency distribution diagram of peaks parameters, including $t_{rise}$, $t_{1/2}$, $t_{fall}$, $I_{max}$, $N_{release}$, for control (grey) and 25 μM $Rg_1$-treated (red) PC12 cells.

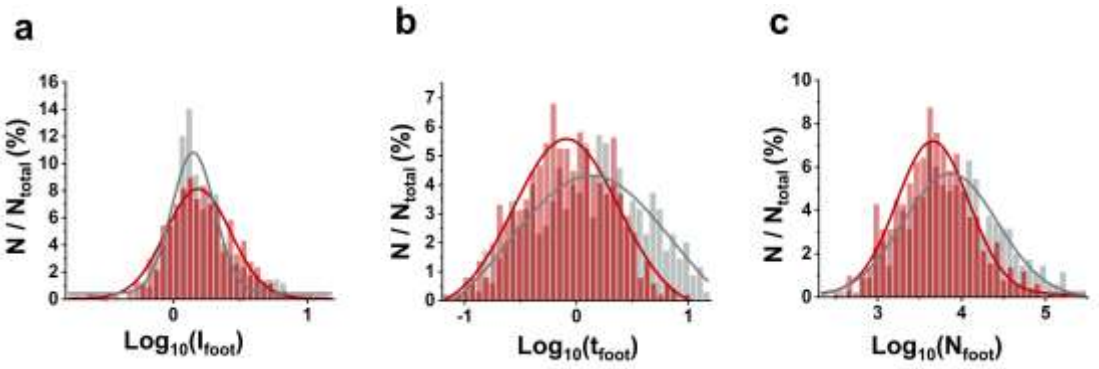


**Fig. S3** Normalized frequency distribution diagram of pre-spike foot parameters, including $I_{foot}$, $t_{foot}$, and $N_{foot}$, for control (grey) and 25 μM $Rg_1$-treated (red) PC12 cells.

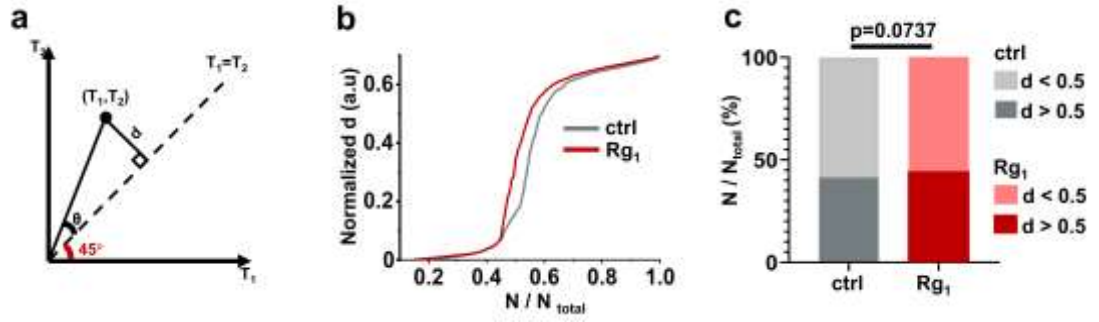


**Fig. S4** Single vs. double exponential analysis of the decay time of exocytotic spikes ($t_{fall}$). (a) Schematic diagram defining the normalized distance d to the "$T_1 = T_2$" line used in $t_{fall}$ analysis. (b) Cumulative histogram of d obtained in PC12 cells treated without (grey) or with (red) 25 μM $Rg_1$ ($n_{d>0.5}/n_{d<0.5}$ = 330/468 for control group vs. $n_{d>0.5}/n_{d<0.5}$ = 651/787 for $Rg_1$-treated cells). (c) Stacking percentage histogram plots describing the number of spikes with $d > 0.5$ or $d < 0.5$. Data are compared with the Chi-Square test.

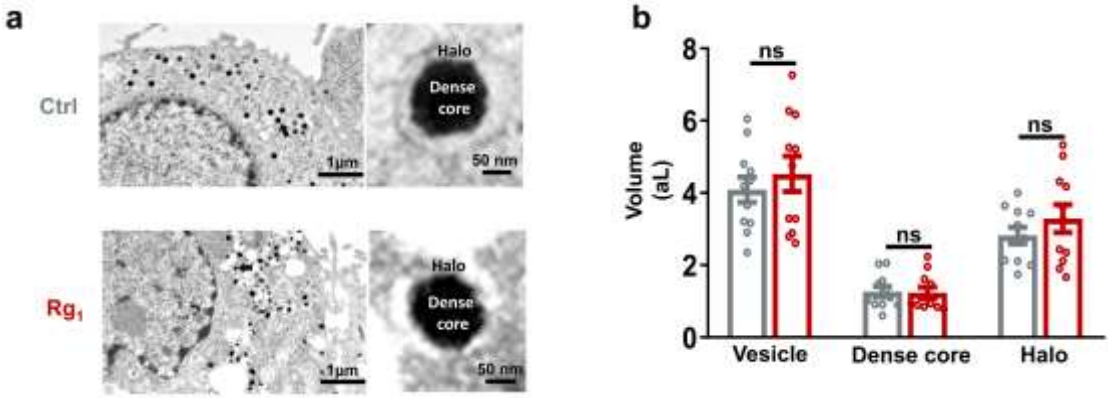


**Fig. S5** Vesicle size in $Rg_1$-treated and control PC12 cells. (a) The TEM image of vesicles from $Rg_1$-treated and control PC12 cells. The right panels depicted the enlarged views of vesicles marked with black arrows in control and $Rg_1$-treated groups, respectively. (b) Histogram plots describing the volume of the vesicle, dense core, and halo. Error bar, SEM. *ns*, no significant difference.

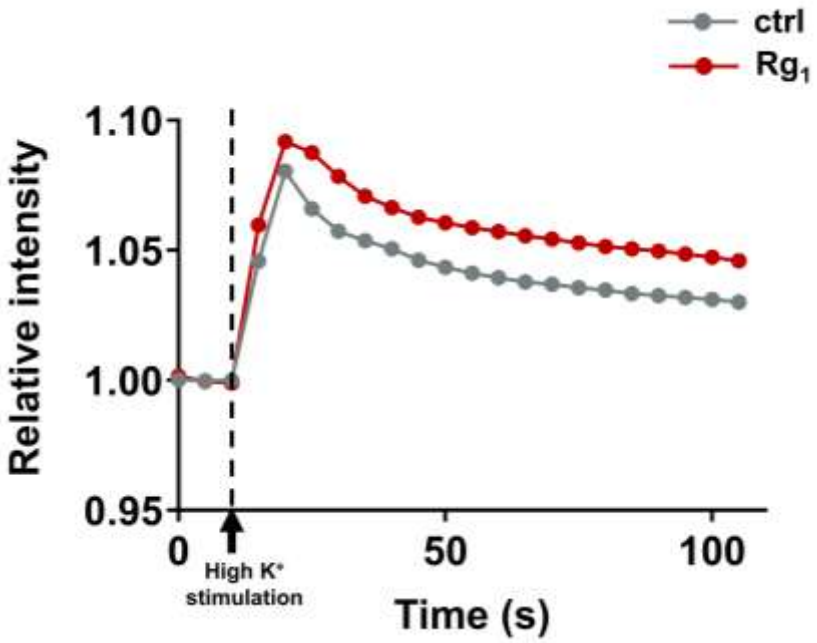


**Fig. S6** Calcium influx of PC12 cells stimulated with 70 mM $K^+$. Cells were treated with (red) and without (grey) 25 μM $Rg_1$. The relative intensity is calculated by dividing the fluorescence intensity after 70 mM $K^+$ stimulation by that before stimulation.

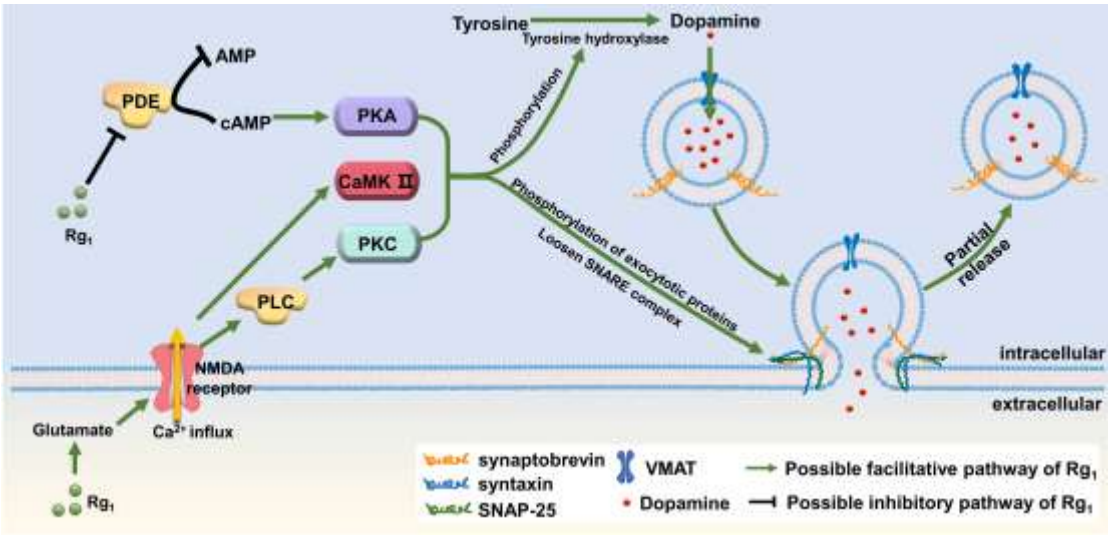


**Fig. S7** Potential biological mechanism of ginsenoside $Rg_1$ effect on neurotransmitter synthesis, storage and release.

**Equation S1:**

$$f(t) = A_1 e^{-(t-t_0)/T_1} + A_2 e^{-(t-t_0)/T_2}$$

where $T_1$ and $T_2$ are the characteristic decay times (defined with $T_1 \leqslant T_2$).

**Equation S2:**

$$d(T_1, T_2) = \sin\theta = \frac{T_2 - T_1}{\sqrt{2(T_1^2 + T_2^2)}}$$

The spikes with $d > 0.5$ were previously considered more suitable for double exponential fitting.[5,6] θ is the angle formed between the vectors ($T_1$, $T_2$) and (1, 1).

## References:


1. X. Li, S. Majdi, J. Dunevall, H. Fathali and A. G. Ewing, *Angew. Chem. Int. Ed. Engl*, 2015, **54**, 11978-11982.
2. X. Li, J. Dunevall and A. G. Ewing, *Angew. Chem. Int. Ed. Engl*, 2016, **55**, 9041-9044.
3. X. Li, A. S. Mohammadi and A. G. Ewing, *J. Electroanal. Chem.*, 2016, **781**, 30-35.
4. Q. Yue, X. Li, F. Wu, W. Ji, Y. Zhang, P. Yu, M. Zhang, W. Ma, M. Wang and L. Mao, *Angew. Chem. Int. Ed. Engl.*, 2020, **59**, 11061-11065.
5. R. Trouillon and A. G. Ewing, *Chemphyschem*, 2013, **14**, 2295-2301.
6. R. Trouillon and A. G. Ewing, *ACS Chem. Biol.*, 2014, **9**, 812-820.