Electronic Supplementary Material (ESI) for Chemical Science.

Supporting Information

Nanoelectrochemistry reveals how presynaptic neurons regulate vesicle release to sustain synaptic plasticity under repetitive stimuli

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Experimental Methods

Materials

Carbon fiber was bought from Goodfellow Cambridge Limited (Britain). 1B100-4 glass capillaries were bought from World Precision Instruments LLC (Florida, U. S. A.). Epoxy was purchased from Shenzhen Ausbond Co., Ltd (Shenzhen, China). Poly-L-lysine (PLL), dopamine (DA), laminin, nerve growth factor (NGF), and Hoechst 33258 were bought from Sigma-Aldrich (St. Louis, U. S. A.). DMEM/F-12, B-27, penicillin/streptomycin, trypsin, HEPES were purchased from Thermo Fisher Scientific (Massachusetts, U. S. A.). Anti-tyrosine hydroxylase (TH) antibody, anti-synaptophysin (SYP) antibody, and DyLight 488 conjugated AffiniPure goat anti-rabbit IgG were bought from Boster Biological Technology Co., Ltd (Wuhan, China). Fluo-4 AM was purchased from Beyotime Biotechnology Co., Ltd (Shanghai, China). YF555 dye phalloidin conjugate was purchased from Shanghai BioScience Co., Ltd (Shanghai, China). Other chemicals were bought from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). All the chemicals unless special remark were reagent grade and used without further purification, and all aqueous solution were allocated by ultrapure water (resistivity ≥ 18 MΩ/cm).

Dopaminergic neuron culture

Primary dopaminergic (DAergic) neurons were isolated from ventral tegmental area (VTA) of newborn (one day old) SD rats according to previous reports.[1,2] The rats were beheaded, then the brain, cerebral hemispheres, VTA were dissociated in 4°C HBSS buffer in sequence. The collected VTA were transported into 0.125% trypsin and incubated in cell incubator (HERAcell 150i, Thermo Fisher Scientific, Massachusetts, U. S. A.) for 3 min. The trypsinization was terminated by adding isovolumetric serum-free culture medium, and then the mixture was centrifuged (5424 R, Eppendorf, Hamburg, Germany) at 2000 rpm for 10 min. Subsequently, supernatant was discarded and cells were resuspended in DMEM/F-12 culture medium adding 2% B-27, 100 U/mL penicillin and 100 μg/mL streptomycin, and 10 ng/mL NGF. Finally, cell suspension was planted on PLL/laminin pretreated coverslips and cultured at 37°C and 5% CO_2. The culture medium was replaced every two days and cell experiments were carried out after 4-6 days culture.

Fabrication of carbon fiber nanoelectrodes

Carbon fiber nanoelectrodes (CFNEs) were manufactured in the same method reported before.[2-4] First, a cleaned carbon fiber (7 μm) adhered on a copper wire went through flame etch. To be specific, the tip of carbon fiber was inserted into alcohol burner flame repeatedly until a standard conical tip (diameter down to 50 nm) could be observed by microforge (MF200, World Precision Instruments LLC, Florida, U. S. A.) through cone angle, diameter change rate,

light and shadow change at tip edge. Subsequently, conical carbon fiber was inserted into a glass capillary with 1 μm tip diameter pulled by laser-based micropipette puller (P-2000/G, Sutter Instrument, California, U. S. A.). Finally, the tip of conical carbon fiber was insulated in glass capillary by a heater wire (H4, World Precision Instruments LLC, Florida, U. S. A.), and the junction between copper wire and glass capillary was fixed by epoxy resin. The complete CFNEs performed as the length and tip diameter of exposed conical carbon fiber being *ca.* 1-2 μm and 50 nm respectively. All CFNEs were tested in 1 mM $K_3[Fe(CN)_6]$/1 M KCl with an electrochemical workstation (CHI660E, CH Instrument, Shanghai, China), and only electrodes with standard cyclic voltammetry curve were used for exocytosis amperometric detection in synaptic cleft. Morphology characterization of CFNEs was performed by field emission scanning electron microscope (SEM, SIGMA, Carl Zeiss, Oberkochen, Germany).

Amperometric measurements and data analysis

During amperometric measurement, neurons were placed in an electrical stimulation pool surrounding by extracellular solution with 2 mM Ca^{2+} and observed by an inverted microscopy in room temperature. Usually, we chose *en passant* synapse formed by varicosity on axon and a soma from another neuron. When a synapse is successfully identified under the microscope, the tip of the CFNE was firstly located nearby the intersection of the single varicosity by a micromanipulator (TransferMan 4R, Eppendorf, Hamburg, Germany), and then was slowly wedged along the axon into the synapse by horizontal travelling till a slight distortion of the synapse occurred. During penetration of conical CFNE into synapse, the synaptic cleft is gradually stretched while maintain leakproofness. And tip of CFNE was tightly surrounded by pre- and post-synaptic membrane. Since docked vesicle replenishment requires more than 10 s and endocytic pathway takes place ~ 20 s after exocytosis,[5,6] periodic electrical field stimulus was set as 70 s interval stimulus started with 10 s resting time, 1 ms 10 V cm^{-1} stimulus and later 60 s resting time. In the course of periodic stimulus, amperometric spikes were continuously recorded with patch clamp amplifier (EPC-10, HEKA Elektronik GmbH, Ludwigshafen, Germany) at a constant potential at 800 mV, with an Ag/AgCl wire as reference/counter electrode. Signals were sampled at 50 kHz, and Bessel filtered at 2.9 kHz by “Patch Master” software, with whole detection system placed in a Faraday cage and all apparatuses grounded through a common ground. Data analysis was realized by Igor Pro software kindly provided by Dr. E. V. Mosharov from Columbia University,[5] without filter and only signals 5 times bigger than noise were chosen and analyzed.

Intracellular calcium ion concentration measurement

According to previous research,[7,8] electrical field stimulus (EFS, 1 ms 10 V cm^{-1} stimulus) was inflicted on target neurons by stimulus isolator (ISO-Flex, A. M. P. Instruments, Jerusalem, Israel) and patch clamp amplifier. We used calcium fluorescent probe Fluo-4 AM to confirm

whether EFS succeeded in triggering AP with Ca^{2+} concentration elevation in neurons. The neurons were gently rinsed with PBS for 3 times before incubated with 2 μM Fluo-4 AM at 37°C for 30 min. After washed off residual dye, neurons were further incubated for 30 min to make sure intracellular Fluo-4 AM has already been cleaved by endogenous esterases to form Fluo-4. Fluorescence microphotographs were recorded before and after stimulus to exhibiting intracellular Ca^{2+} concentration change by an inverted fluorescent microscopy (AxioObserver Z1, Carl Zeiss, Oberkochen, Germany). Besides, cell bath was changed into extracellular solution (containing 2 mM $CaCl_2$), exciting light intensity and exposure time were kept identical during fluorescent experiment.

Immunofluorescence

The cultured cells were first fixed in 4% paraformaldehyde (in PBS) for 20 min in room temperature. Then cells were permeabilized in 0.2% Triton X-100 (in PBS) for 20 min, and subsequently blocked by 1.5% normal goat serum (in PBS) for 30 min in room temperature. Each step was proceeded after 3 times gentle PBS wash-out. To label TH or SYP, neurons were incubated with corresponding antibody (1:400, in PBS) overnight at 4°C, then washed with PBS for 3 times and incubated with fluorescein-labeled secondary antibody (1:100, in PBS), Hoechst (1:1000, in PBS) to label nucleus and phalloidin (1:40, in PBS) to label F-actin for 1 h at 37°C in corresponding experiment. Immunofluorescence was visualized with an inverted fluorescent microscopy while keeping exciting light intensity and exposure time identical.

Transmission electron microscope (TEM) image

Because the brain of newborn rat has not differentiated completely, we chose 7 d old rats as TEM experiment object. We used same way as shown above to isolate VTA, and inflicted EFS towards brain tissue before fixed in 2.5% glutaraldehyde. To be specific, VTA of same 7 days SD rat was isolated and divided into two groups. In experimental group, VTA were placed in an electrical stimulation pool surrounding by extracellular solution with 2 mM Ca^{2+} and suffered 9 times stimuli; in control group, VTA were placed in another electrical stimulation pool surrounding by extracellular solution with 2 mM Ca^{2+}, but without any stimulation. After stimuli towards experimental group finished, two tissue were fixed at same time as soon as possible. After rinsed with PBS for 3 times, the tissue was post-fixed in 1% osmic acid for 2 h, dehydrated in an ascending ethanol series, and embedded in epon araldite resin. Brain tissue sections with 50-100 nm thickness were cut using ultramicrotome (EM UC7, Leica Microsystems, Wetzlar, Germany) and post-stained with premixed solutions of uranyl acetate and lead citrate. The tissues were imaged at 80 kV by transmission electron microscope (HT7800, Hitachi Limited, Tokyo, Japan).

References

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Supplementary Figures and Tables

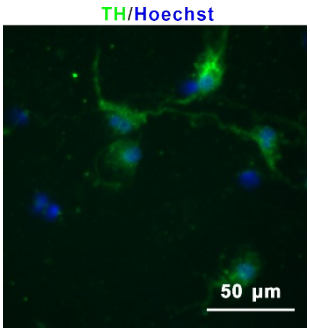

Figure S1. Representative microscopic image of DAergic neurons labeled with TH (DAergic neuronal marker, green) and Hoechst (cell nucleus marker, blue).

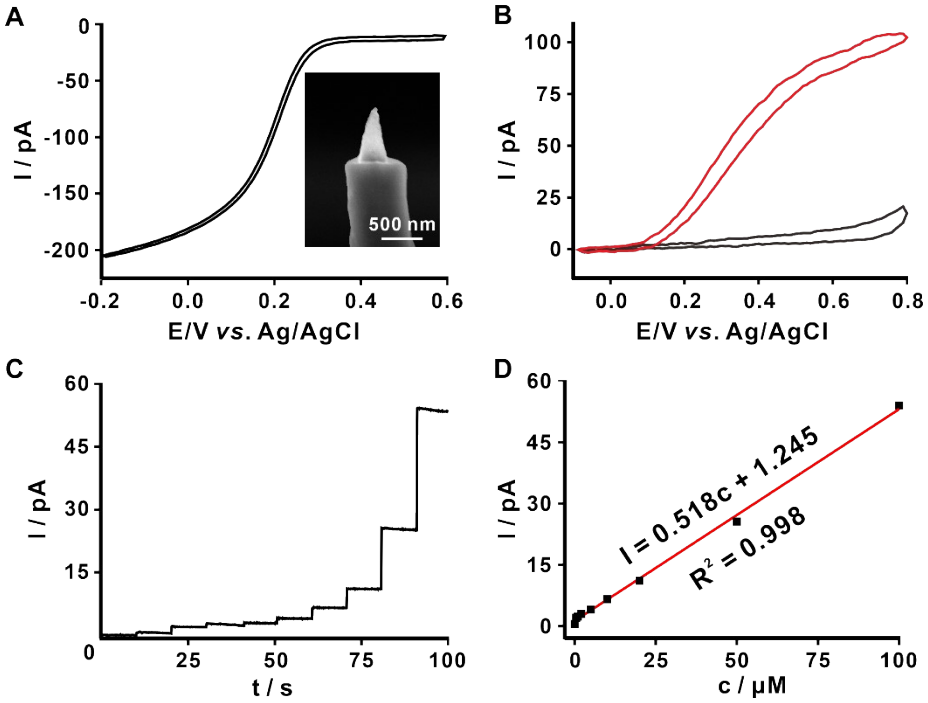

Figure S2. Electrochemical characteristics of CFNE. (A) Cyclic voltammogram of CFNE in 1 mM $K_3[Fe(CN)_6]$; SEM image of a CFNE tip is shown in the inset. (B) Cyclic voltammograms of CFNE in PBS with 100 μM DA (red) or without DA (black). (C, D) Amperometric curve (C) of CFNE at +800 mV *vs.* Ag/AgCl response to a serial concentration of DA (50 nM, 500 nM, 1 μM, 2 μM, 5 μM, 10 μM, 20 μM, 50 μM and 100 μM) in PBS and the corresponding calibration curve (D).

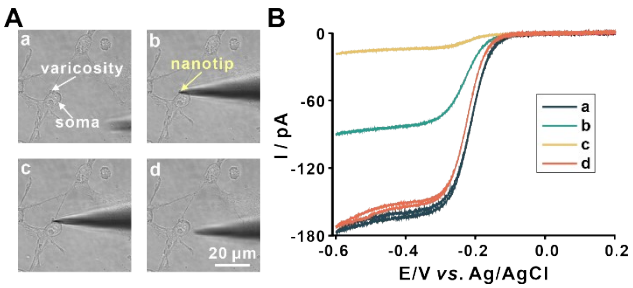

Figure S3. Bright field microscopic images (A) and corresponding cyclic voltammograms (B) with 1 mM $Ru(NH_3)_6^{3+}$ added in PBS showing the inserting and withdrawing process of CFNE tip inside a DAergic synaptic cleft. And CFNE is whole outside (a), half inside (b), whole inside (c) and total withdrawal from synapse (d), respectively.

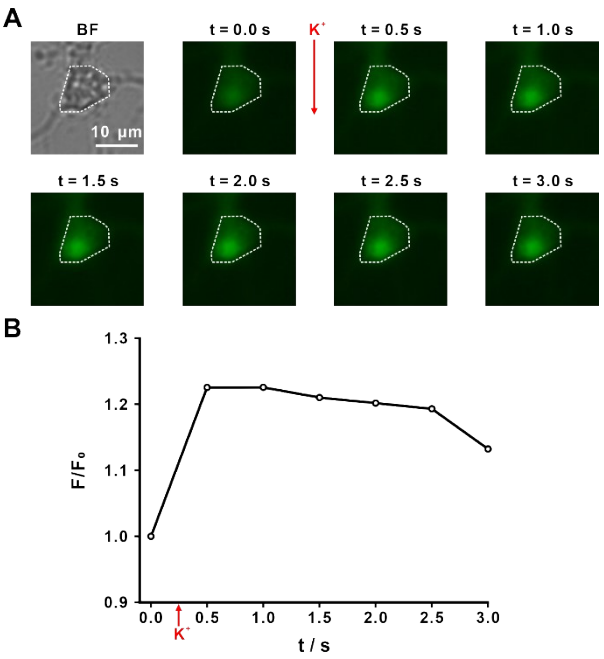

Figure S4. Microscopic images (A) and corresponding statistical analysis (B) of a DAergic neuron showing the change of intracellular Ca^{2+} concentration revealed by Fluo-4 AM green fluorescence before or after high K^+ solution stimulus, red arrow shows the moment of high K^+ solution addition.

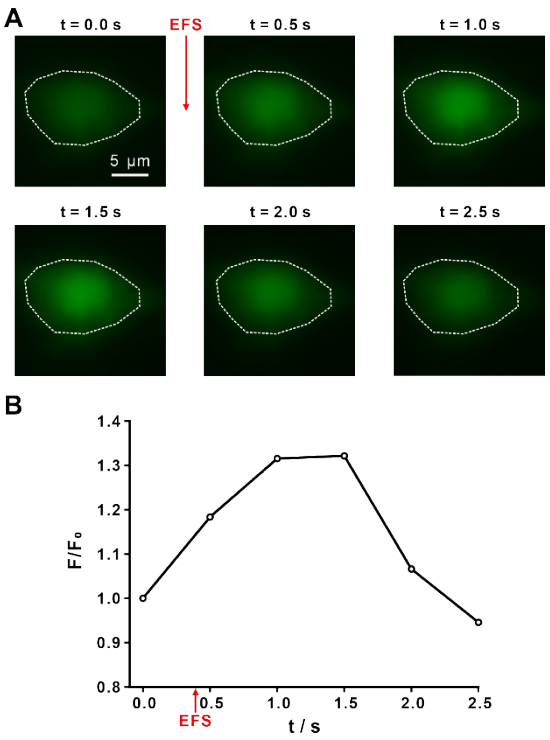

Figure S5. Microscopic images (A) and corresponding statistical analysis (B) of a DAergic neuron showing the change of intracellular Ca^{2+} concentration revealed by Fluo-4 AM green fluorescence before or after electric field stimulus (EFS), red arrow shows the moment of EFS. EFS was almost simultaneously applied as fluorescence sampling point at t = 0.5 s, Hence, the Ca^{2+} influx was just beginning at this timing, and the fluorescence intensity still increased between t = 0.5 s - 1.0 s.

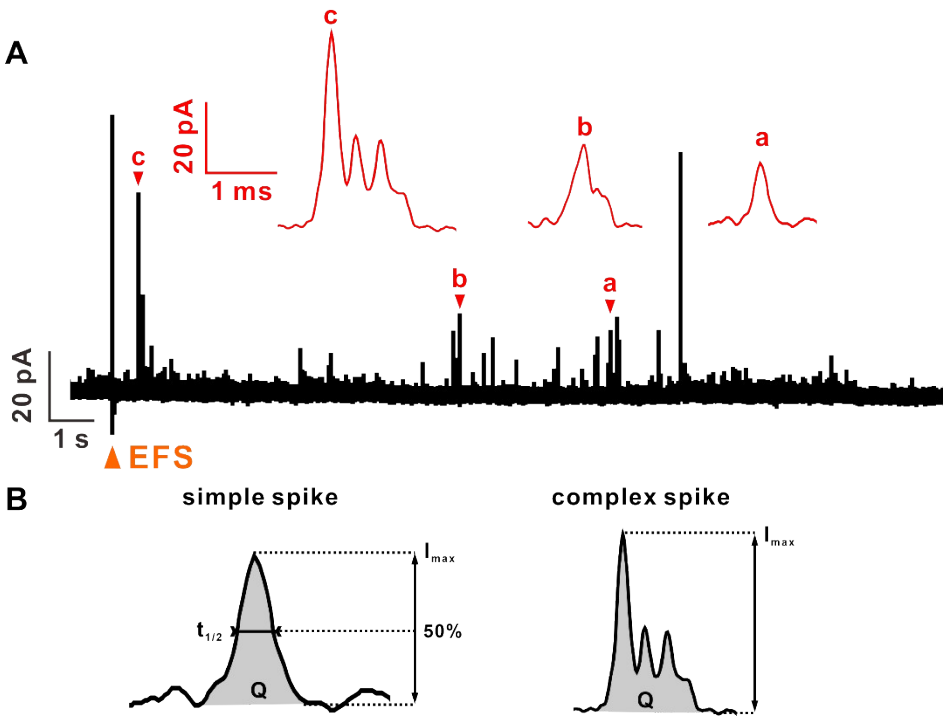

Figure S6. (A) Representative amperometric trace showing a sequence of exocytotic events inside a DAergic synaptic cleft with EFS; three typical amperometric spike shapes represent simple events (a) and complex event (b and c), respectively; (B) Typical parameters of simple spike including half maximum, $t_{1/2}$; peak amplitude, I_{max}; the charge of peak area, Q. And parameters of complex spike including peak amplitude, I_{max}; the charge of peak area, Q.

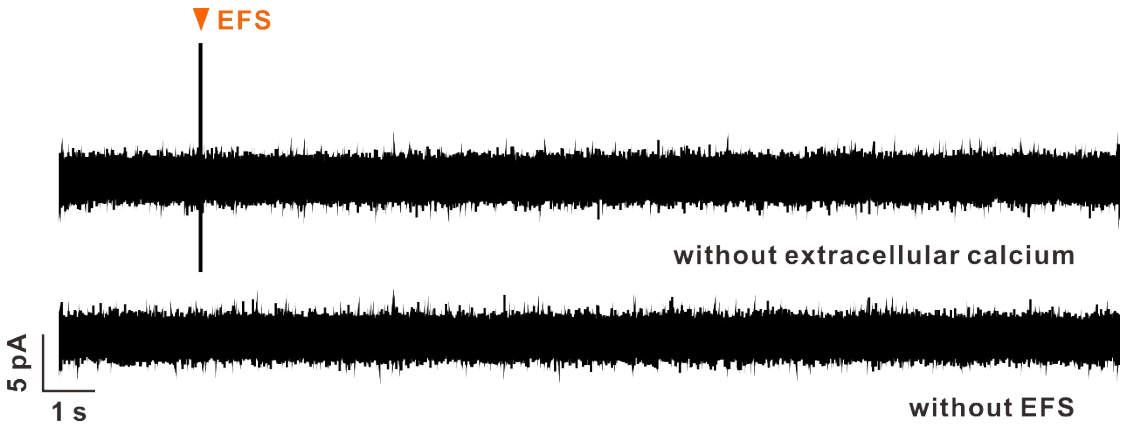

Figure S7. Representative amperometric trace showing no exocytotic events happened inside a DAergic synaptic cleft without extracellular calcium or EFS, respectively.

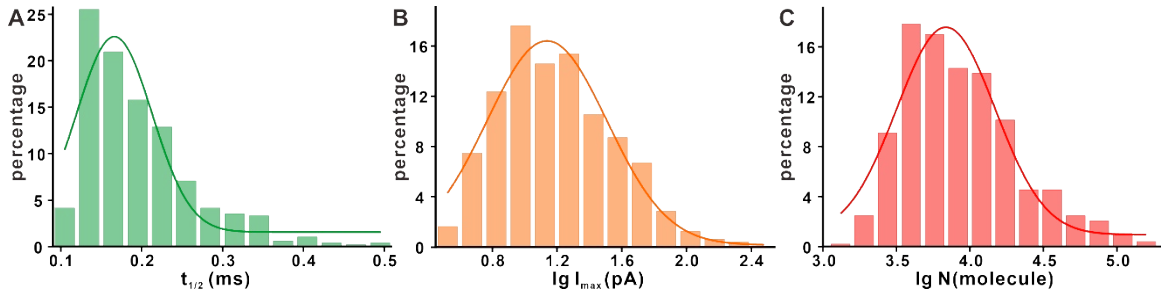

Figure S8. Normalized frequency histograms describing the distribution of time width at half maximum, $t_{1/2}$ (A), peak amplitude, I_{max} (B), and the number of DA molecules, N (C) of simple events (n = 494 from 12 cells) after one-time EFS inside a DAergic synaptic cleft.

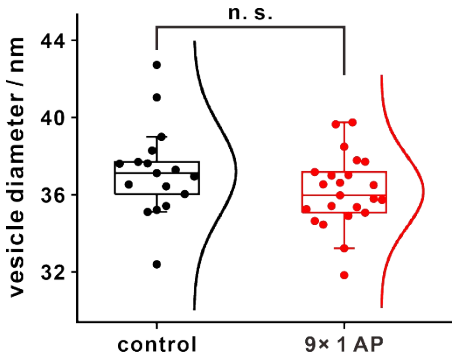

Figure S9. Statistical analysis of vesicle diameter from TEM images of VTA synapses before or after 9 times EFS, where each dot means average diameter of vesicles in one synapse; n_{cell} = 17 in control group and n_{cell} = 23 in experimental group, n.s.: no significant.

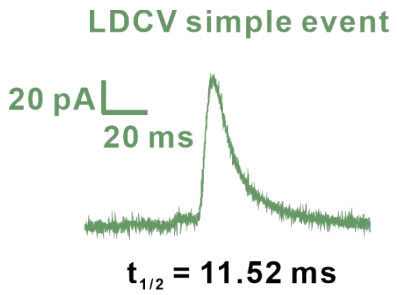

Figure S10. Typical spike of simple LDCV exocytosis event from rat adrenal chromaffin cell detected by carbon fiber microelectrode.

Table S1. Event parameters of DCVs amperometrically detected inside synapse under repetitive stimuli, where stimuli time means in which trace DCV was observed, I_{max}, $t_{1/2}$, N mean peak amplitude, distribution of time width at half maximum and number of released DA molecules of DCV-like long-duration simple events, respectively.

	Stimuli time	I_{max} **(pA)**	$t_{1/2}$ **(ms)**	***N***
synapse 1	13	42.3	0.60	92830
synapse 7	4	11.5	0.63	44560
synapse 8	6	28.3	1.14	122300
	6	46.2	2.62	509700
synapse 9	10	8.2	2.71	119600
synapse 10	9	46.2	0.73	124000