Supporting Information

Combined electrochemistry and mass spectrometry to interrogate the mechanism of action of modafinil, a cognition-enhancing drug, at the cellular and sub-cellular level

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Material and Methods

Materials, reagents and solutions

Modafinil and all other chemicals were of analytical grade and purchased from Sigma–Aldrich, unless otherwise stated. The HEPES physiological saline with the final osmolality of 310 mOsm/kg contained NaCl (150 mM), KCl (5 mM), $MgCl_2$ (1.2 mM), glucose (5 mM), HEPES (10 mM), $CaCl_2$ (2 mM), pH 7.4. Stimulation of the cells was fulfilled by a solution containing NaCl (55 mM), KCl (100 mM), $MgCl_2 \cdot 6H_2O$ (1.2 mM), $CaCl_2$ (2.0), glucose (5.0 mM), HEPES (10 mM), pH 7.4. All solutions were prepared in MQ water produced from a Purelab Classic purification system (ELGA, Sweden), and were filtered (0.2 μm) prior to use. T-25 flasks (vented, polystyrene, Falcon) were purchased from Fisher Scientific, Sweden.

Cell culture and treatment

PC12 cells were obtained as a gift from Lloyd Greene (Columbia University). The cells were grown in RPMI-1640 media (PAA Laboratories, Inc. Australia) supplemented with 10% donor equine serum (PAA Laboratories) and 5% fetal bovine serum Gold (PAA Laboratories). The cells were cultured on mouse collagen coated cell culture flasks (collagen type IV, BD Biosciences, Bedford, MA) in a 100% humidified incubator at 37 °C and 7% CO_2 and were sub-cultured every 7-9 days. The media was replaced every 2 days throughout the lifetime of all cultures.

Modafinil solutions at four different concentrations (0, 1, 10, or 100 μM in 0.2 % DMSO:RPMI 1640 media) were prepared for the treatment of PC12 cells. For electrochemical experiments, PC12 cells were sub-cultured on mouse collagen coated culture dishes (type IV, BD Biosciences, Bedford, MA) 4−6 days before the experiment and cell media were replaced every day. The cells were incubated with a predetermined concentration of modafinil for 3 h before experiments. The incubation time of 1 h was also tested, but the changes observed were

insignificant. Prior to single-cell analysis, the media was rinsed three times and replaced with HEPES physiological saline. The experiments were repeated with 3 generations of cells.

Electrode fabrication

The carbon fiber micro-disk electrode was fabricated as previously described (Li et al., 2016). Briefly, carbon fiber with 5.0-μm diameter was aspirated into a borosilicate capillary (1.2 mm O.D., 0.69 mm I.D., Sutter Instrument Co., Novato, CA, U.S.A.). Then the capillary was pulled by a micropipette puller (Model P-1000, Sutter Instruments Co., Novato, CA, U.S.A.) and the carbon fiber was cut at the glass junction. The tip was immersed in a solution of epoxy (Epoxy Technology, Billerica, MA, U.S.A) to seal the gap between the carbon fiber and glass. The glued electrodes were treated in an oven at 100°C overnight to complete the sealing step. The sealed electrodes were beveled at 45° angle (EG-400, Narishige Inc., London, UK). To fabricate the nanotip electrode 50 to 100 μm of the aspirated fiber extending from the glass was cut and etched in the flame (for less than 2 s) to obtain a needle-sharp fiber tip. Prior to using the electrodes for the main experiments, the individual electrode's response was tested by investigating the cyclic voltammogram (-0.2 to 0.8 V vs. Ag|AgCl, at 100 mV/s) of the electrode in a solution of dopamine (100 μM, in PBS, pH 7.4). Only electrodes showing good reaction kinetics and stable steady-state diffusion limited currents were used for the experiment.

Electrochemical experiments

Prior to amperometric (SCA and IVIEC) experiment, cells were rinsed by replacing the RPMI medium of the cell culture dish with a pre-warmed (37 °C) HEPES physiological saline. The cells remained at 37°C on the microscope stage during the entire experiment. An inverted microscope (IX81, Olympus, Japan), located in a Faraday cage, was employed to perform the

electrochemical experiments of the single PC12 cells. Amperometric traces were recorded by an Axon 200B potentiostat (Axopatch 200B, Molecular Devices, Sunnyvale, CA) with a working potential of +700 mV versus a homemade Ag/AgCl reference electrode positioned nearby in the surrounding buffer solution. SCA, investigating the exocytosis dynamic, was performed using a carbon fiber micro-disk electrode (Figure S1A). The electrode was gently settled on the membrane of a PC12 cell by a Patch-Clamp Micromanipulator (PCS-5000, Burleigh Instruments, Inc., USA) to avoid damaging the cell membrane. The target cell was stimulated once by a 5 s injection pulse (20 psi) of K^+ stimulating solution, causing docked vesicles to fuse to the cell membrane and a fraction of vesicular catecholamine content to be released and oxidized at the surface of micro-disk electrode (Figure S1B). For this purpose, a glass micropipette containing K^+ stimulating solution, was coupled to a microinjection system (Picospritzer II, General Valve Corporation, Fairfield, NJ), and placed in 40 μm distance from the cell. The current from catecholamine oxidation results in an amperometric spike (Figure S1E) a trace of spikes is shown in Figures S1C and S1D. After establishing a spike baseline, the spike parameters can be evaluated. In Figure S1E the area of the spike above the baseline gives the total charge transferred (Q), and the number of transmitter molecules (N) can be calculated based on the Faraday's law: $N = Q/nF$, where F is Faraday constant (96480 coulomb/mole) and n is the number of electrons donated by oxidized catecholamine ($2e$). To obtain further information about the influence of modafinil on the dynamics of exocytosis, peak characteristics of individual spikes including the spike amplitude (I_{max}, the maximum current), the spike width ($t_{1/2}$, the width of the amperometric peak at 50% of amplitude), the spike rising phase (t_{rise}, the time from 25% to 75% of the maximum amplitude at the rising part of the peak), and the spike falling phase (t_{fall}, the time from 75% to 25% of the maximum amplitude at the falling region of the peak) can be used.

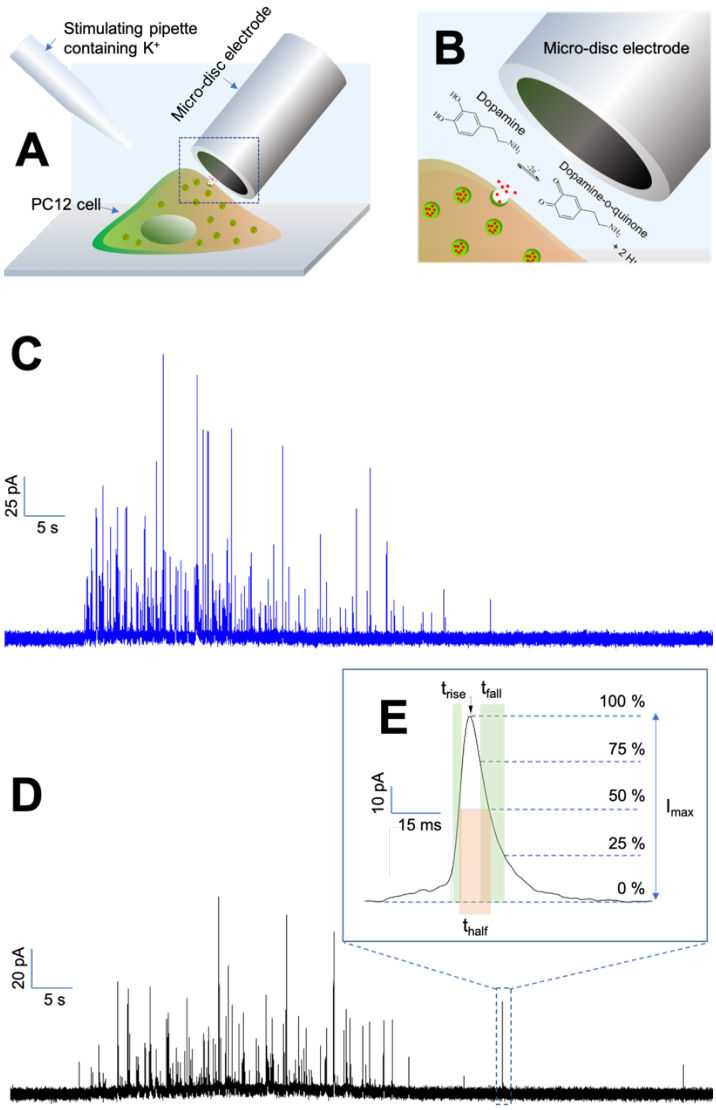

Figure S1. (A) SCA technique schematic; (B) Oxidative reaction of dopamine at the surface of a micro-disc electrode during exocytosis; (C) a typical amperometric trace for exocytosis events at a single PC12 cell; (D) a typical amperometric trace for exocytosis events at a modafinil-treated single PC12 cell; (E) a typical transient spike and its parameters.

Amperometric data acquisition, processing and analysis

The signal output was filtered at 2 kHz with a 4-pole Bessel filter and digitized at 10 kHz with a Digidata model 1440 A (Molecular Devices, CA, USA) and Axoscope 10.4 software (Axon Instruments Inc., Sunnyvale, CA, U.S.A). The amperometric traces were processed in IgorPro 6.22 (Wavemetrics, Lake Oswego, OR). A binomial smoothing filter (1 kHz) was applied for the current. The limit of peak detection was set to five times the standard deviation of the noise (I_{max} ~ 2 pA). The traces were manually inspected after peak detection and false peaks were

manually rejected. The individual spikes parameters (number of molecules (N), I_{max}, t_{rise}, t_{half}, and t_{fall}) in each trace were pooled and the median of each parameter was calculated. Each treatment result was compared with control by a two-tailed Mann-Whitney rank-sum test, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

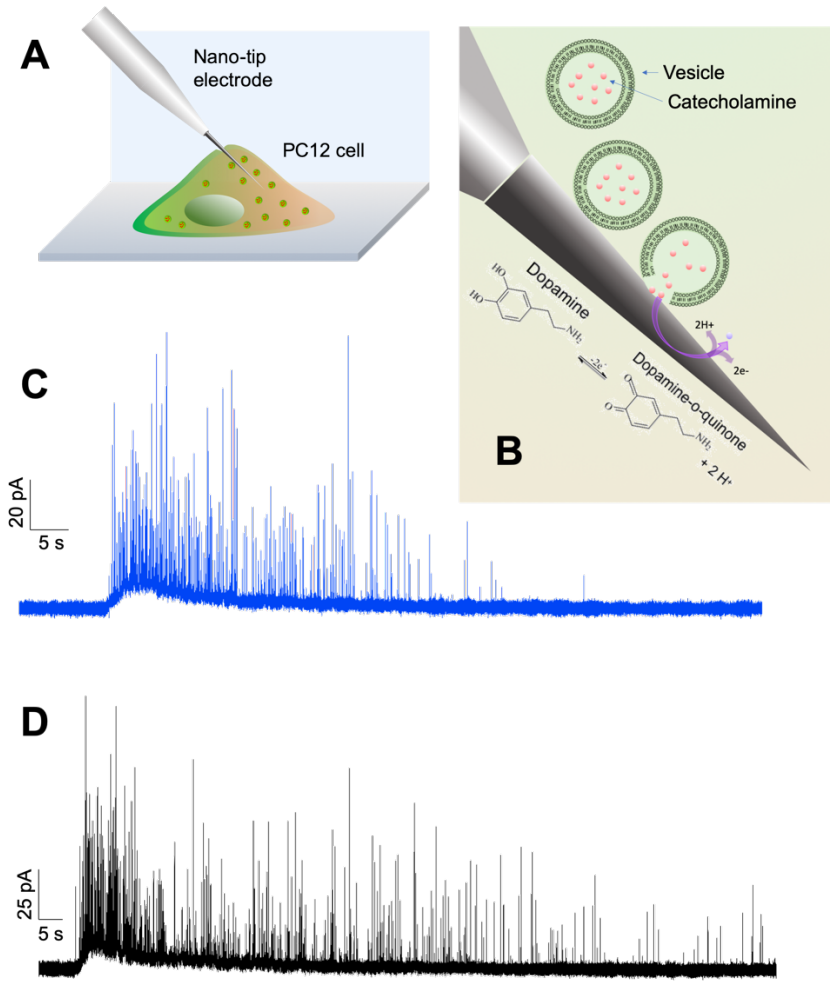

Figure S2. (A) IVIEC technique schematic; (B) Oxidative reaction of dopamine at the surface of nano-tip electrode during electroporation of vesicle; (C) A typical amperometric trace of vesicle content in a control PC12 cell; (D) A typical amperometric trace of vesicle content in a modafinil-treated cell.

Sample preparation for the SIMS experiments

For SIMS experiments, PC12 cells were grown onto a poly-D-lysine-coated silicon wafer. After growing for 3 days, cell medium was removed and the cells were incubated in the 0.2 % DMSO:RPMI 1640 media containing different concentrations of modafinil as mentioned above. After treatment, the cells grown on silicon wafers were rinsed with warm 150 mM ammonium acetate pH 7.4 (37 ℃) for three time to remove cell medium and salts. The silicon wafers were fast-frozen in the pre-cooled propane and thereafter freeze-dried. The samples were introduced into the TOF-SIMS chamber for analysis. The experiments were repeated with 3 generations of cells.

Mass spectrometry imaging

The ToF-SIMS analysis was performed by a TOF.SIMS V instrument (ION-TOF GmbH, Münster, Germany) equipped with a 25 keV bismuth liquid metal ion gun as a primary ion source. The data were recorded in the positive and negative ion modes, and the spectra were acquired using a Bi_3^+ ion beam. The pulsed ion current was 0.3 pA with a maximum ion dose density below the static limit. The high current bunched mode was used to obtain high mass resolution with a measured mass resolution of 5000 at *m/z* 58. An analysis area with 500 × 500 μm^2 was scanned on the sample surface to acquire enough cells.

All TOF-SIMS spectra and images were recorded, processed and analyzed by the SURFACELAB 6 software (version 6.3, ION-TOF GmbH). The mass spectra were internally calibrated to the fragmental peaks of CH_3^+, $C_2H_5^+$, $C_3H_7^+$ and $C_5H_{15}PNO_4^+$ for the positive ion mode and C^-, C_2^-, C_3^-, C_4^- for the negative ion mode. The mass spectra of regions of interest (ROI) comprising cells were extracted to remove the interferences of substrate. To calculate the relative abundance, the intensity of individual peaks was normalized to the number of selected pixels and the total intensity of the peaks in the range of m/z 20-200.

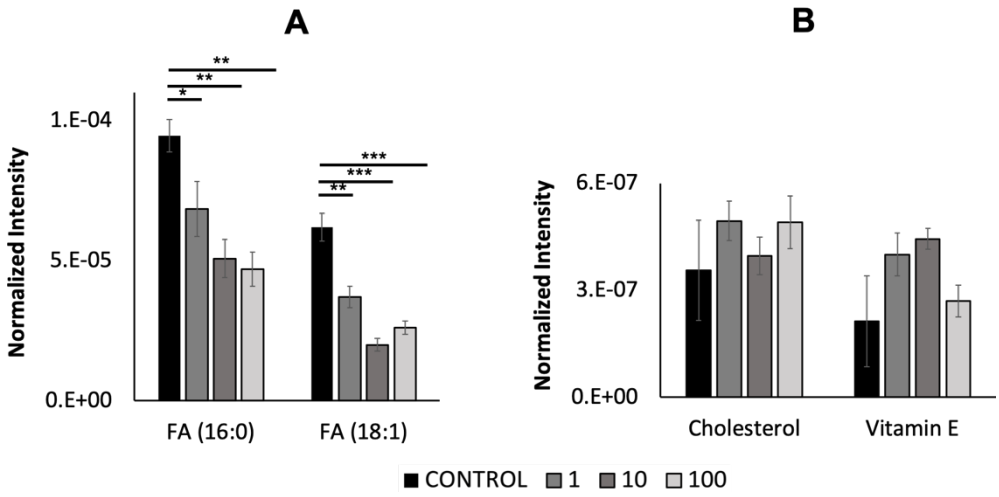

Figure S3. (A) Normalized intensity for the negative lipid head groups at m/z 255.2 for FA (16:0) and m/z 281.2 for FA (18:1); (B) normalized intensity for the positive lipid head groups at m/z 369.3 for cholesterol and m/z 430.4 for vitamin E. Each treatment result was compared with control by a two-tailed Mann-Whitney rank-sum test, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

References

Li X, Dunevall J and Ewing AG (2016) Using Single-Cell Amperometry To Reveal How Cisplatin Treatment Modulates the Release of Catecholamine Transmitters during Exocytosis. *Angew. Chem. Int. Edit.* **128**, 9187-9190.