Supporting Information

Single-Vesicle Electrochemistry Following Repetitive Stimulation Reveals a Mechanism for Plasticity Changes with Iron Deficiency

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Experimental Section

Chemicals

All components of the isotonic solution (150 mM NaCl, 5 mM KCl, 1.2 mM MgCl₂, 2 mM CaCl₂, 5 mM glucose and 10 mM HEPES), the 100 mM K⁺ stimulation solution (55 mM NaCl, 100 mM KCl, 1.2 mM MgCl₂, 2 mM CaCl₂, 5 mM glucose and 10 mM HEPES), and the phosphate-buffered saline (PBS) solution (3.0 mM KCl, 10.0 mM NaH₂PO₄, 2.0 mM Na₂SO₄, 1.2 mM MgCl₂, 131.25 mM NaCl, and 1.2 mM CaCl₂) were purchased from Sigma-Aldrich (Stockholm, Sweden). The pH of all solutions was adjusted to 7.4 using concentrated HCl or NaOH. All aqueous solutions were prepared using 18 MΩ.cm Milli-Q water from Purelab Classic purification (ELGA, Sweden) and filtered using vacuum filtration system with a membrane pore size of 0.45 µm (VWR, Sweden).

A 10 mM stock solution of dopamine (Sigma-Aldrich, Sweden) was prepared in 0.1 M HClO₄ and stored at -20 °C. A 100 µM dopamine solution was prepared daily in PBS buffer for testing electrodes. 12.5 mM stock solutions of deferoxamine mesylate (DFOM) salt (Sigma-Aldrich, Sweden) and ferric ammonium citrate (FAC) (Sigma-Aldrich, Sweden) were prepared in water and stored at -20 °C. 100 µM DFOM salt solution and 100 µM FAC solution were prepared daily in RPMI 1640 medium supplemented with 10% donor horse serum and 5% fetal bovine serum.

Cell culture

PC12 cells were received as a gift from Lloyd Greene at the Columbia University. They were maintained in RPMI 1640 medium (Sigma-Aldrich, Sweden or Gibco, Fisher Scientific, Sweden) supplemented with 10% donor horse serum (Sigma-Aldrich, Sweden) and 5% fetal bovine serum (Sigma-Aldrich, Sweden) in a 100% humidified incubator with 7% CO₂ at 37 °C. The cells were grown on collagen type IV coated cell culture flasks (Corning BioCoat, Fisher Scientific, Sweden) and sub-cultured every 7 days. The medium was replaced every 2 days. For single cell experiments, cells were sparsely seeded on 60 mm collagen type IV coated cell culture dishes (Corning BioCoat, Fisher Scientific, Sweden) for 4 days prior to the experiments. Cells were treated with 100 µM DFOM for 24 h for iron deficiency studies or 100 µM DFOM for 24 h followed by 24 h rescue with treatment of 100 µM FAC for iron repletion studies. Before the experiments, the medium was removed, and the cells were washed 3 times with isotonic solution and kept in isotonic solution during the entire experiments.

Calcium imaging

PC12 cells were seeded on Ibidi μ-slide 8 well plates (Ibidi GmbH, Germany) for calcium imaging experiments. The fluorescence dye used for calcium imaging was Fluo-4. To prepare the Fluo-4 stock solution, 100 µL dimethyl sulfoxide (DMSO) was added to one vial of Fluo-4 (50 µg, Invitrogen, Fisher Scientific, Sweden). Before incubation with the fluorescence dye, cell medium was removed from each well and isotonic solution was used to wash the cells. Fluo-4 incubation was carried out by adding the Fura-4 stock solution to the isotonic solution to reach a final concentration of 0.4 µM. The cells were then incubated for 20 min at 37 °C. Isotonic solution was used to wash the cells to remove the fluorescence dye and the cells were kept in the isotonic solution during the entire experiments. Calcium imaging was performed using an LSM 700 laser scanning microscopy (Zeiss GmbH, Jena, Germany) equipped with a Plan-Apochromat 20x objective (numerical aperture (NA) = 0.8). Fluo-4 was excited at the wavelength of 488 nm and emission signals over 500 nm were collected. After a baseline of 10 s, exocytosis was triggered by injecting the 100 mM K⁺ stimulation solution and the total recording time was 90 s. Images were acquired with Zen microscope software (Zeiss) and the analysis was done in the software by
choosing the cells as regions of interest. The average calcium changes in cells in different groups were analyzed using Matlab (The MathWorks Inc.).

Fabrication of Carbon Fiber Electrodes and Electrochemical Measurements

Carbon-fiber electrodes were fabricated by aspirating a 5 µm-diameter carbon fiber into a glass capillary (O.D.: 1.2 mm, I.D.: 0.69 mm, 10 cm length, Sutter Instrument Co., Novato, CA) and pulling it into two separate electrodes with a vertical micropipette puller (model PE-21, Narishige, Inc., Japan). For disk electrodes, the extended carbon fiber was cut with a scalpel to the edge of the glass and the fiber/glass interface was sealed with epoxy (G A Lindberg ChemTech AB, Sweden). Electrodes were cured at 100 °C overnight and were then beveled with a commercial beveller (EG-400, Narishige Inc., London, UK) at a 45° angle. For nanotip electrodes, the extended carbon fiber was cut to a length of 100-150 µm and then flame etched with a butane gas burner (Clas Ohlson, Sweden) to obtain a thin needle shape tip with 50-100 nm diameter and 30-100 µm length. The electrodes were then sealed with epoxy. Excess epoxy was rinsed with acetone and the electrodes were subsequently cured at 100 °C overnight. Both disk and nanotip electrodes were tested with cyclic voltammetry (-0.2 V to 0.8 V versus Ag/AgCl, 100 mV/s) in 100 µM dopamine solution. Electrodes that showed good response to dopamine and stable steady-state currents were used for the experiments.

Amperometry was used to detect exocytotic release and vesicular content. Electrochemical measurements were performed on or in single cells on an inverted microscope (IX81 or IX71, Olympus). The electrodes were held at a constant potential of +700 mV versus an Ag/AgCl reference electrode with an Axopatch 200B potentiostat (Molecular Devices, Sunnyvale, CA, USA). The signal output was filtered at 2 kHz and digitized at 5 kHz. Exocytosis was stimulated with 100 mM K+ stimulation solution using a microinjection system (Picospritzer II, General Valve Corporation, Fairfield, NJ, USA) and each injection was triggered for 5 s with a 20-psi injection pulse.

Data Analysis and Statistics

All data were converted in Matlab (The MathWorks Inc.) and analyzed with an Igor Pro 6.7 script (from David Sulzer lab, Columbia University). A 1-kHz (binomial sm.) filter was applied to all amperometric traces and the threshold for peak detection was 3 times the standard deviation of the noise. Amperometric traces were inspected manually after peak selection to avoid false positives. All statistics were performed in GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA) with Wilcoxon matched-pairs signed rank test or unpaired Mann-Whitney rank sum test. Statistical significance was designated at p < 0.1 and all data are presented as mean of median ± SEM.
Fig. S1. Average number of molecules released per exocytotic event as observed by SCA for 4 repetitive stimulation. (A) Control cells: number of molecules released during exocytosis is significantly increased after 3 and 4 repetitive stimulation (p = 0.0017 and p = 0.070, respectively) in control cells. (B) DFOM-treated cells: number of molecules released during exocytosis is significantly decreased after 4 repetitive stimulation (p = 0.010), opposite to control. (C) DFOM + FAC treated cells: number of molecules released during exocytosis is not significantly different during repetitive stimulation. Pairs of data sets were compared with a Wilcoxon matched-pairs signed rank test. n > 18 cells.

Fig. S2. Average number of exocytotic events for 4 repetitive stimulation obtained by SCA from (A) control cells, (B) DFOM treated cells, and (C) DFOM + FAC treated cells. Pairs of data sets were compared with a Wilcoxon matched-pairs signed rank test. n > 18 cells. *p < 0.1, **p < 0.01, ***p < 0.001, and ****p < 0.0001. p values are listed in Table S10.
Fig. S3. Average intracellular calcium levels before, during and after the 1st stimulation in control cells (red), DFOM treated cells (black), and DFOM+FAC treated cells (blue). Cells were stimulated with 100 mM K⁺ stimulation solution for 10 s after the measurement started. n > 27 cells were imaged for each condition and the shaded areas around the lines represent SEM.

Fig. S4. Normalized average number of IVIEC events without stimulation. The number of IVIEC events were used to estimate the number of intracellular vesicles. Significant reductions of the number of vesicles were overserved in iron-deficient cells (p < 0.0001) and iron depleted cells, compared to the control (p = 0.053). Number of IVIEC events was significantly increased in iron-depleted cells compared to the iron-deficient cells (p = 0.036). Pairs of data sets were compared with a Mann-Whitney rank-sum test. n > 18 cells. *p < 0.1, **p < 0.01, ***p < 0.001, and ****p < 0.0001.
Table S1. p values for comparison of the number of molecules released during exocytosis and stored in vesicles obtained by SCA and IVIEC, respectively, from control, DFOM, and DFOM+FAC-treated cells.

| Number of molecules       | Control | DFOM   | DFOM+FAC |
|---------------------------|---------|--------|----------|
| Control (SCA)             | x       | 0.023  | 0.72     |
| DFOM (SCA)                | x       | 0.037  | (*)      |
| DFOM+FAC (SCA)            | x       |        | (*)      |
| Control (IVIEC)           | x       | 0.060  | (*) 0.39 |
| DFOM (IVIEC)              | x       | 0.36   |          |
| DFOM+FAC (IVIEC)          | x       |        |          |

Table S2. p values for comparison of the number of molecules released during exocytosis obtained from SCA for the 1st and the 4th repetitive stimulation from control, DFOM, and DFOM+FAC-treated cells.

| Number of molecules       | Control (1) | Control (4) | DFOM (1) | DFOM (4) | DFOM+FAC (1) | DFOM+FAC (4) |
|---------------------------|-------------|-------------|----------|----------|--------------|--------------|
| Control (1)               | X           | 0.070 (*)   | 0.023 (*)| 0.72     |              |              |
| Control (4)               | 0.012 (*)   |            | 0.037 (*)| 0.30     |              |              |
| DFOM (1)                  | X           | 0.0090 (**)| 0.30     | 0.24     |              |              |
| DFOM (4)                  | X           | 0.30       |          | 0.71     |              |              |
| DFOM+FAC (1)              | X           | 0.24       |          | 0.71     |              |              |
| DFOM+FAC (4)              |             |            | X        |          |              |              |
Table S3. p values for comparison of the number of molecules stored in vesicles obtained by IVIEC without stimulation and after 3 repetitive stimulation from control, DFOM, and DFOM+FAC-treated cells.

| Stimulation number | Control (no stim.) | Control (after 3 stim.) | DFOM (no stim.) | DFOM (after 3 stim.) | DFOM+FAC (no stim.) | DFOM+FAC (after 3 stim.) |
|--------------------|-------------------|------------------------|-----------------|----------------------|---------------------|-------------------------|
| Control (no stim.) | X                 | 0.025 (*)              | 0.060 (*)       | 0.39                 |                     |                         |
| Control (after 3 stim.) | X             |                        | 0.0007 (***)    | 0.012 (*)            |                     |                         |
| DFOM (no stim.)    |                   |                        | 0.89            | 0.36                 |                     |                         |
| DFOM (after 3 stim.) |                 |                        | X              | 0.65                 |                     |                         |
| DFOM+FAC (no stim.) |                   |                        |                 | X                    | 0.68                |                         |
| DFOM+FAC (after 3 stim.) |               |                        |                 |                      |                     | X                       |

Table S4. P values for comparison of $I_{\text{max}}$ obtained from SCA for the 1$^{\text{st}}$ and the 4$^{\text{th}}$ repetitive stimulation from control, DFOM, and DFOM+FAC-treated cells.

| Stimulation number | $I_{\text{max}}$ | Control (1) | Control (4) | DFOM (1) | DFOM (4) | DFOM+FAC (1) | DFOM+FAC (4) |
|--------------------|-----------------|-------------|-------------|----------|----------|--------------|---------------|
| Control (1)        | X               | 0.23        | 0.86        | 0.10     |          |              |               |
| Control (4)        |                 | X           |             | 0.098 (*) | 0.10     |              | 0.94          |
| DFOM (1)           |                 |             | X           | 0.019 (*) | 0.058 (*) |              |               |
| DFOM (4)           |                 |             |             | X        | 0.12     |              |               |
| DFOM+FAC (1)       |                 |             |             | X        | 0.47     |              |               |
| DFOM+FAC (4)       |                 |             |             |          | X        |              |               |
### Table S5. p values for comparison of $t_{\text{half}}$ obtained from SCA for the 1$^{\text{st}}$ and the 4$^{\text{th}}$ repetitive stimulation from control, DFOM, and DFOM+FAC-treated cells.

| Stimulation number | $t_{\text{half}}$ | Control (1) | Control (4) | DFOM (1) | DFOM (4) | DFOM+FAC (1) | DFOM+FAC (4) |
|--------------------|------------------|-------------|-------------|----------|----------|-------------|-------------|
| Control (1)        | X                | 0.0027 (**) | 0.88        | 0.45     |          |             |             |
| Control (4)        |                  | X           | 0.045 (*)   | 0.63     |          |             |             |
| DFOM (1)           |                  | X           | 0.019 (*)   | 0.69     |          |             |             |
| DFOM (4)           |                  | X           | X           | 0.081 (*)|          |             |             |
| DFOM+FAC (1)       |                  |             | X           | 0.47     |          |             |             |
| DFOM+FAC (4)       |                  |             | X           | X        |          |             |             |

### Table S6. p values for comparison of $t_{\text{rise}}$ obtained from SCA for the 1$^{\text{st}}$ and the 4$^{\text{th}}$ repetitive stimulation from control, DFOM, and DFOM+FAC-treated cells.

| Stimulation number | $t_{\text{rise}}$ | Control (1) | Control (4) | DFOM (1) | DFOM (4) | DFOM+FAC (1) | DFOM+FAC (4) |
|--------------------|------------------|-------------|-------------|----------|----------|-------------|-------------|
| Control (1)        | X                | 0.0083 (**) | 0.34        | 0.97     |          |             |             |
| Control (4)        |                  | X           | 0.67        | 0.72     |          |             |             |
| DFOM (1)           |                  | X           | 0.49        | 0.42     |          |             |             |
| DFOM (4)           |                  | X           |             | 0.51     |          |             |             |
| DFOM+FAC (1)       |                  |             | X           | 0.20     |          |             |             |
| DFOM+FAC (4)       |                  |             | X           | X        |          |             |             |
Table S7. p values for comparison of \( t_{fall} \) obtained from SCA for the 1\textsuperscript{st} and the 4\textsuperscript{th} repetitive stimulation from control, DFOM, and DFOM+FAC-treated cells.

| Stimulation number | \( t_{fall} \) | \( t_{fall} \) | \( t_{fall} \) | \( t_{fall} \) | \( t_{fall} \) |
|--------------------|----------------|----------------|----------------|----------------|----------------|
|                    | Control (1)    | Control (4)    | DFOM (1)       | DFOM (4)       | DFOM+FAC (1)   | DFOM+FAC (4)   |
| Control (1)        | X 0.058 (*)    | 0.23           | 0.22           |                |                |
| Control (4)        |                | X 0.40         |                | 0.54           |                |
| DFOM (1)           |                |                | X 0.35         | >0.99          |                |
| DFOM (4)           |                |                |                | X 0.97         |                |
| DFOM+FAC (1)       |                |                |                | X 0.13         |                |
| DFOM+FAC (4)       |                |                |                |                | X              |

Table S8. p values for comparison of the average number of exocytotic events obtained from SCA for the 1\textsuperscript{st} and the 4\textsuperscript{th} repetitive stimulation from control, DFOM, and DFOM+FAC-treated cells.

| Stimulation number | Number of exocytotic events | Number of exocytotic events | Number of exocytotic events | Number of exocytotic events | Number of exocytotic events |
|--------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|                    | Control (1)                 | Control (4)                 | DFOM (1)                    | DFOM (4)                    | DFOM+FAC (1)                | DFOM+FAC (4)                |
| Control (1)        | x <0.0001 (***)             | <0.0001 (***)               | 0.050 (*)                   |                |                |
| Control (4)        | x                           | 0.0027 (***)                | 0.23                        |                |                |
| DFOM (1)           | x                           | <0.0001 (***)               | 0.0086 (***)                |                |                |
| DFOM (4)           | x                           |                             | 0.019 (*)                   |                |                |
| DFOM+FAC (1)       | x                           |                             | <0.0001 (***)               |                |                |
| DFOM+FAC (4)       |                             |                             |                             | x              |                |
Table S9. p values for comparison of the fraction of release for the 1\textsuperscript{st} and the 4\textsuperscript{th} repetitive stimulation from control, DFOM, and DFOM+FAC-treated cells.

| Stimulation number | Fraction of release | Stimulation number |  
|--------------------|---------------------|---------------------|
|                   | Control (1)         | Control (4)         | DFOM (1) | DFOM (4) | DFOM+FAC (1) | DFOM+FAC (4) |
| Control (1)       | x 0.029 (*)&        | 0.49                | 0.029    | (*)      | 0.029        | (*)          |
| Control (4)       | x                   |                      | 0.029    | (*)      | 0.029        | (*)          |
| DFOM (1)          | x                   | 0.029 (*)&           | 0.49     |          |              |              |
| DFOM (4)          | x                   |                      |          | 0.057    | (*)          |              |
| DFOM+FAC (1)      | x                   |                      |          |          | 0.49         |              |
| DFOM+FAC (4)      | x                   |                      |          |          |              |              |

Table S10. p values for comparison of the average number of exocytotic events for 4 repetitive stimulation obtained from SCA from control, DFOM, and DFOM+FAC-treated cells.

| Stimulation number | Number of exocytotic events |
|--------------------|----------------------------|
|                   | 1   | 2   | 3   | 4   |
| Control 1         | x   | 0.0012 (**) | <0.0001 (****) | <0.0001 (****) |
| Control 2         | x   | 0.025 (*)   | 0.0020 (**)    |
| Control 3         | x   | 0.018 (*)   |
| Control 4         | x   |   |   |   |
| DFOM 1            | x   | 0.011 (*)   | 0.0004 (**)    | <0.0001 (****) |
| DFOM 2            | x   | 0.38        | 0.0010 (**)    |
| DFOM 3            | x   | 0.013 (*)   |
| DFOM 4            | x   |   |   |   |
| DFOM+FAC 1        | x   | 0.012 (*)   | 0.0001 (****)  | <0.0001 (****) |
| DFOM+FAC 2        | x   | 0.0005 (***)| <0.0001 (****) |
| DFOM+FAC 3        | x   | 0.0062 (**) |
| DFOM+FAC 4        | x   |   |   |   |