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pHluorin assays, analysis, and fluorescence microscopy

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ABSTRACT

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Vesicle recycling measurements

1 Neurons expressing sypHy were imaged at 12-14 DIV. Experiments were conducted in standard extracellular solution containing (in mM): NaCl 150, KCl 3,Glucose 20, HEPES 10, $CaCl_2$ 2, $MgCl_2$ 3, pH adjusted to 7.35. To block recurrent network activity, experiments were conducted in the presence of 10µM DNQX (6,7-Dinitroquinoxaline-2,3 (1H,4H-dione, Merck) and 50µM APV (DL-2-Amino-5-phosphonopentanoic acid, Merck). After each experiment, the bath was perfused with saline in which 50 mM NaCl was replaced with NH_4Cl to visualize the total vesicle population.

2 For imaging, cultured neurons were placed in a stimulation chamber between parallel platinum wires (RC-49MFSH, Warner Instruments). Stimulation (300 bipolar pulses of 10 V/cm, each of a duration of 1 µs, at 20 Hz for 15 s), was delivered using a high-power stimulus-isolation unit (SIU-102B, Warner Instruments) driven by an isolated pulse-stimulator (2100, A-M Systems). 50 images were obtained (43 at 0.2 Hz and then 7 images at 0.125 Hz) per experiment. At least 30 synaptic regions of interest (ROIs) were analyzed in each case. The baseline sypHy fluorescence (F_0) in each synapse was the average value measured in 6 pre-stimulation images. The fluorescence increment at time t [$DF(t)=F(t)-F_0$] was normalized by the baseline value for each synapse. Synaptic $DF(t)/F_0$ values were averaged across synapses in each experiment (shown as symbols in bar-chart graphs). These were averaged to obtain mean values for each experimental condition. Non-responding synaptic puncta were excluded. Experiments were performed using at least three independent cultures on different days. To confirm the presence of h-α-syn-mCherry and tagBFP-synapsins, fluorescent-tagged proteins were imaged before each experiment. All pHluorin assays were performed at room temperature. Fluorescence measurements were performed on a Nikon TiE inverted microscope driven by the NIS-elements software package (Nikon). The microscope was equipped with an Andor Neo 5.5 sCMOS camera (Oxford Instruments), a 40X 0.75 NA Plan Fluor objective, EGFP and Cy3 filter cubes (TE-series, Chroma), BFP, mCherry and Cy5 filter cubes (Semrock), and a perfect-focus mechanism (Nikon).

Quantification of endocytosis rates

3 Endocytosis rates were assessed based on the decay of sypHy fluorescence after the termination of stimulation. Data were fit with a single-exponential decay-function (32 data points, 160 seconds) starting 5 seconds after stimulation cessation. The function is:
(eq. 1)

$$y = y_0 + Ae^{\frac{-t}{\tau}},$$

where A is an amplitude, y_0 is an offset and t is the time constant,assuming stimulation starts at t=0 for all traces.Fit results were discarded if t was longer than 160 seconds (the duration of the data being fit).

Measurement of the recycling pool relative size

4 The relative size of the recycling pool was calculated based on imaging of cumulative exocytosis. Cumulative exocytosis was achieved by blocking SV reacidification by adding 1 µM bafilomycin A1 (Enzo Life Sciences Cat# BML-CM110-0100) to the bathing medium itemized above. Neurons were imaged at 0.2 Hz throughout the experiment. 6 baseline images were acquired, and stimulation was applied at t=0 for 2 minutes at 20 Hz (2400 action potentials), until saturation. The fluorescence of the total vesicle population (F_{max}) was measured at the end of each experiment by perfusing the chamber with NH_4Cl-saline.

Synaptic sypHy signals were measured from at least 30 ROIs as explained above, subtracting from each its mean baseline value and normalizing it by F_{max}.

The relative size of the recycling pool was defined as the ratio of the mean of the last three data points (at saturation, before NH_4Cl exposure) and F_{max}.

Evaluation of width of SV distribution

5 Neurons were fixed using 4% paraformaldehyde (EMS) in phosphate buffered saline (Biological Industries) for 10 minutes, washed thoroughly with PBS and permeabilized with PBS supplemented with 0.1% triton X100 (Merck) for 1 minute and washed three times. Blocking solution (PBS with 5% skim milk powder; Merck) was applied for 1 hour. The coverslips were incubated for 1 h with the indicated primary antibodies (see above) in blocking solution at RT, washed X3 and then incubated with secondary antibodies in blocking solution for 1 hour at RT. Finally, the preps were washed X3 and mounted using immumount (Thermo Scientific). Neurons were imaged using a 60X 1.4 NA oil-immersion Apochromat objective (Nikon). Linear profiles were drawn manually along axonal segments and through synaptic puncta in the vGlut1 channel using NIS elements (Nikon). The intensity profiles were imported into Origin (2022) (RRID:SCR_014212) http://www.originlab.com/index.aspx?go=PRODUCTS/Originand fit individually with Gaussian functions. The standard deviation parameter (s) of the fit was extracted, and the FWHM was calculated thus:

(eq. 2) $$FWHM = 2\sqrt{ln(4)}\sigma = \sim 2.355\sigma$$

Semi quantitative determination of synaptic fluorescence int...

6 Synaptic puncta were detected by an in-house thresholding algorithm in which the threshold is iteratively decreased, detected objects are filtered based on their area and roundness (>0.7), saved, and then blanked to not be chosen again. Subsequently, objects that the user judges by eye not to represent synaptic puncta, or those which are out of focus are removed manually. The peak fluorescence at the center-of-mass (2X2 pixels in size) in each punctum is recorded. Synaptic intensity values were averaged per image. All experimental conditions of fluorescence intensity experiments were performed and processed; in each imaging session, the mean intensity value of the control condition was used to normalize all recorded values, to reduce inter-session variability. Normalized intensity values were then averaged across sessions.

Measurement of synaptic enrichment

7 Synaptic enrichment was measured as described previously (4). Neurons were transduced at 5 DIV with either sypHy or sypHy-E-domain, h-α-syn-mCherry and soluble tagBFP as a measure of local volume. At 14 DIV, the neurons were fixed and immunostained with anti-vGlut1 antisera (secondary: NL-637) to visualize synaptic puncta. Analysis lines (at least 30) were drawn in each image, starting in the axon, through a synapse, and into the surrounding background. The intensity profiles corresponding to the h-α-syn-mCherry and tagBFP channels were fit with a Gaussian function to determine the axonal (F_{axon}) and synaptic (F_{syn}) intensity values of each color thus:

(eq. 3) $$F = F_{axon} + F_{syn}e^{\frac{(x-x_c)^2}{2w^2}},$$

where x_c is the center of the Gaussian (the synaptic center) and w is its width.

The percentage of synaptic enrichment ($E\%$) of h-α-syn-mCherry is defined thus:

(eq. 4) $$E\% = \left(\frac{F_{syn}(red)/F_{axon}(red)}{F_{syn}(blue)/F_{axon}(blue)} - 1\right) * 100$$