Moss et al., http://www.jgp.org/cgi/content/full/jgp.200910314/DC1

SUPPLEMENTAL MATERIALS AND METHODS

TRANSFECTIONS

Cells were plated 16 h before transfection (Table S1, Step 1). The mass of cDNA in Table S1 is for the vector pcDNA3.1(+)mGAT1. The wild-type mGAT1 plasmid differs in molecular weight from the empty pcDNA3.1(+) vector or the fluorescent mGAT1 constructs. The x axis of Fig. 1 (A–C) refers to the former, and equimolar amounts of the other plasmids were used. Before transfection, cDNA was diluted in DMEM in one tube to which PLUS reagent was subsequently added (Table S1, Step 2). In a second tube, Lipofectamine was diluted in DMEM (Table S1, Step 3). The tubes were briefly vortexed and incubated at room temperature for 15 min. The diluted Lipofectamine was subsequently added to the cDNA/PLUS reagent dilution and vortexed (Table S1, Step 4). The transfection mixes were incubated for an additional 15 min at room temperature while the pre-plated cells were washed once with DMEM to remove residual sera from the culture medium, and then placed in the appropriate volume of DMEM to perform the transfection (Table S1, Step 5). The cDNA/PLUS/Lipofectamine mixes were then added to the cells (Table S1, Step 6), which were placed in the incubator for 16 h. The transfection cocktail was then replaced by complete N2a medium. When cDNA quantity was the experimental variable, the amount transfected is stated in the relevant Results section. However, cell density and amount of Lipofectamine and PLUS reagents were kept constant for each size of culture dish/well; conditions remained within the manufacturer's recommended cDNA/transfectant ratio in any experiment. The α4YFP and/or β2CFP plasmids were transfected into N2a cells at levels of 250 ng per plasmid in a 35-mm dish. Equimolar amounts of the appropriate nonfluorescent constructs were included in transfections that used only one of the fluorescent nAChR subunits.

For GABA uptake experiments performed in HEK 293T cells, we plated 2×10^5 cells per well in 12-well plates precoated with PEI 16 h before transfection. Otherwise, the transfection was performed as for N2a cells.

[^{3}H]GABA UPTAKE ASSAY

Uptake assays were performed in 12-well plates 48 h after transfection. Culture medium was aspirated and cells were equilibrated for 10 min at room temperature in 1 ml Krebs-Ringer's (KRH) buffer containing the following (in mM): 130 NaCl, 1.3 KCl, 2.2 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 10 glucose, and 10 HEPES, pH 7.4 (Ramamoorthy et al., 1998). GABA uptake was initiated on aspiration of KRH buffer and the addition of 500 µl of 2.5 or 80 µM GABA at room temperature. 25 nM [^{3}H]GABA was included in 2.5 µM GABA solution, and 40 nM [^{3}H]GABA was included in the 80-µM GABA solutions. Uptake was terminated by four rapid washes with KRH plus GABA using the same total GABA concentration as used during the uptake step. Cells were lysed in 1 ml 2% SDS in PBS. [^{3}H]GABA accumulation was assayed by liquid scintillation spectrometry. Specific uptake was normalized to the total protein in each well as determined using the bicinchoninic acid protein assay.

Concentration–response experiments for the fluorescent mGAT1 constructs described in this paper were performed on N2a cells in 12-well plates transfected under the nonsaturating conditions as described above. GABA uptake was initiated on aspiration of KRH buffer and the addition of 500 µl of 1, 2.5, 5, 10, 30, or 80 µM GABA at room temperature. 25 nM [^{3}H]GABA was included in 1–10 µM GABA solution, and 40 nM [^{3}H]GABA was included in the 30- and 80-µM GABA solutions. [^{3}H]GABA uptake was terminated after 3 min as described above.

SURFACE BIOTINYLATION

48 h after transfection (according to Table S1), N2a cells in 60-mm dishes were incubated in Hank's balanced salt solution (HBSS) containing 1 mg/ml biotin on ice for 30 min with gentle shaking. Excess biotin was removed by rapid washing twice in

Table S1

Cell density, transfection reagent, and cDNA quantities required for mGAT1 expression in N2a cells optimized not to exceed either the cells' synthetic machinery or trafficking mechanisms

Dish/plate	Area (cm^2)	Relative area	Cells/well	cDNA	Plus reagent	Final diluted volume in serum-free DMEM	Lipofectamine reagent	Volume media on cells (DMEM)	Final transfection volume
				(ng)	*(µl)*	*(µl)*	*(µl)*	*(ml)*	*(ml)*
Protocol steps			Step 1	Step 2	Step 2	Steps 2 and 3	Step 3	Step 5	Step 6
96-well	0.47	0.05	1.75×10^4	12.5	1	10	0.5	0.05	0.07
48-well	0.95	0.10	3.50×10^4	25	2	17.5	0.75	0.1	0.125
24-well	1.90	0.20	7.00×10^4	50	4	25	1	0.2	0.25
12-well	3.80	0.40	1.37×10^5	100	5	50	2	0.4	0.50
6-well/35 mm	9.62	1.00	3.50×10^5	250	6	100	4	0.8	1.0
60-mm	28.27	2.94	1.03×10^6	750	8	250	12	2	2.5
100-mm	78.54	8.16	2.86×10^6	2,000	20	750	30	5	6.5

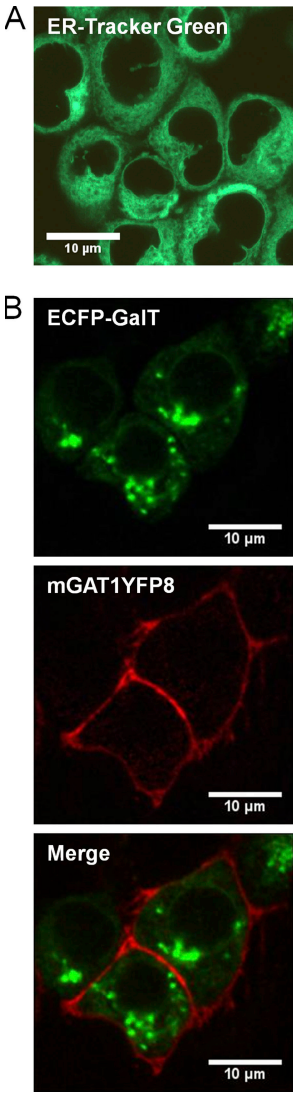

Figure S1. Subcellular localization of cell organelles in N2a cells. (A) ER labeled in live N2a cells with ER-Tracker Green (Invitrogen) according to the manufacturer's instructions. (B) The trans-Golgi was labeled by ECFP-GalT (green) in live N2a cells, and fluorescence is strongly localized in the perinuclear region of the cell. The same cells were cotransfected with mGAT1YFP8 (red), which primarily localized in the cell periphery. The merged image is shown in the final panel. Bars, = 10 µM.

HBSS. Biotin was then quenched for 15 min on ice with 100 mM glycine dissolved in PBS/Ca^{2+}/Mg^{2+} (PBS plus 0.1 mM $CaCl_2$ and 0.2 mM $MgCl_2$). The cells were lysed with 500 µl radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS, supplemented with one protease inhibitor cocktail tablet per 10 ml) at 4°C for 60 min, shaking vigorously. The cell lysates were centrifuged at 20,000 *g* at 4°C for 15 min to remove cell debris. 50 µl of cell lysate was saved for the quantification of total mGAT1 protein. The rest of the sample was mixed with 100 µl of immobilized monomeric avidin beads and incubated on a rotating platform at 4°C overnight. The lysate was separated from the beads and kept for quantification of the intracellular mGAT1 fraction. The beads were then washed three times with ice-cold RIPA buffer, and protein adsorbed by the beads (the plasma membrane–bound fraction) was eluted with 100 µl SDS loading buffer (62.5 mM Tris-Cl, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol) at room temperature for 30 min on a rotating platform. Protein samples were quantified by standard Western blotting procedures using a 1:1,000 dilution of AB1570W anti-GAT1 or a 1:5,000 dilution of anti-GFP rabbit antiserum 132002 for the primary antibodies. Goat anti–rabbit peroxidase–conjugated secondary antibody was applied at a 1:2,000 dilution, and blots were developed using the ECL Plus Western blotting detection reagent and an AlphaImager HP imaging system (Cell Biosciences).

METHOD FOR RADIAL ANALYSIS OF NFRET AMPLITUDE DISTRIBUTIONS

The location of the cell center *c* was determined from the weighted sum of pixel positions in a whole cell ROI. To measure the fractional radius of a single pixel *p*, the vector v_p from *c* to *p* is extended past *p*, in the same direction, until it reaches the cell edge *w*, defining the vector v_w from *c* to *w* (Fig. 13 A). The fractional radius is defined as the ratio $||v_p||/||v_w||$. In this analysis, a pixel located at *c* and one at the absolute periphery have percent distance values of 0 and 100%, respectively.

We used the fractional radius concept in a MATLAB graphical user interface that visualized, on the NFRET image of the cell, the location of all the pixels in a single bin or from a group of selected bins in the histogram of the all-pixel NFRET amplitude distribution for a given ROI. The user examined the NFRET distribution histogram and clicked on color-coded histogram bins or preset NFRET amplitude percentile groups (Fig. 13 A; look-up table in far right panel of Fig. 13 B). The selected pixels were highlighted with the same color in the NFRET image (Fig. 13 B). The routine calculated the percent radial distance of each pixel from *c* and exported the values to files for each NFRET amplitude percentile group. The mean fractional displacement from *c* was calculated from the compiled percentile distance values for the thousands of pixels in each NFRET amplitude percentile group.

REFERENCE

Ramamoorthy, S., E. Giovanetti, Y. Qian, and R.D. Blakely. 1998. Phosphorylation and regulation of antidepressant-sensitive serotonin transporters. *J. Biol. Chem.* 273:2458–2466. doi:10.1074/jbc.273.4.2458