Supplementary Material

Brain sterol flux mediated by CYP46A1 affects membrane properties and membrane-dependent processes

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Supplementary Materials and Methods

Isolation of synaptosomal fractions

The cerebellum-free brains were homogenized on ice in a Dounce homogenizer (14-15 strokes per sample) in Syn-PER™ Synaptic Protein Extraction Reagent (TermoFisher Scientific #87793), containing a cocktail of protease inhibitors (TermoFisher Scientific #A32963). Brain homogenates (10%) were subjected to centrifugation (1,200 g, 4°C, 10 min), and the pellets obtained were resuspended in 2 ml of Syn-PER™ reagent. The suspensions were spun down (1,200 g, 4°C, 10 min), and the supernatants from the 1st and 2nd centrifugation were combined. Synaptosomal fractions were isolated from these combined supernatants by centrifugation (14,000 g, 4°C, 30 min) and subsequent wash of the pellet *via* resuspension in 1 ml of Syn-PER™ reagent and another centrifugation (14,000 g, 4°C, 30 min). The resulting pellet was resuspended in 0.5 mL of Syn-PER™ reagent and used either immediately or aliquoted. The aliquots were flash frozen in liquid nitrogen and stored at -80°C. Protein content in synaptosomal fractions was determined by the Pierce™ BCA protein assay kit (TermoFisher Scientific #23225).

Western blots

Suppl. Table 1. Antibodies Used for Western blots

Antigen	Primary antibody		Secondary antibody	
	Clonality, host, dilution	Vendor, catalog #	Clonality, host, dilution	Vendor, catalog #
Glutamine Synthetase	Monoclonal mouse IgG, 1:1,000	Abcam ab228590	Goat anti-mouse IgG IRDye 680RD, 1:10,000	Li-Cor 925-68070
HDAC2	Monoclonal mouse IgG, 1:2,000	Millipore 05-814		
PSD-95	Monoclonal mouse IgG, 1:4,000	Abcam ab2723		
Synapto-physin	Monoclonal mouse IgG, 1:4,000	Synaptic Systems 101011		
β-Actin	Polyclonal mouse IgG, 1:2,000	Abcam ab8226		
EAAT2	Polyclonal rabbit IgG, 1:2,000	Abcam ab41621	Goat anti-rabbit IgG IRDye 800 CW, 1:10,000	Li-Cor 925-32211
β-Actin	Polyclonal rabbit IgG, 1:2,000	Abcam ab8227		

Construct preparation, expression, and purification of PFO-D4-GFP-GST

The D4 domain of PFO was cloned from the Clostridium perfringens genomic DNA (ATCC #13124D-5) and placed at the N-terminus of the superfolder GFP in the pET LIC vector (Addgene #29772). GST with the thrombin site at the N-terminus was cloned from the pGEX-2TK vector (GE Healthcare Life Sciences #27-1542-01) and placed after GFP. The D434S and C459A mutations were introduced in the PFO-D4 domain using QuickChange Site-Directed Mutagenesis kit (Agilent #200524) according to the manufacturer's instructions. The PFO-D4-GFP-GST construct was then transformed into One ShotTM BL21 (DE3) competent *E. coli* cells (ThermoFisher Scientific #C600003) and selected on an LB-Agar plate containing 0.1 mg/mL of ampicillin (Fisher BioReagents #BP1760-25). A single isolated colony was grown overnight in 1 ml of LB medium containing 100 μg/mL of ampicillin and the next morning was diluted 1:100 into one liter of TB medium containing 100 μg/mL of ampicillin. Cells were grown at 37°C and shaking at 250 rpm until the culture absorbance reached the value of 0.6 at 600 nm. Protein expression was induced by 1 mM isopropyl-1-thio-β-d-galactopyranoside (Research Product International #I56100-5.0) and proceeded for 5 hrs at 37 °C and shaking at 250 rpm. *E. coli* cells were harvested by centrifugation (4,000 g, 4ºC, 20 min) and resuspended in 35 ml of PBS containing protease inhibitors (ThermoFisher Scientific A32963). Cell suspension was incubated with 0.1 mg/mL of lysozyme (Sigma-Aldrich, #L6876) on ice for 30 min followed by addition of 0.05 mg/mL DNase (Sigma-Aldrich #D5025) and 1% Triton X-100 (Sigma-Aldrich T8787). Cells were sonicated on ice with a Branson sonifier model 250 using eight 20-s pulses at 1-min intervals. Cell debris and membranes were removed by the 106,000 g centrifugation for 60 min at 4°C, and the supernatant obtained was applied to the glutathione agarose column (ThermoFisher Scientific #16100) equilibrated with 50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl. The resin was washed with 3 column volumes of the equilibration buffer, and the PFO-D4-GFP-GST was eluted with the equilibration buffer containing 10 mM reduced glutathione (ThermoFisher Scientific #78259). The yellow-colored fraction was collected, diluted 2-fold with the equilibration buffer to remove glutathione and concentrated using the 50,000 kDa cut off Amicon filter (Fisher Scientific #UFC905008). Protein concentration was determined from the absorbance at 488 nm using the molar extinction coefficient of 83,300 M^{-1} cm^{-1} (Pédelacq, Cabantous *et al.*, 2006).

PFO-D4-GFP-GST incubations with synaptosomal membranes

The assay conditions were first optimized for the synaptosomal to PFO-D4-GFP-GST protein ratio by generating the two calibration curves. The first was of the GFP fluorescence intensity *vs* the PFO-D4-GFP-GST concentrations at a fixed concentration of synaptosomal protein (100 μg). The second calibration curve was of the GFP fluorescence intensity *vs* the synaptosomal protein concentrations at a fixed PFO-D4-GFP-GST concentration (15 μg/mL). These experiments enabled the selection of the synaptosomal to PFO-D4-GFP-GST protein ratio so that the fluorescent signal was maximal and linear with the synaptosomal fraction concentration. Synaptosomal fractions (20-30 μL in Syn-PER™ reagent containing 200 μg of protein) were diluted to 1 mL by Locke's solution (30 mM HEPES, pH 7.4, containing 1.8 mM $CaCl_2$, 0.8 mM, $MgCl_2$, 0.8 mM Na_2HPO_4, 5.5 mM glucose, 3 mM $NaHCO_3$, 120 mM NaCl, and 5 mM KCl) and incubated with 15 μg/mL of purified PFO-D4-GFP-GST at room temperature for 35 min. To remove unbound PFO-D4-GFP-GST, synaptosomal fractions were span down by centrifugation (14,000 g, 4°C, 30 min) and washed two times with 1 mL of Locke's solution by resuspending and spinning down the pellet. No fluorescence was detected in the supernatant after the second wash. Synaptosomal pellets were resuspended in 0.5 mL of Locke's solution and allowed to equilibrate for 10 min at 37°C. The fluorescence emission spectra were recorded under constant stirring using a Jobin Yvon-Spex FluoroMax-4 spectrofluorometer (Bensheim) with 1 nm spectral resolution. The GFP fluorescence was excited at 485 nm and measured at 510 nm followed by correction for non-specific fluorescence. The latter was determined from incubations of PFO-D4-GFP-GST with synaptosomal fractions (200 μg of protein) after cholesterol extraction with 30 mM of methyl-β-cyclodextrin (MilliporeSigma #332615) for 40 min at room temperature in 1 mL of Locke's solution. Methyl-β-cyclodextrin was then removed from synaptosomal fractions by centrifugation (14,000 g, 4°C, 30 min), and the final pellet was used for incubations with PFO-D4-GFP-GST. The cholesterol content in these fractions was ~5% of that in the corresponding fractions before cholesterol depletion.

Membrane ordering

Di-4-ANEPPDHQ (10 μM final concentration from 5 mM stock in DMSO) was incubated with synaptosomal fractions (10-15 μL in Syn-PER™ buffer containing 100 μg of protein) in 300 μL of Locke's solution for 35 min at 37°C. The probe was then removed by centrifugation (14,000 g, 4°C, 30 min), and the pellet was resuspended in 300 μL of Locke's solution. The suspension was

allowed to equilibrate for 15 min at 37°C, and emission spectra were recorded at 37°C upon excitation at 473 nm. General polarization (GP) of di-4-ANEPPDHQ was calculated as described (Amaro, Reina *et al.*, 2017): $GP = (I_{560} - I_{650})/(I_{560} + I_{650})$, where I_{560} and I_{650} represents the fluorescence intensity at 560 nm and 650 nm, respectively. Studies utilizing F2N12S (0.2 μM final concentration from 0.2 mM stock in DMSO) were conducted similarly, except the incubation time of the probe with synaptosomal fractions was 10 min at 37°C, and samples were not span down but used for the fluorescence measurements at 37°C. F2N12S was excited at 405 nm, and the ratio between its short- and long-wavelength emission bands was calculated at 485 nm and 575 nm, respectively.

Osmotic resistance

Synaptosomal fractions (10-15 μL in Syn-PERTM buffer containing 100 μg of protein) were diluted to 320 μL by Locke's solution and incubated with different (0 M, 0.05 M, 0.12 M, 0.2 M, and 0.4 M) concentrations of NaCl for 6 min at 37°C. The absorbance of synaptosomal fractions was the measured at 520 nm *vs* the reference cuvette containing the same reagents but no synaptosomal fraction.

Transmission electron microscopy

A part of synaptosomal pellet from the cerebellum-free brain (approximately one tenth) was overlaid with 0.5 mL of a quarter strength of the Karnovsky's fixative (4% paraformaldehyde and 5% glutaraldehyde in 0.1 M Na cacodylate, pH 7.4 (Karnovsky, 1965)) and incubated for 2.5 hrs at room temperature with two 0.5 mL changes of this fixative. Synaptosomal pellets were then washed three times for 5 min with 0.1 PBS, pH 7.4, and sequentially incubated in the following solutions: 1% OsO_4 in 0.1 M Na cacodylate buffer, pH 7.4, for 2 hrs; 1% tannic acid in 0.05 M Na cacodylate buffer, pH 7.4, for 30 min; 1% $NaSO_4$ in 0.05 M cacodylate buffer, pH 7.4, for 5 min; 70% ethanol for 5 min, and 1% paraphenylendiamine in 70% ethanol for 30 min (Guyton and Klemp, 1988). Then, samples were dehydrated with 70% ethanol for 5 min, 75% ethanol for 10 min, 90% ethanol for 10 min, 95% ethanol for 10 min, and 100% ethanol for 10 min. Next, samples were passed through propylene oxide (Millipore Sigma #110205) and placed in an in a Poly/Bed 812 embedding media (Polysciences #08791-500). Thin sections (80 nm) were cut on an RMC MT 6000-XL ultramicrotome (Boeckeler Instrument), mounted on Gilder square 300 mesh nickel

grids (Electron Microscopy Sciences) and sequentially stained with acidified methanolic uranyl acetate followed by a modification of Sato's triple lead stain (Fujioka, Tandler *et al.*, 2012). Sections were coated on a Denton DV-401 carbon coater (Denton Vacuum LLC) and examined with a FEI Tecnai Spirit (T12) electron microscope (ThermoFisher Scientific) equipped with a Gatan US4000 4kx4k CCD (Gatan). Membrane thickness was measured by the ImageJ software (NIH).

Glutamate content

The measurements were carried out using the Glutamate Assay Kit (BioVision #K629-100). To determine the amount of glutamate released in response to addition of sucrose (35 mM final concentration) or KCl (117 mM final concentration), synaptosomal fractions (40-60 μL containing 400 μg of protein) were mixed with the ice-cold assay buffer (536-556 μL), enzyme mix (8 μL), and developer (36 μL) followed by incubation for 20 min at 37°C. The suspensions were then equally divided and placed in the sample and reference cuvettes maintained at 37°C. Sucrose (4.3 μL from a 2.6 M aqueous sucrose stock containing 48 mM $CaCl_2$) or KCl (13 μL from a 3M KCl stock containing 48 mM $CaCl_2$) were added to the sample cuvette, and the same volume of 48 mM $CaCl_2$ was added to the reference cuvette. Absorbance at 450 nm was recorded every 10 s until a plateau was reached. To determine the total amount of glutamate in the sample, synaptosomal fractions (10-15 μL containing 100 μg of protein) were diluted with 0.1 mL of ice-cold H_2O containing 0.1% Triton X-100 and incubated for 10 min to lyse synaptosomes. Then 35 μL of this solution (35 μg of protein) was mixed with 285 μL of the assay buffer containing enzyme mix (4 μL) and developer (18 μL), and incubated for 20 min at 37°C. Absorbance at 450 nm was recorded *vs* assay buffer containing the same amount of the enzyme mix, developer, and a non-lysed synaptosomal fraction. The glutamate content (in nmol) was determined from the calibration curves, which were generated by plotting the absorbance at 450 nm *vs* the increased concentrations of glutamate (0, 4, 12, 20, and 30 nmol). The glutamate content was then normalized to the protein content in the sample.

Inhibition of protein kinases and protein phosphatases

Synaptosomal fractions (40-60 μL containing 400 μg of protein) were mixed with the reagents from the Glutamate Assay Kit (BioVision #K629-10: 536-556 μL of the assay buffer, 8 μL of the enzyme mix, and 36 μL of the developer) and incubated for 20 min at 37°C after the addition of a specific inhibitor (0.8 μL from a stock in DMSO: 14.1 mM (R)-Roscovitine (Enzo Life Sciences #BML-CC205-0005), 7.16 mM butyrolactone I (Enzo Life Sciences #BML-CC210-0200), 7.14 mM SB 216763 (Tocris #1616), 0.714 mM okadaic acid (Tocris #1136); or 23.43 mM cyclosporin A (Tocris #1101)). The solutions were then equally divided and placed in the sample and reference cuvettes maintained at 37°C. Subsequent glutamate release induced by 35 mM sucrose or 117 mM KCl and the measurements of the glutamate content were as described above.

References

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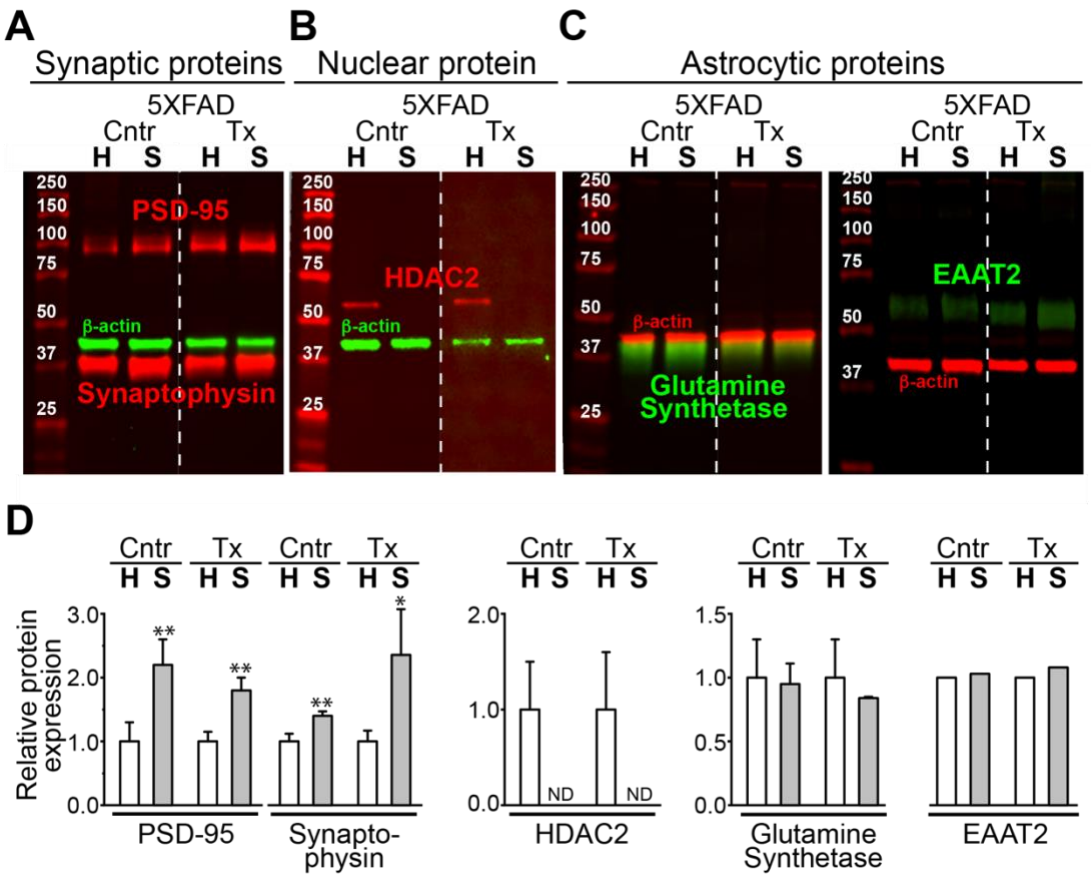
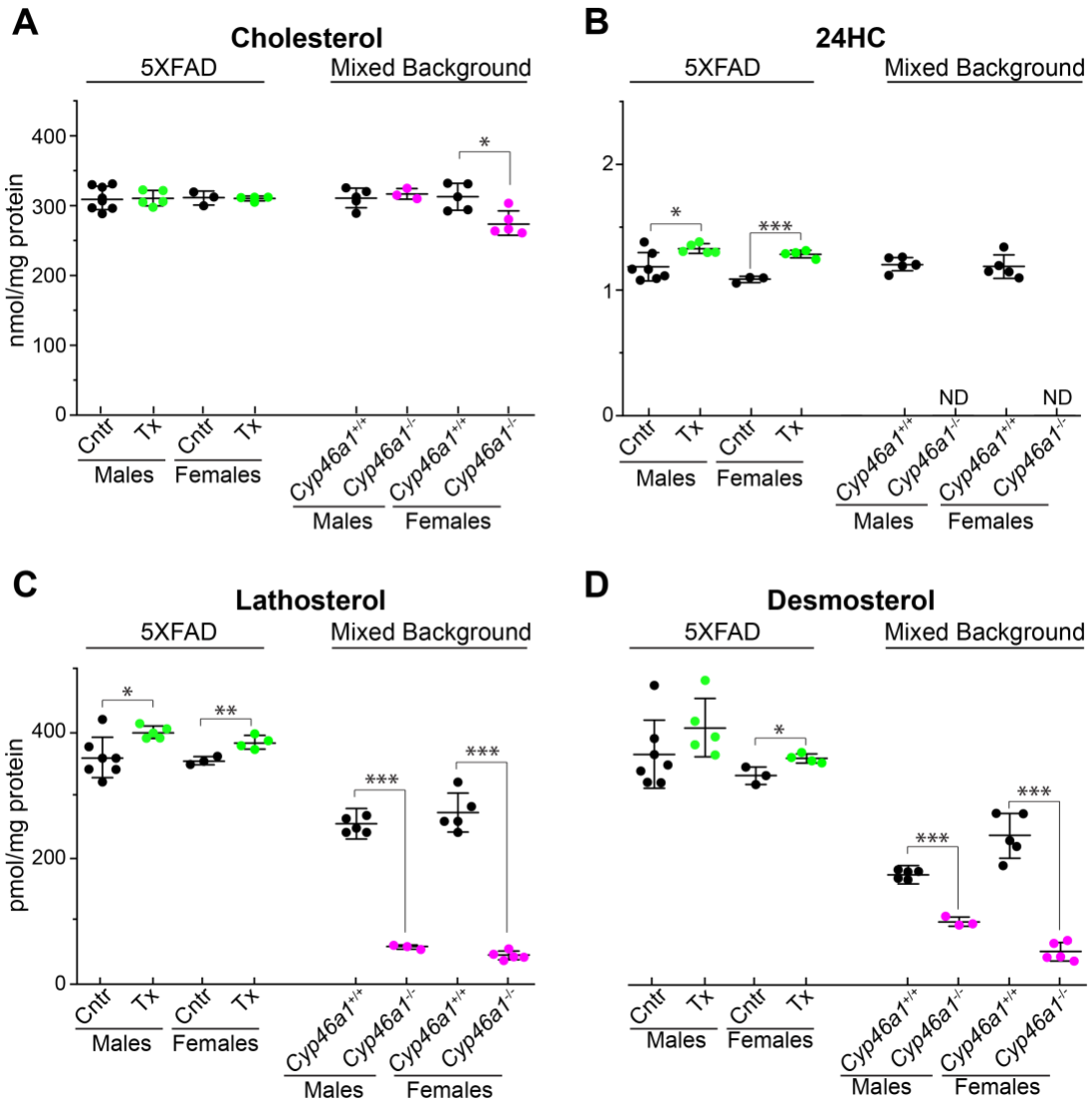
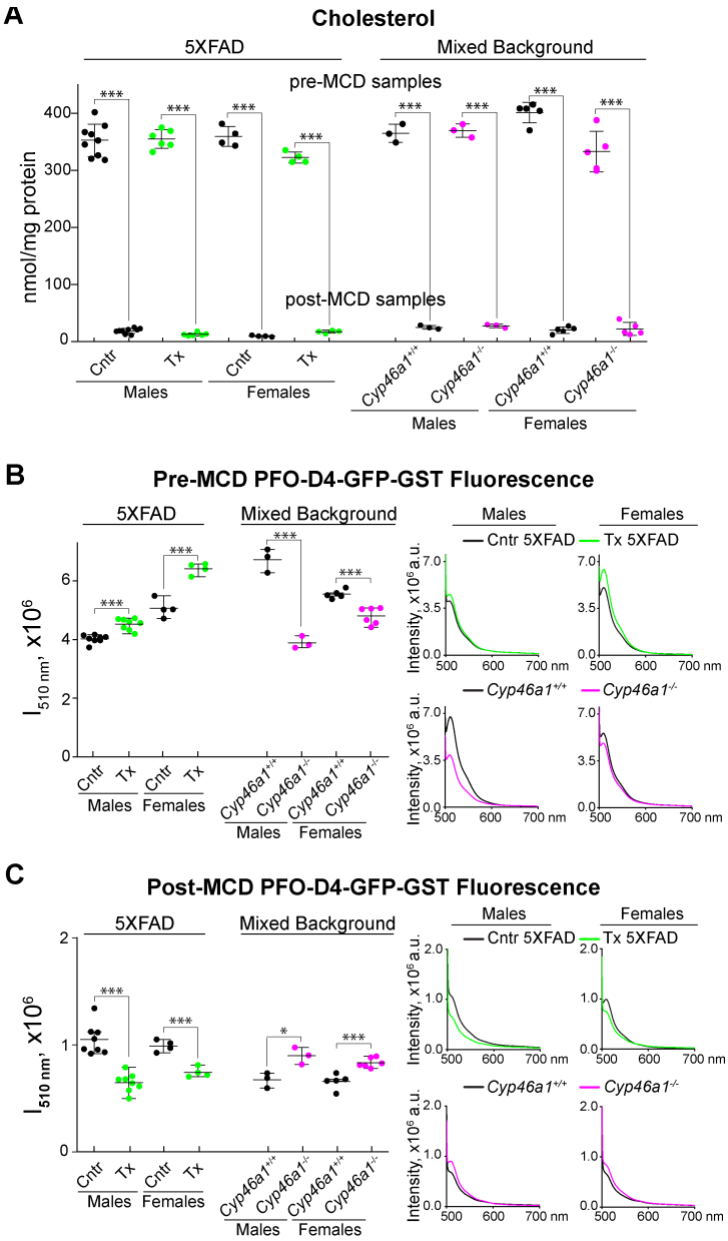


Suppl. Figure S1. Characterization of synaptosomal fractions. Representative Western blots of control (Cntr) and EFV-treated (Tx) 5XFAD mice. (**A)** The levels of synaptic proteins PSD-95 and synaptophysin; (**B)** a nuclear protein HDAC2; and (**C)** astrocytic proteins glutamine synthetase and EAAT2 in the brain homogenates (H) and synaptosomal fractions (S). (**D)** The quantification of the relative protein expression after the normalization to the β-actin signal. The results are the mean ± SD of the measurements in 3-4 mice (PSD-95, synaptophysin, HDAC2, and glutamine synthetase) or the mean of the two measurements (EAAT2). *$P \leq 0.05$, **$P \leq 0.01$ by a two-tallied, unpaired Student's t-test. The vertical white dashed lines separate different gels.



Suppl. Figure S2. Sterol profiles in brain homogenates. (**A**-**D**) The levels of cholesterol, 24HC, lathosterol, and desmosterol, respectively, in the homogenates prepared from the brain of vehicle-treated (Cntr, black circles) and EFV-treated (Tx, green circles) 5XFAD mice as well as wild type mice (*Cyp46a1*$^{+/+}$) on the mixed (C57BL/6J;129S6/SvEv) background (black circles) and *Cyp46a1*$^{-/-}$ mice (magenta circles) on the same background. The results are the mean ± SD of the measurements in individual animals (n=3-7 mice per group and gender. Statistical analysis: a two-way ANOVA followed by Bonferroni post hoc comparisons. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.



Suppl. Figure S3. Labeling of synaptosomal fraction membranes with PFO-D4-GFP-GST. (**A**) The levels of total cholesterol in intact synaptosomal fractions (pre-MCD samples) and synaptosomal fractions after incubation with 30 mM methyl-β-cyclodexrin (MCD, post-MCD samples). (**B)** and (**C)** Fluorescence intensity (I) at 510 nm as a result of PFO-D4-GFP-GST binding to intact (pre-MCD PFO-D4-GFP-GST fluorescence) and cholesterol-depleted (post-MCD PFO-D4-GFP-GST fluorescence) synaptosomal fractions, respectively. The averaged emission spectra of PFO-D4-GFP-GST are shown on the right. All results are the mean ± SD of the measurements in individual animals (n=3-9 mice per group and gender). Statistical analysis: a two-tallied, unpaired Student's t-test. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$. Cntr, control or vehicle-treated 5XFAD mice (black circles and traces); Tx, EFV-treated 5XFAD mice (green circles and traces); *Cyp46a1*$^{+/+}$ mice (black circles and traces) and *Cyp46a1*$^{-/-}$ (magenta circles and traces) on the mixed (C57BL/6J;129S6/SvEv) background.