Supporting Information to Accompany:

Probing the functional limits of the norepinephrine transporter with self reporting, fluorescent stilbazolium dimers

Erika L. Smith, Adrienne S. Brown, Edward Adjaye-Mensah and James N. Wilson

Department of Chemistry, University of Miami, 1301 Memorial Drive, Coral Gables, FL 33124

Characterization of 1 and 2:

(1,1'-(1,6-hexanediyl)bis[4-[(E)-2-(6-hydroxy-2-naphthalenyl)ethenyl]]pyridinium dibromide (1). IR ν_{max}: 3035.5, 2937.9, 2835.8,1640.4,1513.9,1436.6, 1282.2, 1159.6, 973.8, 869.5, 664.2 cm^{-1}. ^{1}H NMR (500 MHz, DMSO/MeOH): δ 1.94 (4H, bs), 4.54 (4H, bs), 7.18 (4H, m), 7.59 (2H, d, *J* = 16 Hz), 7.78 (2H, d, *J* = 9 Hz), 7.85 (4H, d, *J* = 8.5 Hz), 8.09 (2H, s), 8.17 (2H, d, *J* = 16 Hz), 8.28 (4H, d, *J* = 6 Hz), 9.01 (4H, d, *J* = 6.0 Hz), 10.17 (2H, s), 1.36 (4H, bs). ^{13}C NMR (125.74 MHz, DMSO): δ 25.33, 30.68, 59.88, 109.62, 119.98, 122.32, 123.97, 124.30, 127.54, 127.87, 130.17, 130.65, 130.91, 136.24, 141.97, 144.61, 153.61, 157.60. HRMS (ES+) m/z calcd for $C_{40}H_{38}N_2O_2^{2+}$: 289.1467 Found: 289.1457. MP: 323 °C.

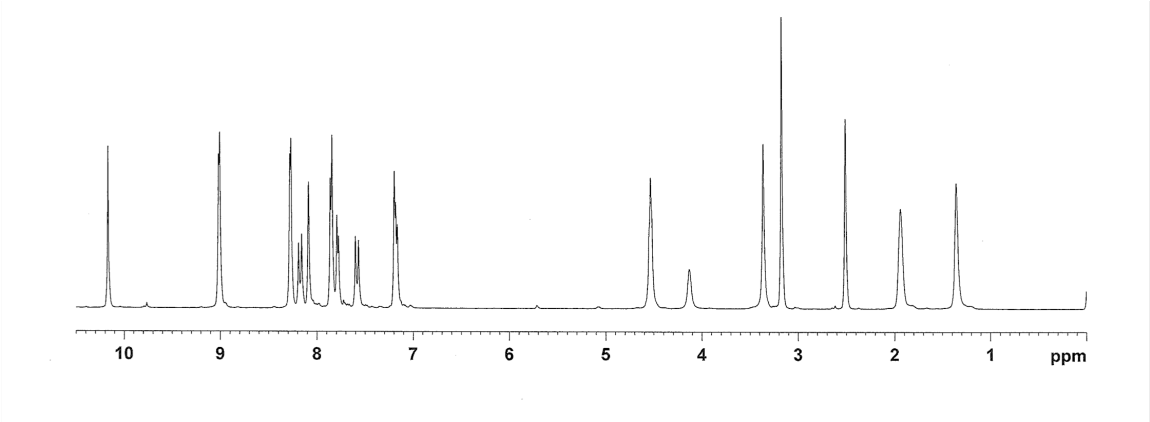

Fig S1: ^{1}H NMR of **1**.

(1,1'-(1,6-hexanediyl)bis[4-[(E)-2-(2-naphthalenyl)ethenyl]]pyridinium dibromide (2). IR ν_{max}: 3031.3, 2936.7, 2858.3, 1637.4, 1510.5, 1463.3, 1365.4, 1164.5, 979.3, 867.2, 762.9, 641.9, 575.1 cm^{-1}. ^{1}H NMR (300 MHz, DMSO): δ 1.37 (4H, s), 1.96 (4H, s), 4.57 (4H, t, *J* = 7.5 Hz), 7.61 (4H, s), 7.72 (2H, d, *J* = 16.3 Hz), 8.00 (8H, d, *J* = 7.5 Hz), 8.25 (4H, d, *J* = 14.0 Hz), 8.34 (4H, d, *J* = 5.3 Hz), 9.07 (4H, d, *J* = 5.2 Hz). ^{13}C NMR (125.74 MHz, DMSO): δ 25.27, 30.69, 59.94, 124.12, 121.18, 124.35, 127.47, 128.01, 128.27, 129.00, 129.25, 130.30, 133.32, 133.38, 134.21, 141.32, 144.31, 153.30. HRMS (ES+) m/z calcd for $C_{40}H_{38}N_2^{2+}$: 273.1518 Found: 273.1526. MP: 274 °C.

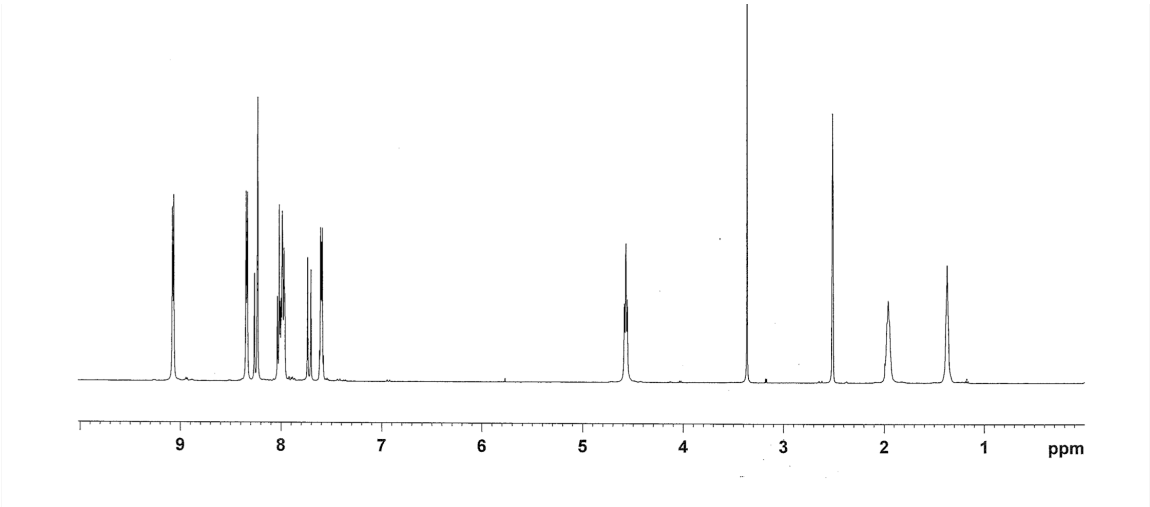

Fig S2: ^{1}H NMR of **2**.

Cell culture and confocal imaging:

HEK293 cells selectively and stably expressing hNET (provided by R. Blakely, Vanderbilt University) were cultured as previously described in sterile T-75 flasks (Nunc #156472). [1,2] Cells were maintained in DMEM (Dulbecco's Modification of Eagle's Medium with 4.5 g/L glucose, without L-glutamine and sodium pyruvate; Cellgro #15-017-CV) containing 10% dialyzed FBS (Fetal Bovine Serum, defined and heat inactivated, Hyclone #SH30070.03), 2mM glutamine (Himedia #TCL-012), 100 units/ml penicillin (Gibco #15140), 100 μg/ml streptomycin (Gibco #15140), and 250 μg/ml Geneticin (Gibco #10131) at 37°C and 5% CO_2. Cells were seeded at a density of 10^5 cell/cm^2 in 96 microwell plates (BD Falcon #353219) and incubated for at least 48 hours until a visible monolayer was established. Prior to imaging, DMEM was removed and L-15 (Leibovitz's L-15 modified, Cellgro #10-045-CV) containing 5% FBS was added to each well.

DMSO stocks of the fluorophores (10 mM) and desipramine (50 mM) were used to prepare working stocks of fluorophores (10 μM) and desipramine (100 μM) in L-15 media containing 5% FBS. Final probe concentrations for cell treatment (500 nM up to 2 μM) were obtained by adding the appropriate volumes to the microwells during imaging experiments. Final DMSO content in the microwells was less than 0.1%.

Imaging was performed on a Leica SP5 confocal microscope housed within the UM Biology Imaging Core Facility. Excitation was achieved using 405 nm, 488 nm and 633 nm lasers with emission collected between 475 and 575 nm for **1** and HNEP$^+$, between 525 and 600 nm for **4** and ASP$^+$, and between 645 and 700 nm for CellMask DeepRed. Images were collected in XYT mode with 2 sec interval sampling for single channel experiments and 5 sec intervals for dual channel experiments. Images were analyzed using Fiji/ImageJA software (NIH, USA).

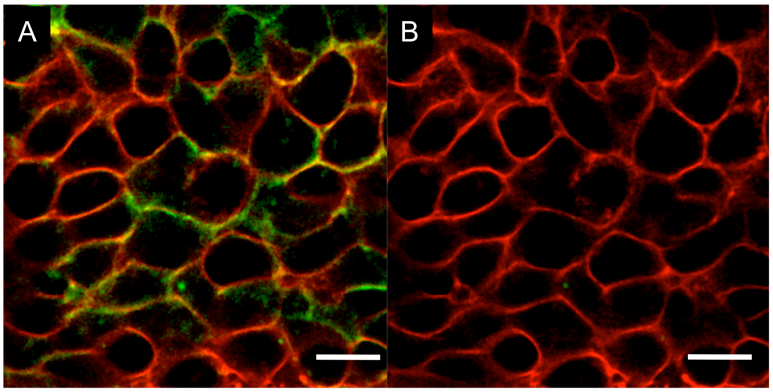

Fig S3: Panel A shows the colocalization of **1** (green) with a cell membrane dye (CellMask Deep Red, red). Introduction of 2 μM desipramine (panel B) results in displacement of **1** indicating NET is the cell membrane target of the dimer probe. Scale bar is 15 μm.

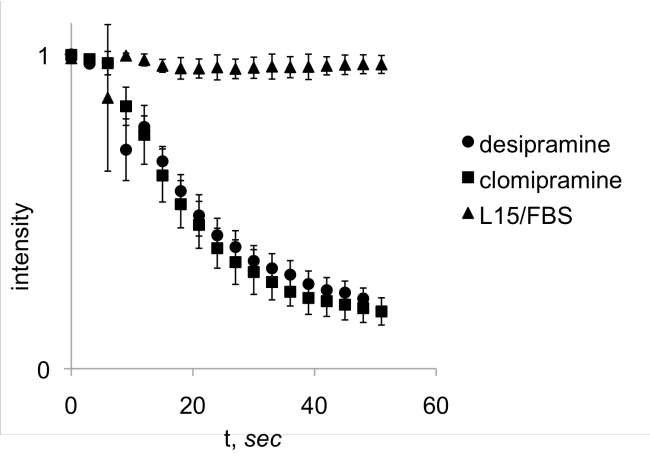

Fig S4: Probe displacement is dependent on the introduction of a competitive inhibitor and is not observed with the introduction of media (L15/FBS) only. hNET-HEK293 cells were pretreated with **4** (2 μM), then 20 μL of desipramine (final concentration = 10 μM), clomipramine (final concentration = 10 μM) or media only were introduced between t = 5 and 10 sec. Data points represent the mean of 4 experiments; error bars show s.e.m. The large error bars between t = 5 and 10 sec are an artifact of solution mixing.

Molecular docking:

Molecular docking was performed using AutoDock Vina.[3] LeuT (PDB ID: 2QJU)[4] was prepared for docking in AutoDockTools (Molecular Graphics Laboratory, Scripps Research Institute) after removal of the small molecules. The grid box was centered on the Leu ligand with 1 Å spacing; dimensions were x = 20 Å, y = 25 Å and z = 12 Å for docking of **1** and **4**. For ASP+ and HNEP+, dimensions were x = 20 Å, y = 16 Å and z = 12 Å. Ligands were first prepared in Spartan '08 with geometries optimized at the HF3-21G* level. Preparation for docking was performed using AutoDockTools with the vinyl bridge torsions restricted.

References:

1. J. W. Schwartz, R. D. Blakely and L. J. DeFelice, *J. Biol. Chem.,* 2003, **278**, 9768.
2. A. Galli, L. J. DeFelice, B. J. Duke, K. R. Moore and R. D. Blakely, *J. Exp. Biol.,* 1995, **198**, 2197.
3. O. Trott and A. J. Olson, *J. Comput. Chem.*, 2010, **31**, 455-461.
4. Z. Zhou, J. Zhen, N. K. Karpowich, R. M. Goetz, C. J. Law, M. E. A. Reith and D.-N. Wang, *Science,* 2007, **317**, 1390.