Supplementary Information

Postendocytic sorting of Constitutively Internalized Dopamine Transporter in Cell Lines and Dopaminergic Neurons

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Running title: Sorting of internalized dopamine transporter

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Supplementary figures

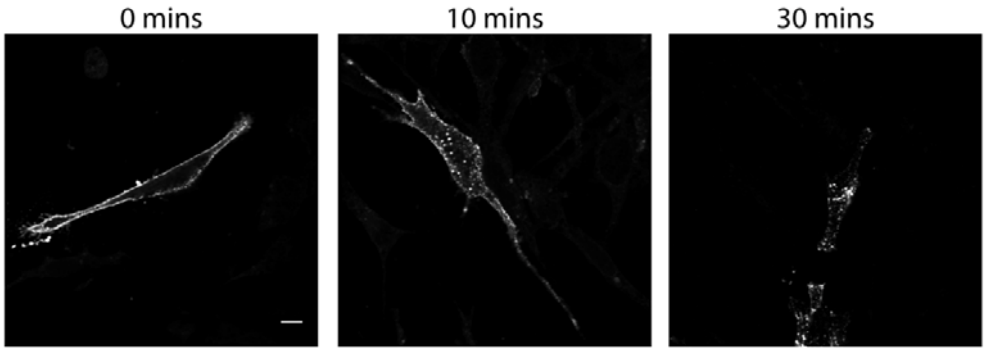

Figure S1: 1Rb3An27 cells expressing FLAG-tagged ß$_2$-adrenergic receptor were labeled with M1 antibody at 4 °C followed by incubation with the agonist isoproterenol (10 μM) for 0, 10 or 30 minutes. After subsequent fixation and permeabilization, the cells were stained with Alexa568 anti-mouse antibody before mounting and analysis by confocal microscopy. Scale bar 10μm.

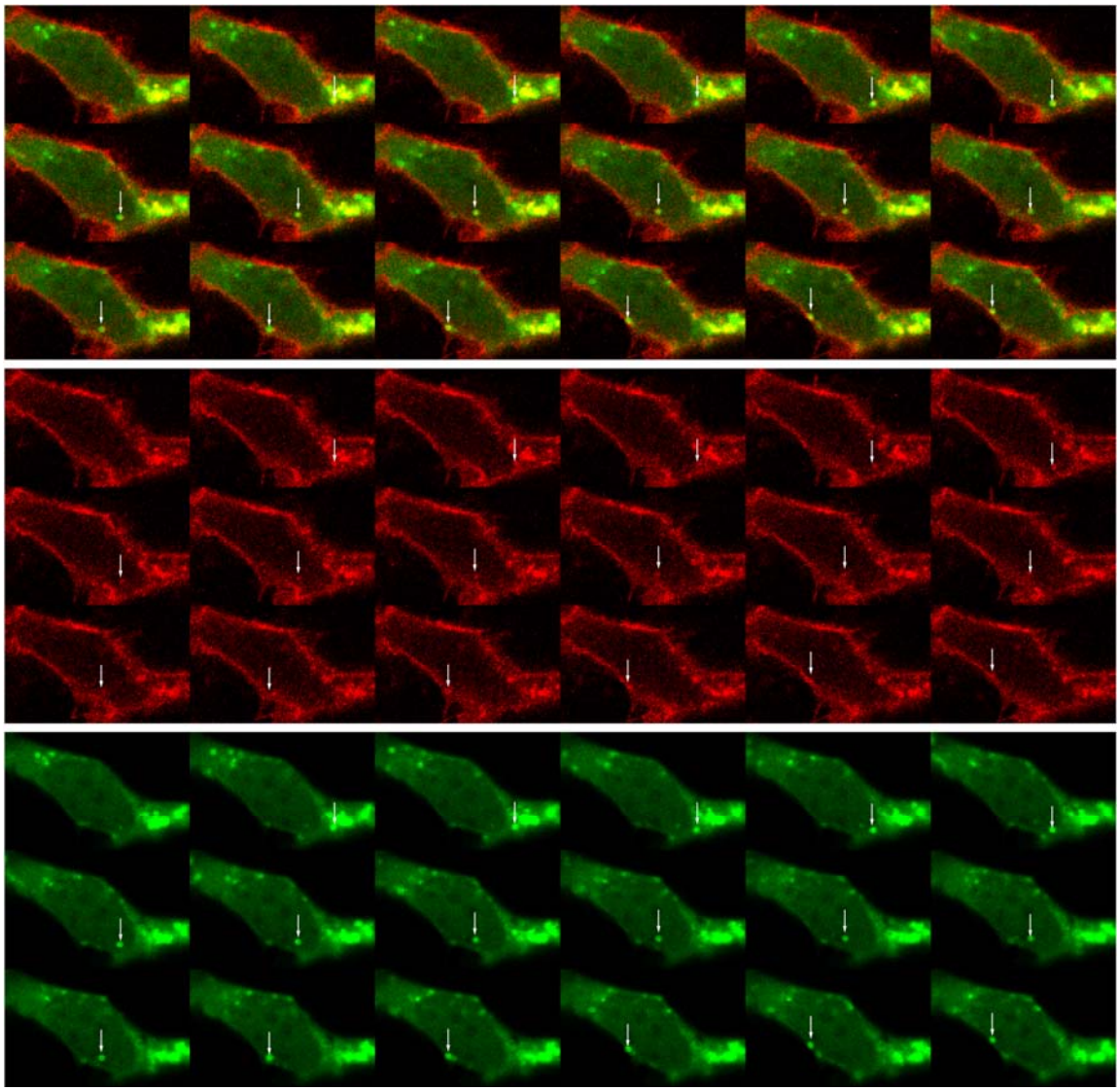

Figure S2: 1Rb3An27 cells expressing DAT and EGFP-Rab7 were labeled with JHC 1-64 as described in Experimental Procedures, and DAT was allowed to internalize for 1 hour before confocal live imaging. The imaging was carried out at room temperature and pictures were taken with time intervals of 8 seconds. The arrow indicates a moving vesicle that is positive both for DAT/JHC 1-64 (red channel) and EGFP-Rab7 (green channel).

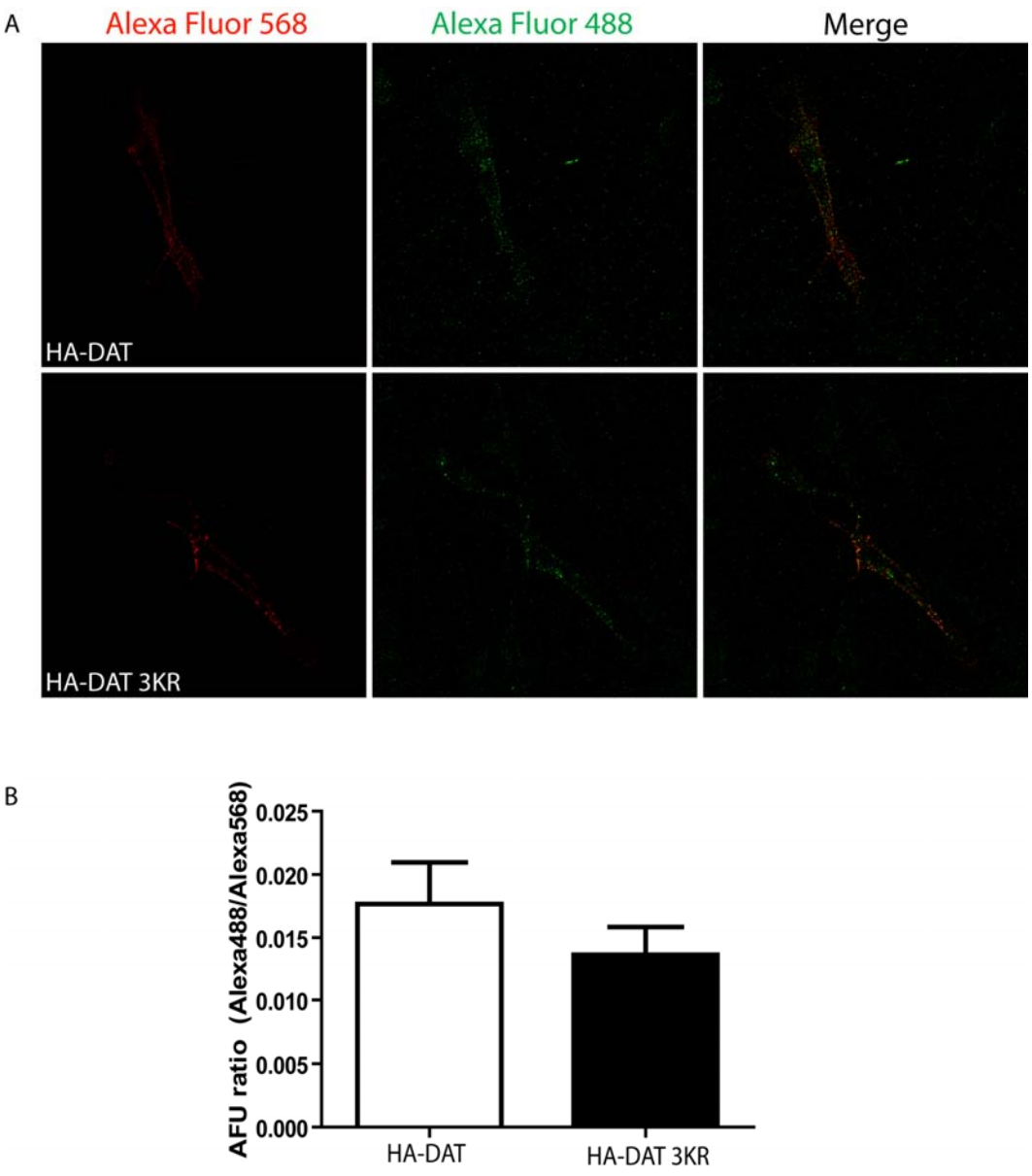

Figure S3: HA-DAT 3KR is similar to HA-DAT constitutively internalized in 1Rb3An27 cells.

A, 1Rb3An27 cells were transiently transfected with HA-DAT (upper panels) or HA-DAT 3KR (lower panels). Cells were incubated with HA.11 antibody for 5 minutes at 18-20 °C to label surface transporters and then incubated for 1 hour at 37 °C to allow internalization. After fixation the cells were labelled with Alexa568 anti-mouse antibody to detect surface transporter (left panels), and after permeabilization the cells were labelled with Alexa488 anti-mouse antibody to detect internalized transporter (middle panels). Right panels show overlay of the two channels.

B, Quantification of constitutively internalized HA-DAT or HA-DAT 3KR (Alexa Fluor 488 signal) relative to surface HA-DAT or HA-DAT 3KR (Alexa568 signal) we observed no difference between HA-DAT and HA-DAT 3KR ($p > 0.05$, t-test). Bars represent means + S.E. of n=18-20 cells. Similar results were obtained in two independent transfections.

Method

The experiment was done modified from (1). Briefly, 1Rb3An27 cells were transiently transfected with HA-DAT or HA-DAT 3KR two days prior to the experiment. Cells were incubated in HA.11 antibody (½-1 µg/ml) in DMEM + 10 % FBS for 5 minutes at 18-20 °C, washed 3 times in DMEM + 10 % FBS and incubated in DMEM + 10 % FBS for 1 hour at 37 °C, keeping one of each transfection on ice for 1 hour. Cells were washed in ice cold PBS and blocked in 0.5 % bovine serum albumin in PBS for 30 minutes, then incubated with 5 µg/ml Alexa568-conjugated goat anti-mouse IgG in blocking buffer for 45 minutes and washed 4 times in PBS. Cells were fixed in 4 % paraformaldehyde in PBS for 20 minutes and washed twice in PBS, then permeabilized 5 minutes in 0.2 % Triton X-100 and incubated in 1 µg/ml Alexa488-conjugated goat anti-mouse IgG in blocking buffer. Finally cells were washed 4 times in PBS and mounted with ProLong Gold Antifade. The cells were imaged with a Zeiss LSM 510 confocal laser scanning microscope using a long pass 505 and long pass 560 filters. Z-stacks were obtained with 370 nm intervals to include the entire cell (with amplifier offset set low to threshold unspecific binding*). Quantification was done with ImageJ software. The cell region of interest was drawn and intensities of fluorescence in the two channels were summed through the Z-stacks, and the Alexa488/Alexa568 ratio taken. The ratio from cells kept at 0 °C was subtracted. 18-20 cells from each condition were analyzed.

1. Sorkina, T., Miranda, M., Dionne, K. R., Hoover, B. R., Zahniser, N. R., and Sorkin, A. (2006) *Journal of Neuroscience* **26**, 8195-8205