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Dopamine transporter is enriched in filopodia and induces filopodia formation

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ABSTRACT

Dopamine transporter (DAT, *SLC6A3*) controls dopamine (DA) neurotransmission by mediating re-uptake of extracellular DA into DA neurons. DA uptake depends on the amount of DAT at the cell surface, and is therefore regulated by DAT subcellular distribution. Hence we used spinning disk confocal microscopy to demonstrate DAT localization in membrane protrusions that contained filamentous actin and myosin X (MyoX), a molecular motor located in filopodia tips, thus confirming that these protrusions are filopodia. DAT was enriched in filopodia. In contrast, R60A and W63A DAT mutants with disrupted outward-facing conformation were not accumulated in filopodia, suggesting that this conformation is necessary for DAT filopodia targeting. Three independent approaches of filopodia counting showed that DAT expression leads to an increase in the number of filopodia per cell, indicating that DAT can induce filopodia formation. Depletion of MyoX by RNA interference resulted in a significant loss of filopodia but did not completely eliminate filopodia, implying that DAT-enriched filopodia can be formed without MyoX. In cultured postnatal DA neurons MyoX was mainly localized to growth cones that displayed highly dynamic DAT-containing filopodia. We hypothesize that the concave shape of the DAT molecule functions as the targeting determinant for DAT accumulation in outward-curved membrane domains, and may also allow high local concentrations of DAT to induce an outward membrane bending. Such targeting and membrane remodeling capacities may be part of the mechanism responsible for DAT enrichment in the filopodia and its targeting to the axonal processes of DA neurons.

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1. Introduction

Dopamine (DA) signaling in the central nervous system is involved in various aspects of locomotor activity, emotions, reward, and affect (Iversen and Iversen, 2007). Dysfunctions of the DA system are implicated in a spectrum of abnormalities, such as Parkinson's disease, schizophrenia, bipolar disorder, attention deficit hyperactivity disorder, and psychostimulant drug abuse (Hyman et al., 2006; Ueno, 2003). In the mouse brain the somatodendritic compartments of DA neurons are located in the midbrain, in particular in the substantia nigra and ventral tegmental area. During development these neurons project axons mainly to striatum to form a highly elaborate axonal network. The amplitude and duration of the dopaminergic neurotransmission are controlled by the regulation of extracellular DA concentrations via reuptake by the plasma membrane DA transporter (DAT) (Gether et al., 2006; Schmitz et al., 2003). DAT is expressed exclusively in DA neurons with the highest concentration in striatal axons. The mechanisms responsible for targeting of DAT, that is synthesized in the somatodendritic compartments of DA neurons in the midbrain, to the axonal processes of these neurons in the striatum and maintaining high levels of DAT in axons are unknown.

DAT functions in the plasma membrane, and therefore, subcellular localization and trafficking of DAT are important for its activity. For instance, regulation of DAT surface levels by endocytosis has been proposed (Melikian, 2004). In addition, DAT activity is regulated by its sub-compartmentalization in specialized domains of the plasma membrane (Adkins et al., 2007; Foster et al., 2008; Hong and Amara, 2010). We have previously demonstrated DAT localization in filopodia-like membrane protrusions in non-neuronal cells and primary mesencephalic cultures of DA neurons (Rao et al., 2012; Sorkina et al., 2009). Interestingly, DAT was less mobile in these membrane protrusions than in other regions of the plasma membrane (Rao et al., 2012; Sorkina et al., 2009). We then hypothesized that targeting to membrane protrusions serves to retain functional DAT at the cell surface.

In the present study we addressed three main questions. First, are DAT-containing structures actually the filopodia or other types of membrane protrusive structures? Filopodia are broadly defined as thin (0.1–0.3 μm), finger-like structures that are filled with tight parallel

Abbreviations: F-actin, filamentous actin; DA, dopamine; DAT, dopamine transporter; EGFR, epidermal growth factor receptor; HA, hemagglutinin epitope; MyoX, myosin X or myosin 10; VASP, vasodilator-stimulated phosphoprotein; GFP, RFP and YFP, green, red and yellow fluorescent proteins, respectively.

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bundles of filamentous (F)-actin (Mattila and Lappalainen, 2008). In neurons filopodia have important roles in axonal outgrowth, branching, cell migration, dendritic spine formation and interneuron communication (Gallo, 2011; Gallo, 2013). Second, is DAT enriched in the filopodia-like protrusions? In other words, is concentration of DAT higher in these protrusions than in other areas of the membrane? Third, if DAT is enriched in filopodia-like protrusions, is it targeted to pre-existing protrusions or capable to induce de novo formation of membrane protrusions and maintain these structures? To address these questions, the localization of DAT and its mutants was analyzed using fluorescence microscopy imaging in comparison with subcellular distribution of other membrane proteins, F-actin and resident filopodia proteins.

2. Materials and methods

2.1. Reagents

Antibodies were purchased from the following sources: mouse monoclonal antibody to hemagglutinin epitope HA11 (16B12, cat# MMS-101P) and HA11 conjugated with Alexa488 (HA11-A488, cat# A488-101L) were from Covance (Berkley, CA); rabbit polyclonal myosin X (MyoX) antibody (working dilution 1:100–500 and 1:1000 in immunofluorescence and western blotting experiments, respectively) from Sigma Aldrich (St. Louis, MO) (cat# HPA024223); rabbit polyclonal antibody to α-actinin from Cell Signaling Technology (cat# 3134S; working dilution 1:1000). Mouse monoclonal antibody to epidermal growth factor receptor (EGFR) Mab528 was from American Type Cell Culture Collections, Inc. (Manassas, VA) (cat# HB-8509). Mouse monoclonal antibody to vasodilator-stimulated phosphoprotein (VASP) (BD Biosciences, cat# 610447, working dilution 1:100) was a kind gift from Dr. Partha Roy (McGowan Institute for Regenerative Medicine, Pittsburgh). Donkey anti-mouse, anti-rat and anti-rabbit antibodies conjugated with Alexa488, Cy5 or Cy3 (working dilution 1:250–500) were from Jackson Immuno Research (West Grove, PA); IRDye-800 and IRDye-680-conjugated goat anti-rabbit antibodies were purchased from LI-COR Biosciences (Lincoln, NE) (working dilution 1:10,000). Protein G-Sepharose was purchased from Invitrogen (Carlsbad, CA). Cell Mask Deep Red Plasma Membrane Stain (CellMask, cat# C10046) and phalloidin-Alexa680 (cat# A22286) (working dilution 1:500) were from Invitrogen. Paraformaldehyde was from Electron Microscopy Sciences (Hatfield, PA). Tissue culture reagents were purchased from Invitrogen. Triton X-100, protease Inhibitors and most other reagents were purchased from Sigma Aldrich.

2.2. DNA constructs

Wild-type and mutants (W63A and R60A) of yellow fluorescent protein (YFP) and hemagglutinin epitope (HA) tagged human DAT (YFP-HA-DAT) were described previously (Sorkina et al., 2009). To generate RFP (red fluorescent protein)-HA-DAT, HA-DAT sequence from the YFP-HA-DAT construct was inserted into the tagRFP-C1 vector (provided by Dr. V. Verkhusha, Albert Einstein College of Medicine) using *Kpn*I restriction site at the 5′-end and a *Sma*I site at the 3′-end. Lifeact-RFP described in Berepiki et al. (2010) was provided by Dr. Lichius (University of Edinburgh, Edinburgh, UK). EGFR mutant construct lacking the entire cytoplasmic domain (EGFR-ΔC) was described previously (Miranda et al., 2004). GFP (green fluorescent protein)-MyoX described in Berg and Cheney (2002) was provided by Dr. R. E. Cheney (University of North Carolina). RFP-CAAX construct, which consisted of monomeric RFP with the membrane-targeting H-Ras sequence KLNPPDESGPGCMSCKCVLS attached in frame to the C-terminus of RFP and cloned into pCS2 + vector (Wallingford et al., 2000), was provided by P. K. Umasanker (University of Pittsburgh).

2.3. Cell culture, transfections and RNA interference

Parental and DAT-expressing porcine aortic endothelial (PAE) cells were grown in F12 medium with 10% fetal bovine serum (FBS). PAE cells constitutively expressing YFP-HA-DAT (PAE/YFP-HA-DAT) were described previously (Sorkina et al., 2009). PAE cells constitutively expressing RFP-HA-DAT (PAE/RFP-HA-DAT) were generated by selection in the presence of G418 (400 μg/ml). HEK293T and HeLa cells were grown on DMEM with 10% FBS. All cell lines were regularly checked for mycoplasma using Lonza (Allendale, NJ) mycoplasma detection kit. For live imaging cells were grown on collagen-coated glass bottom 35 mm dishes (Mat-Tek, Ashland, MA) or Poly-D-Lysine (Sigma)-covered glass coverslips, and for immunofluorescence staining experiments the cells were grown on glass coverslips.

The cells were transfected with DNA plasmids using Lipofectamine (Invitrogen) or Effectene (Qiagen, Valencia, CA) kits following manufacturer's protocols.

ON-TARGET SMARTpool siRNA to MyoX (cat# J-007217-05) and non-targeting siRNA (cat# 1027310) were purchased from Thermo Fisher Scientific (Pittsburgh, PA) and Qiagen, respectively. siRNA transfections were performed with DharmaFECT® Transfection Reagent #1 from Thermo Fisher Scientific according to manufacturer's recommendations and as described previously (Sorkina et al., 2013). Typically, cells were assayed 72 h after siRNA transfection. Efficiency of MyoX knock-down was determined in each experiment by immunofluorescence and Western blotting.

2.4. DA neuronal cultures

Primary mesencephalic postnatal cultures were prepared from HA-DAT knock-in mice as previously described (Ding et al., 2004; Rao et al., 2012). Experiments were performed on neurons at days in vitro (DIV) 6–10. Cultures were grown on glass coverslips pre-covered with Poly-D-Lysine and laminin. All animal studies were performed according to an approved IACUC protocol.

2.5. Fluorescence microscopy

For live-cell imaging, the cells grown on Mat-Tek dishes or glass coverslips were placed onto the stage of the spinning disk confocal imaging system based on a Zeiss Axio Observer Z1 inverted fluorescence microscope (with 63× Plan Apo PH NA 1.4), equipped with a computer-controlled Spherical Aberration Correction unit, Yokogawa CSU-X1, Vector photomanipulation module, Photometrics Evolve 16-bit EMCCD camera, HQ2 cooled CCD camera, environmental chamber and piezo stage controller and lasers (405, 445, 488, 515, 561, and 640 nm) (Intelligent Imaging Innovations, Inc., Denver, CO), all controlled by SlideBook software (Intelligent Imaging Innovation, Denver, CO). To obtain high resolution 3D images of the cells, a z-stack of 10–20 confocal images at 200–400 nm z-stepsize was acquired with 37 °C, humidity and 5% CO_2 atmosphere maintained throughout the duration of the imaging. Image acquisition settings were identical during imaging of all experimental variants in each experiment.

For immunofluorescence staining, cells on coverslips were washed with ice-cold PBS (Invitrogen) and fixed with freshly prepared 4% paraformaldehyde for 15 min at room temperature. To detect surface EGFR (transient expression of EGFR-ΔC), the cells were incubated with EGFR antibody Mab528 and subsequently with the secondary anti-mouse antibody conjugated to Cy5. To detect MyoX and other intracellular proteins, fixed cells were permeabilized with 0.1–2% Triton X-100 for 3 min and incubated with appropriate primary and secondary antibodies in PBS containing either 0.5% BSA or 0.05% Tween-20. Each antibody incubation was followed by three 2-min wash in the same buffers. Both primary and secondary antibody solutions were pre-cleared by centrifugation at 100,000 ×*g* for 20 min. Coverslips were mounted in ProLong Gold (Invitrogen).

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