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ORIGINAL ARTICLE / ÖZGÜN ARAŞTIRMA

Determination of spatial proximity between the N-terminus and cocaine binding site of the dopamine transporter by FRET

Dopamin taşıyıcıların kokain bağlama bölgesi ile N-ucu arasındaki uzaysal yakınlığının FRET ile belirlenmesi

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ABSTRACT

Objective: The dopamine transporter (DAT) mediates uptake of dopamine from the synaptic cleft and provides rapid termination of neurotransmission. It is the site of action for different drugs of abuse, including cocaine and amphetamine. Serine 7 and 12 at the N-terminus were found to be critical residues in phosphorylation. Here, we addressed spatial proximity site relationship of N-terminal extension with respect to cocaine binding in wild type and Ser7/12Asp and Ser7/12Ala mutants.

Materials and Methods: The yellow-fluorescent protein (YFP) and mutations were introduced into the N-terminus of the hSynDAT by a two-step PCR. Dopamine uptake assays were performed and Förster resonance energy transfer (FRET) distances were determined using donor bleach method in a confocal microscope.

Results: All N-terminally YFP-tagged DAT constructs were properly trafficked to the cell surface. K_m values were 0.76, 0.75 and 1.24 μM for wild-type, Ser7/12Ala and Ser7/12/Asp constructs, respectively. FRET efficiency value was found to be 0.15 for YFP-labeled DAT while corresponding estimated distance was calculated as 68.4 Å. FRET efficiencies of other constructs were negative and no energy transfer was detected.

Conclusion: Our data show that extreme end of N-terminus is in FRET distance from cocaine binding site and mutation of critical serine residues to the phosphorylation-mimicking form (Ser7/12Asp) have increased this distance.

Keywords: Dopamine transporter (DAT), Cocaine binding, Förster resonance energy transfer (FRET), N-terminus of DAT, Phosphorylation of DAT

ÖZET

Amaç: Dopamin taşıyıcılar (DAT) dopaminin sinaptik geri-alımından ve böylelikle sinirsel iletinin çabuk sonlanmasından sorumludur. Bu proteinler alışkanlık yapıcı kokain, amfetamin gibi maddelerin de etki merkezlerini oluşturur. Proteinin N-ucunda bulunan Serin 7 ve 12'nin fosforilasyon bakımından kritik amino asitler olduğu bilinmektedir. Bu çalışmada N-ucu ile kokain bağlama bölgesi arasındaki uzaklık, yaban tip ve Ser7/12Asp ve Ser7/12Ala mutantlarında belirlenmiştir.

Gereç ve Yöntem: Sarı-floresan protein (YFP) ve mutasyonlar DAT'ın N-terminal ucuna 2-aşamalı PCR ile yerleştirilmiştir. Dopamin geri-alım testleri yapılmış ve konfokal mikroskopta verici-ağartma yöntemi ile Förster rezonans enerji transferi (FRET) mesafeleri belirlenmiştir.

Bulgular: Tüm N-ucundan YFP-etiketlenmiş DAT proteinleri düzgün olarak hücre yüzeyine taşınmıştır. K_m değerleri sırasıyla yaban tip, Ser7/12Ala and Ser7/12/Asp mutantları için 0.76, 0.75 ve 1.24 μM olarak saptanmıştır. FRET etkinliği yaban-tip DAT için 0.15 olarak bulunmuş ve FRET uzaklığı 68.4 Å olarak bulunmuştur. Diğer konstraktlarda FRET etkinlikleri negatiftir ve enerji transferi saptanmamıştır.

Sonuç: Veriler bize N-ucu bölgesinin kokain bağlama bölgesi ile FRET mesafesinde olduğunu ve kritik serin rezidülerinin mutasyonlarının (Ser7/12Asp) bu mesafeyi arttırdığını göstermektedir.

Anahtar kelimeler: Dopamin taşıyıcı (DAT), Kokain bağlama, Förster rezonans enerji transferi (FRET), DAT amino terminal, DAT fosforilasyonu

Introduction

The concentration of dopamine in the synaptic cleft is elaborately controlled by dopamine transporters (DAT) which are responsible for the uptake of dopamine from the cleft back into the presynaptic terminal [1, 2]. The dopamine transporter belongs to a family of Na^+/Cl^- -dependent

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solute carriers, which also include other closely related neurotransmitter transporters such as norepinephrine (NET) and serotonin transporters (SERT). The transport mechanism couples substrate translocation to the electrochemical gradients of Na^+ and Cl^- therefore, they are also referred to as sodium symporters.

Since this family of molecules manages fast removal of biogenic amines and amino acids from the synaptic cleft, dysfunction of them results in multiple emotional and cognitive disturbances, including depression, Parkinson and epilepsy. The reward stimulating effects of commonly abused drugs like cocaine and amphetamine (AMPH) is shown to be mainly due to inhibition of DAT and prevention of uptake mechanism [3].

The high hydrophobicity due to twelve membrane spanning domains hampered high-resolution structure analysis of these proteins for years and our knowledge of function were mostly from indirect biochemical methods. After intensive years of research, crystallization of a bacterial homolog leucine transporter (LeuT) from *Aquifex aeolicus* was achieved at 1.65 Å resolution and provided valuable insight to the structure-function relationship of the protein [4]. However, amino acid sequence alignment of LeuT and DAT shows that amino terminus of bacterial homolog aligns with the 56th position of DAT and flexibility of this region puts constraints to our understanding of its functional interactions. Therefore conformational changes of N-terminus are still focus of interest [5].

In this study, we aimed to address the possible involvement of extreme N-terminus and its phosphorylated forms of DAT in substrate binding. Förster resonance energy transfer (FRET) was used as a tool to analyze proximities between N-terminal mutants and substrate binding pocket. Phosphorylation of serine 7 and 12 residues was previously found to be responsible from trafficking, amphetamine-induced efflux and cocaine binding of dopamine transporters [6-8] . Therefore, two serine residues were mutated to either aspartic acid or alanine to test the effect of phosphorylations and FRET efficiencies between the yellow-fluorescent protein (YFP) and a fluorescently labeled cocaine analog were determined. We observed that neither the N-terminus, nor its phospho-mimicking forms approach to the substrate binding site upon cocaine analog binding and the distance between N-terminus and ligand binding site becomes greater in constructs involving serine mutations.

Materials and Methods

Constructs

The cDNA encoding the human dopamine transporter and specifically engineered with artificially introduced restriction sites (hSynDAT) was cloned into hygromycine resistant mammalian expression vector pCIHygro. The YFP was added to the N-terminus of the hSynDAT cDNA by two-step polymerase chain reaction (PCR) using Phusion polymerase (Finnzymes, Espoo, Finland). SynDAT and its forms containing either S7A-S12A or S7D-S12D mutations were used as templates. All constructs were confirmed by restriction enzyme digestion and by automated DNA sequence analysis (MWG Biotech, Ebersberg, Germany).

Cell culture and transfection

Human embryonic kidney (HEK) 293 cells were maintained at 37°C in 5% CO_2 grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 0.01 mg/ml gentamicin (Invitrogen). The cells were seeded at a density of 1-1.5 x 10^6 for stable, 3-3.5 x 10^6 for transient cultures per 75 cm^2 flask. Transfection was performed using either lipofectamine or lipofectamine 2000 reagent (Invitrogen). Briefly, 2 µg of DNA was combined with 8 µl of lipofectamine for 30 min. at room temperature and then this mixture was added to the previously seeded cells. Transfection was allowed to occur during 5 hour incubation at 37°C in CO_2 incubator.

[^{3}H] Dopamine uptake experiments

Transfected cells were plated on the poly-D-lysine coated 24-well dishes two days before the uptake test as 100000 cells/well. Prior to the experiment, cells were washed with freshly prepared uptake buffer (25 mM Hepes, 130 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM L-ascorbic acid and 5 mM glucose, pH:7.4). Unlabelled dopamine was added to the cells in varying concentrations, and uptake was initiated by the addition of 10 nM [^{3}H] dopamine. After 10 min. incubation, cells were lysed and and counted by Wallac Tri-Lux β-scintillation counter (Wallac). Nonspecific uptake was determined in the presence of 1 mM non-labeled dopamine. All tests were performed in triplicate.

Confocal Microscopy

Cells were seeded on 8-well Lab-Tek chambers as 40.000 cells/well two days before the experiment. Donor bleach method was applied in which protection of YFP from bleaching (488 nm, 60% laser power) was an indicator of FRET between donor YFP and acceptor rhodamine-labeled cocaine analogue JHC 1-064 (kindly provided by Amy

H. Newman from NIDA-IRP, Baltimore, MD). 488 nm at 10% power has been used as an excitation wavelength, while emission spectrum is confined using BP 505-530 filter. 4xzoom was applied during all measurements. Cells were bleached in the YFP channel by scanning a region of interest (ROI) 60 times using the 514 argon laser line. All determinations were performed at least in 8 repeats or more. Bleaching time constants (τ_{bl}) of donor only (τ_d) or donor and acceptor ($\tau_{d,a}$) labeled cells were calculated using GraphPad one phase exponential decay analysis. Energy transfer efficiences were calculated according to the following formula: ET(%)=[1 - (τ_d / $\tau_{d,a}$)] [9].

Results

N-terminally YFP-tagged DAT (NTp1-YFP DAT) and double serine mutants to mimic phosphorylation/ dephosphorylation forms (S7A-S12A and S7D-S12D YFP DAT), were constructed as described in Methods (Figure 1) and transiently expressed in HEK 293 cells using the bicistronic vector pcHygro. NTp1 YFP DAT was properly trafficked to the cell surface. Introducing YFP-protein to the amino terminus of DAT did not cause to a substantial change in either expression or binding properties of DAT. Uptake assays showed that dopamine binding capacities of all constructs were similar (Figure 2a). Surface expression of all constructs was as efficient as wild type, as demonstrated by confocal microscopy analysis (Figure 2b). There was a small decrement in V_{max} values. In YFP DAT, V_{max} was nearly 50% of nonlabeled construct, but for the phosphor/ dephospho-mimetic mutants (S to A and D conversions), this value increased up to 85%, close to the wild type transport parameters (Table I).

Table I. [^{3}H] dopamine uptake and binding characteristics of human SynDAT and its YFP-labelled mutants.

	EC_{50} (μM) [95% CI]	**V_{max} (fmol/min/10^5 cells ± SE)**	**K_m (μM) [95% CI]**
SynDAT	1.45 [1.01-2.08]	20673±2676	1.45 [1.01-2.08]
NTp1YFPSynDAT	0.64 [0.55-0.74]	11283±1278	0.76 [0.58-0.99]
NTp1YFPSynDATStoA	0.91 [0.87-0.95]	14716±998	0.75 [0.68-0.83]
NTp1YFPSynDATStoD	1.0 [0.97-1.12]	17644±1546	1.24 [1.0-1.5]

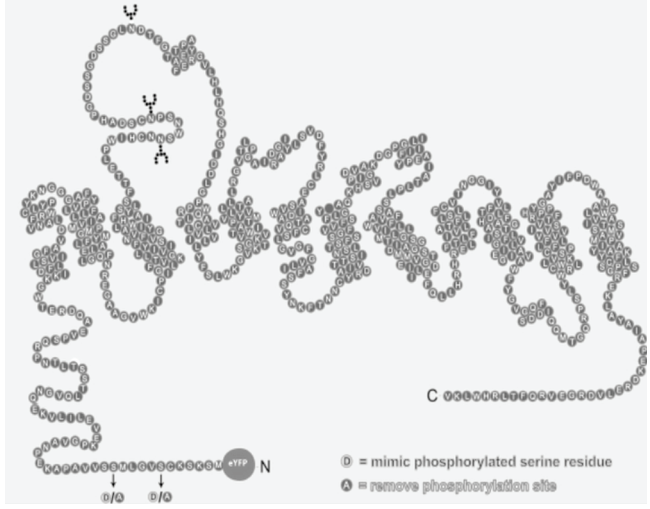

Figure 1. Schematic two dimensional representation of hDAT with introduced modifications.

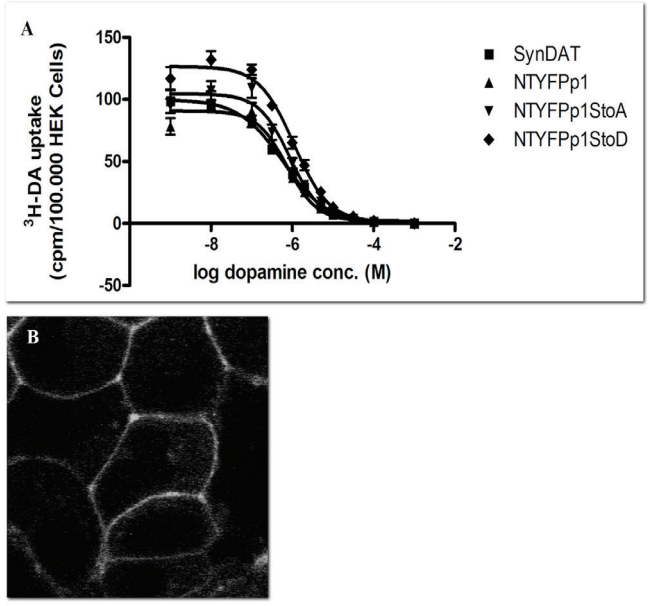

Figure 2. (a) [^{3}H] dopamine uptake of all constructs and **(b)** surface expression of NTp1-YFP DAT. YFP was placed on position 1 of dopamine transporter by two-step PCR-based mutagenesis in the background of SynDAT template as described in Methods. The effect of phosphorylation at two serine positions was examined by establishing Ser / Ala (S7A and S12A) and Ser / Asp (S7D and S12D) mutations. All constructs were functional and showed surface expression similar to NTp1-YFP DAT (data not shown).

Spatial proximity was examined using fluorescently labeled cocaine analog JHC 1-064 as substrate (Figure 3). JHC 1-064 was previously shown to preferentially label surface expressed dopamine transporter [10]. The average lifetime of YFP in the absence and presence of the ligand was 2.13±0.21 and 2.51±0.39 s for N-terminus YFP

labeled DAT, 2.42±0.43 and 2.16±0.25 s for double serine to alanine mutant, 2.65±0.17 and 2.44±0.2 s for double serine to aspartic acid mutant**,** respectively. Half-lives as determined by one-phase exponential decay analysis were not significantly different for three constructs (Table II).

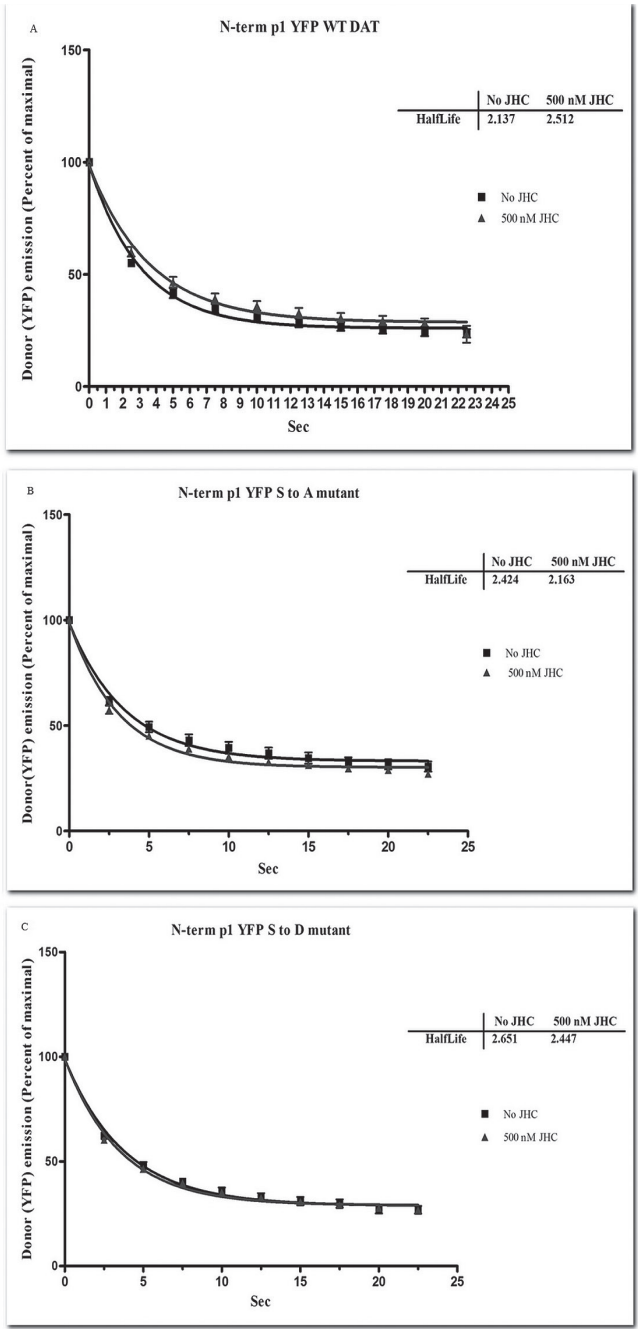

Figure 3. FRET curves of hDAT constructs with YFP introduced at position 1 for **(a)** WT, **(b)** S7A-S12A and **(c)** S7D-S12D forms. Cocaine analog JHC1-64 – induced intensity changes were measured during photobleaching recovery period. Data are averaged from 8-12 independent experiments. Curves represent summate of all experiments after exponential decay analysis.

Table II. Comparison of bleaching recovery half-lives as determined by one-phase exponential decay analysis. Comparison of half-lives (n=8) between substrate bound / unbound forms and three-types of constructs.

	No JHC 1-64 [95% CI]	**JHC 1-64 (500 nM) [95% CI]**	**P -value[a]**	**P -value[b]**
NTp1YFPSynDAT	2.13 [1.9 – 2.45]	2.51 [2.2 – 2.99]	0.119	
				0.85
NTp1YFPSynDATStoA	2.42 [2.0 – 2.95]	2.16 [1.97 – 2.39]	0.266	
NTp1YFPSynDATStoD	2.65 [2.36 – 3.0]	2.44 [2.2 – 2.7]	0.058	

P<0.05 was considered statistically significant.
[a] Comparison of substrate bound / unbound forms
[b] Comparison of N-terminal YFP labeled WT, StoA and StoD DAT

To correct for spectral bleed-through, background measurements were taken in the donor channel (DD; excitation and emission wavelengths for YFP are 514 and 527 nm respectively) and in the FRET channel (DA; excitation wavelength for YFP is 514 where emission wavelength for rhodamine is 585 nm) separately and all values were corrected with respect to background. Distances were determined by using efficiency values according to the formula: $r = R_0 \{ (1 / E – 1) \}^{1/6}$, where R_0 was calculated as 51.29 Å. FRET efficiency value was found to be 0.15 for YFP DAT while corresponding estimated distance between donor-acceptor pair in correlation with efficiency was 68.4 Å. Both S7A-S12A and S7D-S12D YFP DAT were out of FRET distance with negative transfer efficiencies.

Discussion

The possession of twelve transmembrane segments has overshadowed structural studies on the family of Na^+/Cl^--dependent solute carriers for years, until the crystal structure of their bacterial homolog leucine transporter was released in 2005 [4]. Large amount of information gathered since then by experimental and computational efforts have enlightened many details of their mechanism of action, but many aspects of dynamic regulations in mammalian counterparts still remain elusive [11, 12].

Though high similarities exist between primary structures of bacterial and mammalian transporters, especially in their transmembrane segments and some functionally important residues, the leucine transporter shares only 20-25%

overall sequence identity with its eukaryotic counterparts. The N-terminus of the crystallized leucine transporter aligns with the 56th position of the dopamine transporter and the flexible structure of N-terminal extensions in general is not suitable for high resolution determinations. Therefore indirect biochemical approaches such as FRET are still important tools to examine functional properties of membrane proteins.

There are accumulating reports on the importance of the N-terminus in DAT function. The N-terminus and the first transmembrane domain of the transporter play important role in substrate binding and function of the protein [5]. Furthermore, the N-terminal region of transporter contains phosphorylation sites, which are targets for physiologically important kinases such as protein kinase C (PKC) or calcium-calmodulin-dependent protein kinase II (CaMKII). The serine cluster located at the N-terminus was found to be a substrate for PKC phosphorylation. There are many studies which reveal role of phosphorylation in transporters, as summarized by Kristensen et al [13]. Serine 7 mutation was indicated to reduce ability of phorbol 12-myristate 13-acetate (PMA) action and thereby cause the loss of PKC-stimulated phosphorylation, whereas serine 12 was involved in mitogen-activated protein (MAP) kinase pathways [6]. In addition, N-terminal phosphorylation was found to affect amphetamine-stimulated dopamine efflux, a process which leads to the reverse transport of dopamine and depletion of transmitter stores in the presynaptic terminal [7]. The same study especially showed that Ser 7 and Ser 12 phosphorylation is essential for AMPH-induced efflux current and mutation of these residues significantly impaired efflux. It also suggested that the phosphorylation of these residues drive DAT to the accessible state for efflux, without affecting the uptake. DAT is subject to PKC-mediated stimulation through the N-terminus, but truncation of the N-terminus did not change PKC-mediated endocytosis of DAT [8,14]. The importance of N-terminal serine residues were further investigated by a recent study by Moritz et al. [15]. In this study, phosphorylation of N-terminal serines (Ser-4, Ser-7 and Ser-13) by various kinases were confirmed and it was found that the mutation of Ser-7 strongly reduced the affinity of rat DAT for a cocaine analog, β-CFT ((−) -2-β-carbomethoxy-3-β-(4-fluorophenyl) tropane).

To determine dynamic spatial changes in distal N-terminus upon cocain binding, we applied site-directed mutagenesis to generate different dopamine constructs of DAT and used FRET method. FRET is a useful tool to estimate intra- or inter- molecular distances and can be used as molecular ruler. Donor photobleaching FRET based on the principle that the bleaching rate of the donor fluorophore is affected by FRET. Here we observed protection from bleaching by using YFP and rhodamine as FRET pairs. Acceptor background correction was achieved by measuring YFP emission in the absence and presence of ligand, since the signal was weakened due to the high background from unbound JHC 1-064. FRET pair consist of YFP at the extreme N-terminus of transporter and rhodamine at the cocaine binding site exhibited very low level of FRET interaction and calculated distance was inside the limits of FRET distance for wild type (68.4 Å). However, there was almost no detectable energy transfer in phospho-mimicking mutants, neither in S7A and S12A nor in S7D and S12D conversions, of N-terminally YFP-labeled dopamine transporter (half-life values of donor-acceptor pair were shorter compared to donor alone). The low FRET signal observed in wild type was probably the result of the higher order multimeric structure of the transporters.

Overall, our results show that YFP-labeling at the extreme N-terminus did not cause a dramatic change in the affinity of the transporter to substrate and in its surface expression, but reduced the V_{max} value nearly to half of that of the wild type. V_{max} values were similarly reduced in other mutants, although the S to D form had closer values to those of YFP-DAT. Our data support the observations that S7/12A conversion slightly decreases affinity to cocaine analogs [8]. The S7/12D mutation had similar effect, but did not result in a major influence on function [15]. FRET signals imply a conformational convergence between N-terminus and substrate binding site of DAT for wild type. On the other hand, there was no such approach when serine residues were mutated. These results suggest an extended form for the N-terminus of DAT similar to that of the leucine transporter and also show that phosphorylation caused a slight alteration, but did not induce a dramatic change in the conformation of the transporter upon cocaine binding.

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