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Impact of disruption of secondary binding site S_2 on dopamine transporter function

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Abstract

The structures of the leucine transporter, drosophila dopamine transporter, and human serotonin transporter show a secondary binding site (designated S_2) for drugs and substrate in the extracellular vestibule towards the membrane exterior in relation to the primary substrate recognition site (S_1). The present experiments are aimed at disrupting S_2 by mutating Asp476 and Ile159 to Ala. Both mutants displayed a profound decrease in [^{3}H]DA uptake compared with wild-type associated with a reduced turnover rate k_{cat}. This was not caused by a conformational bias as the mutants responded to Zn^{2+} (10 μM) similarly as WT. The dopamine transporters with either the D476A or I159A mutation both displayed a higher Ki for DA for the inhibition of [3H]CFT binding than did the WT transporter, in accordance with an allosteric interaction between the S_1 and S_2 sites. The results provide evidence in favor of a general applicability of the two-site allosteric model of the Javitch/Weinstein group from LeuT to DAT and possibly other monoamine transporters.

Keywords

dopamine binding; transporter point mutants; cocaine analog binding; CFT; WIN 35,428; Zinc

Introduction

The dopamine, serotonin, and norepinephrine transporters accumulate monoamine substrate against its concentration gradient with the required energy provided by the electrochemical gradient for Na^+ (Schmitt & Reith 2010). Structural information for the monoamine transporters comes from the crystals of the leucine transporter (LeuT), drosophila DAT (Yamashita *et al.* 2005, Zhou *et al.* 2007, Penmatsa *et al.* 2013), and recently the human serotonin transporter (Coleman *et al.* 2016), showing an additional secondary site (S_2) in the extracellular vestibule to a primary binding site (S_1) for substrates and various types of inhibitors (Yamashita et al. 2005, Zhou et al. 2007, Penmatsa et al. 2013, Coleman et al. 2016, Zhou *et al.* 2009). It was proposed by the Javitch/Weinstein group that the S_2 site in

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LeuT plays a crucial role in substrate transport: Binding of substrate to the S_2 site triggers the release of Na^+ and substrate from the S_1 site (Shi *et al.* 2008). On the other hand, studies by the Gouaux group suggested that LeuT has only one binding site for leucine measured by various biophysical methods (Piscitelli et al., 2010); S_1-mutation F253A-LeuT still only showed leucine binding to S_1 (Wang & Gouaux 2012); and LeuT structures obtained in bicells in the absence of S_2-interfering n-octyl-β-D-glucopyranoside did not contain S_2-bound substrate (Piscitelli *et al.* 2010, Wang *et al.* 2012b, Wang & Gouaux 2012). The Javitch/Weinstein group, however, showed destruction of the S_2 site by protein preparation procedures used for crystallography, resulting in a substrate: LeuT stoichiometry of 1 (Quick *et al.* 2012). In a convincing computational study, the two-site substrate transport model has been successfully applied to human (h) DAT (Shan *et al.* 2011), and a functional role for the S_2 site has been firmly established in the serotonin transporter where it allosterically interacts with the S_1 site (Zhu *et al.* 2016, Plenge *et al.* 2012). The S_2 and S_1 sites are separated by 11–13 Å, and can be bridged by bivalent substrate ligands in our computational models (Schmitt *et al.* 2010). Enhanced binding affinities were observed with ligands bearing two active heads of DA, or heterologous combinations of DA, β-phenethylamine and amphetamine heads, up to two orders of magnitude over the original monovalent substrate (Schmitt et al. 2010).

The present experiments are aimed at studying the role of the S_2 site in transport of DA by hDAT. To this end, the S_2 site was compromised in D476A and I159A in the computational model (Shan et al. 2011). Ile159 corresponds to Ile111 in LeuT, known to be crucial in interaction with the substrate (Shi et al. 2008) and Asp476 in a dDAT-based computational model of hDAT forms a salt-bridge with Arg85 at the bottom of the S_2 site as part of the closed external gate in the occluded form of DAT with DA bound to the S_1 site (Cheng *et al.* 2015). We hypothesized a major impact of S_2 disruption on DAT function, pointing to an important role of the S_2 site in the substrate translocation process. In order to better understand the two-site model for DA transport, we studied (i) the capability of the two S_2 mutants to transport DA, (ii) their conformational bias with micromolar concentrations of Zn^{2+} known to promote outward-facing DAT (see Discussion), and (iii) their interaction with DA as measured by inhibition of binding of the cocaine analog [^{3}H]CFT.

Materials and methods

Site-directed mutagenesis and heterologous expression of hDAT mutant constructs

The mutants I195A and D476A were generated (WT-hDAT cDNA is from Dr. Jonathan Javitch at Columbia University) with the Quick-change site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's protocol and confirmed by DNA sequencing (Genewiz Inc., South Plainfield, NJ, USA). The pair of primers for I159A were 5'-cttcttttacaacgtcgccattgcctgggctctcc-3' and 5'-ggagagcccaggcaatggcgaxcgttgtaaaagaag-3' while primers for D476A were 5'-gaatctacgtgttcaccctcctggaccacttcgctgccggcagcac-3' and 5'-gtgccggcagcgaagtggtccaggagggtgaacacgtagattc-3'.

Culturing and stable transfection of Lewis Lung Carcinoma-Porcine Kidney (LLC-PK_1) cells (gift from Dr. Roxanne Vaughan at University of North Dakota) with human dopamine

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transporter (hDAT) constructs by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was carried out as in our previous work (Chen *et al.* 2001). Stable cells were maintained in minimum-essential medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 200 μg/mL G418 with 5% CO_2 at 37°C in a cell incubation chamber. Cells were grown to about 70% confluence in culture plates prior to experimentation. To enhance the expression of hDAT I159A, sodium butyrate (NaBt) was added into cell culture plates at final concentration of 5 mM and incubated for 24 hrs before each experiment. Constitutional approval applied to the work with cloned human transporters in heterologous cell lines and bacterial amplification (Biosafety Level 2).

[^{3}H]CFT binding assay and [^{3}H]dopamine uptake assay

[^{3}H]CFT (CFT=2β-carbomethoxy-3β-[4-fluorophenyl]-tropane, 85.9 Ci/mmole) and [^{3}H]DA (48 Ci/mmole) are from Perkin Elmer (Boston, MA, USA). Binding of [^{3}H]CFT (Chen et al. 2001) and [^{3}H]DA uptake assays (Ng *et al.* 2014) to stably transfected LLC-PK_1 cells were measured by procedures as described by us previously. Briefly, intact cell suspensions were incubated with [^{3}H]CFT (4 nM final concentration) and varying concentrations of non-labelled CFT or DA spanning their IC_{50} values for 20 minutes at 21°C. A final concentration of 10 μM CFT was used to define nonspecific binding. [^{3}H]DA (5nM final concentration) and non-labelled DA were added into 24-well cell culture plates and incubation continued for 5 min at 21°C.

Immunoblotting studies

Surface DAT was assessed with sulfo-NHS-SS-biotin as described by us previously (Ng *et al.* 2014). Total lysates and biotinylated proteins were resolved on 8% Tris-Glycine mini gels and probed with polyclonal anti-DAT antibody against the N-terminal of DAT (Millipore, Billerica, MA, USA), followed by HRP-conjugated goat anti-rabbit antibody (Thermo scientific, Waltham, MA, USA). Polyclonal anti–β-actin antibody (Sigma-Aldrich, St. Louis, MO, USA) was used as an internal control for loading. The transporter signal was visualized using Thermo Scientific SuperSignal West Pico Chemiluminescent Substrate solution (Thermo Scientific, Waltham, MA, USA) and quantified using Image-J (from website of National Institute of Heath).

Data analysis

The Michaelis-Menten constant (K_m) and maximal velocity (V_{max}) of [^{3}H]dopamine uptake and the binding affinity (K_d) and the maximum binding site (B_{max}) of [^{3}H]CFT binding were calculated by non-linear regression with the Radlig software (KELL program). Half maximal inhibitory concentration (IC_{50}) was estimated by logistic fitting of data by the ORIGIN software (Origin Lab Co.) and converted to inhibition constant (K_i) by the Cheng-Prusoff equation. Statistical analyses were done with SigmaPlot software (Systat Software Inc. San Jose, CA, USA). Results are expressed as the Mean±SEM averaged from at least three independent experiments.

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Statistical analysis

Multiple comparisons of group means were performed by one-way ANOVA followed by Dunnett's Method. The Student's t-test was used for comparing two group values, and the one-sample t-test for comparing effects with a theoretical effect of 0%, two-tailed in both cases.

In preparing the present work for publication, the guide for ethical behavior in publishing research was followed as described in the COPE Report 2003 (available from the Committee on Publication Ethics (COPE)).

Results

Effect of mutations on DAT expression and function

Surface expression of hDAT expressed in LLC-PK_1 cells was evaluated by [^{3}H]CFT binding and biotinylation. The B_{max} of [^{3}H]CFT binding at intact cells reflects surface DAT (Chen *et al.* 2004b) and indeed a close correspondence was observed between the B_{max} values (WT > D476A > I159A, Table 1) and the biotinylation density values (WT > D476A > I159A, Fig. 1A). D476A and I159A displayed 55.2% and 23.9% of the B_{max} vaIue of WT, and 64.8% and 19.3% of the biotinylation density value of WT. It should be noted here that in order to enhance the surface expression (Liang *et al.* 2013, Chen & Reith 2007) of I159A cells, 5 mM NaBt was applied; without NaBt the expression of I159A was extremely low. No differences between the constructs were observed for the [^{3}H]CFT binding K_d, in contrast to the affinity of DA as measured by competition for [^{3}H]CFT binding. Both mutants displayed much weaker affinities for DA than WT: DA's K_i was increased sixteen-fold in D476A (from 4.82 to 83.6 μM) and five-fold in I159A (to 26.1 μM) (Table 1, top panel).

Disruption of the S_2 site in D476A greatly decreased the [^{3}H]DA uptake activity, mostly due to a decrease in V_{max} (an order of magnitude) and to a lesser extent to an increase in K_m (4-fold) (Table 2). The V_{max} was also decreased (order of magnitude), along with increased K_m (2-fold), for I159A. The V_{max} effect is necessarily a function of the low membrane expression of hDAT. In order to take that out of the equation, DA turnover k_{cat} (V_{max} / B_{max}) was computed, and it was appreciably reduced in the mutants (3- to 9-fold) (Table 2). According to the simplified carrier model as used by others (Schomig *et al.* 1988) and ourselves (Chen et al. 2001), knowledge of the values of the parameters in Table 2 and additionally the DA K_i and [^{3}H]CFT binding B_{max} as listed in Table 1, allows the calculation of the forward-rate constant for loaded-transporter k_2 and the reorientation-rate constant for empty-transporter k_3 (Table 2). As noted in our previous work with multiple mutants of hDAT, k_{cat} largely reflects k_3 (Chen et al. 2001), consonant with reorientation of the empty transporter being the rate-limiting step in transport. Noteworthy, k_3 shows the same 3- to 9-fold reduction in the mutants compared with WT as k_{cat} does (Table 2). However, there were also notable reductions in k_2 (Table 2).

"Equilibrium" binding experiments and the effect of Zn^{2+}

Zn^{2+} (10 μM) increased the affinity (decreased K_d) of [^{3}H]CFT binding to a similar extent in D476A as in WT; a somewhat smaller decrease in I159A (Table 1 bottom panel). This is in

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contrast to the enhanced effect seen in inward-facing mutants (Liang *et al.* 2009, Chen *et al.* 2004a). Moreover, Zn^{2+} (1 and 10 μM) inhibited DA uptake equally in WT and D476A; somewhat smaller effects were displayed by I159A (Fig. 2); in contrast, an inward-facing mutant generally responds with an *increase* in DA uptake (Loland *et al.* 2002, Loland *et al.* 2004).

Discussion

This report presents k_2 and k_3 values for dopamine's forward translocation by loaded transporter and reorientation by empty transporter, respectively, based on existing kinetic models (Chen et al. 2001, Li *et al.* 2015). However, it should be considered here that the concentration of DA at the S_1 site during the translocation cycle may not be equal to the outside DA in an intact-cell uptake assay with a permeation pathway separating the S_2 and S_1 sites. Yet, it is this $[DA]_{outside}$ that is used for calculating values for K_i and K_m which, in turn, informs k_2 and k_3. Serious doubt regarding this calculation is raised by the present results for D476A. Thus, when the transporter is fully inward facing (open-to-inside), the external gate, one pair of which is salt-bridge Asp476–Arg85, is normally closed and will open up upon reorientation of the transport to outward facing (Shan et al. 2011). In the D476A mutant, reorientation does not need to open the Asp476–Arg85 bond, and therefore one would expect the reorientation-rate k_3 to be, if anything, enhanced, rather than reduced as observed (table 2). Based on this argument, the true magnitude of k_3 may well be different from what we calculate; the k_2 results, which are based on calculations using the same $[DA]_i$, also need to be viewed with great caution. We do not therefore attach much significance to the relatively small decreases (1.5- to 2-fold) observed for the forward transport rate constant k_2 by D476A or I159A mutation (table 2). Decreases of greater magnitude would be likely if the S_2 site plays a major role in forward transport of DA.

Micromolar concentrations of Zn^{2+} are known to promote outward-facing conformations of DAT (Chen et al. 2004a, Liang et al. 2009, Li et al. 2015, Stockner *et al.* 2013). With Zn^{2+}, DAT spends more time in open-to-out conformations in which D79 is in a salt-bridge with R85 and unavailable for participating in binding to DA (see Cheng et al. 2015). Zn^{2+} (10 μM) increased DA's K_i in WT (Table 1 compare top- and bottom-panel). The results, then, can be understood by taking into account that the preferential DAT conformation with DA bound in the S_1 site is the occluded form with the external gate closed (Shan et al. 2011, Cheng et al. 2015). In both mutants, the lower affinity for DA (Table 1 top-panel) remained low with Zn^{2+} (bottom-panel). In a longer-lasting binding experiment, we reasoned that the ambient concentration of DA exposed to S_1 can be equated with outside [DA], and therefore the higher K_i truly indicates a lower affinity of the S_1 site for DA.

Zn^{2+} effects on DA transport are more complex, involving membrane potential (Meinild *et al.* 2004) with changes in both uptake and efflux (Scholze *et al.* 2002). Most recently, a role for internal $[Na^+]$ has been uncovered, such that Zn^{2+} enhances DA uptake in systems with low $[Na^+]_i$ but lowers uptake at high $[Na^+]_i$ (Li et al. 2015). Evidently, our uptake system is in the latter category. Regardless of whether we consider Zn^{2+} effects on uptake or binding in the present experiments, the mutants responded in a similar way to Zn^{2+} as WT (Table 1 and Fig. 2), suggesting S_2 site disruption did not introduce a conformational bias.

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Computational and biochemical experiments in LeuT indicate that binding of a second substrate molecule to the S_2 site triggers intracellular release of Na^+ and the first substrate molecule from the S_1 site thereby affording substrate translocation (Shi et al. 2008). A similar transport mechanism has been proposed for the DAT (Shan et al., 2011). Crucial in the model is the allosteric interaction between the S_2 and S_1 sites, with ligand occupancy in one affecting the functionality of the other. Such allosterism has been firmly established for the serotonin transporter (Zhu et al. 2016, Plenge et al. 2012). The results of the present study on hDAT provide two lines of evidence in favor of a model with a crucial role of the S_2 site in DA transport. First, DA transport is severely impaired in hDAT constructs with mutations of two residues crucial for S_2 site functionality: D476 and I159, as evidenced by the considerable reduction in DAT turnover rate (k_{cat}) (table 2). Impairment of substrate transport by disruptions in the S_2 site has also been observed for a closely related transporter, hNET (Wang *et al.* 2012a): mutation of many of its S_2 residues to alanine disrupts NE transport, notably mutation of W80, R81, Y152, F317, F388, and D473 (equivalents in hDAT are W84, R85, Y156, F320, F391, and D476, respectively). Second, both D476A and I159A display a higher K_i for DA in inhibiting [^{3}H]CFT binding than WT (Table 1 top panel). As mentioned above, this provides evidence for an allosteric effect of S_2 site disruption on the functional state of the S_1 site, which is one crucial property of two-site-mediated substrate transport (Shi et al., 2008). Although it could be thought that the sole role of the S_2 site is to help along the permeation of DA through its trajectory from out to in by providing low-affinity DA binding along the way, the reduced affinity of DA binding to S_2 site-impaired DAT constructs argues in favor of a general applicability of the two-site allosteric model of the Javitch/Weinstein group from bacterial LeuT to DAT and possibly other mammalian monoamine transporters.

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The abbreviations used are

DA	dopamine
DAT	dopamine transporter
CFT	(-)-2-β-carbomethoxy-3-β-(4-fluorophenyl)tropane

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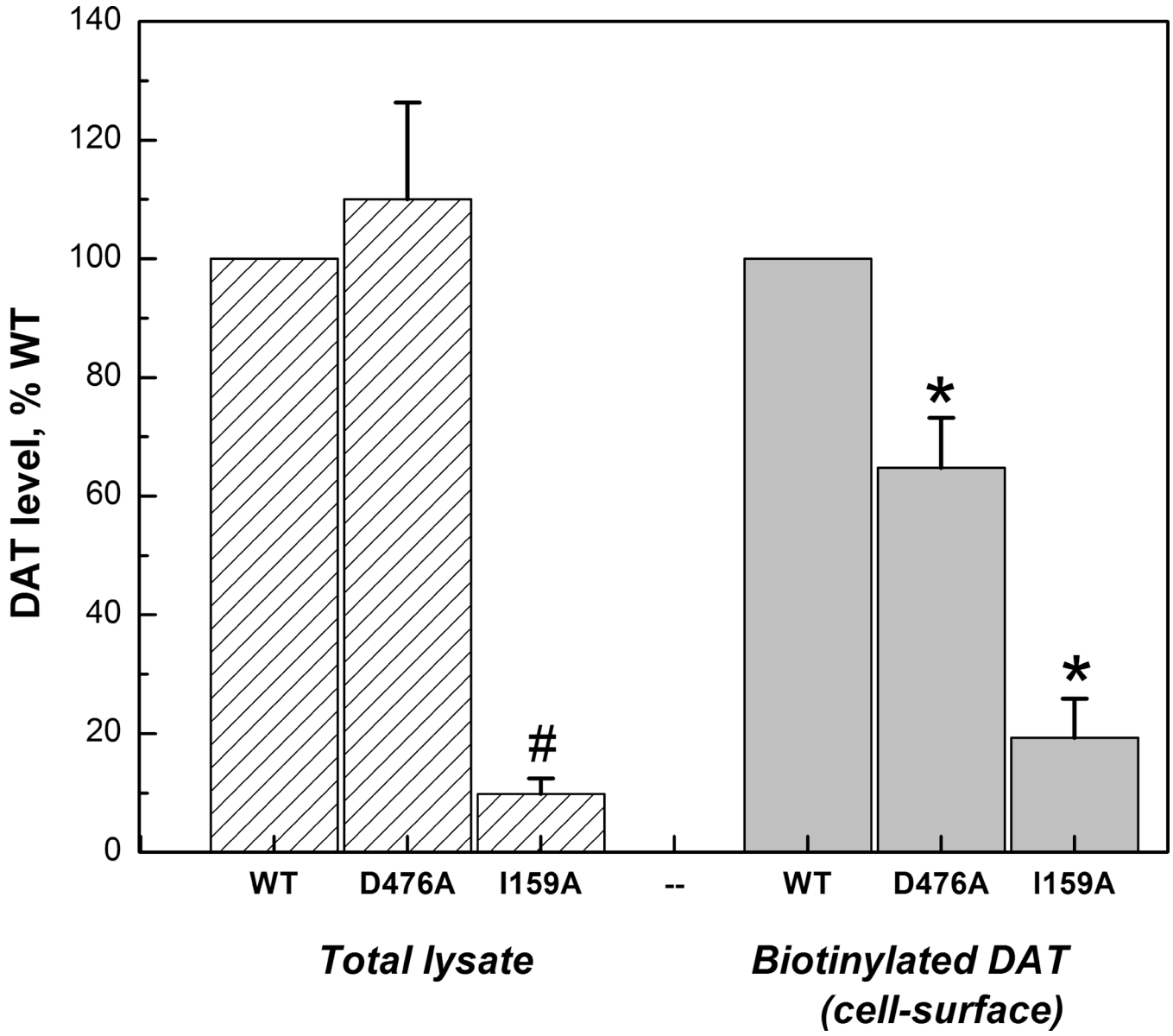

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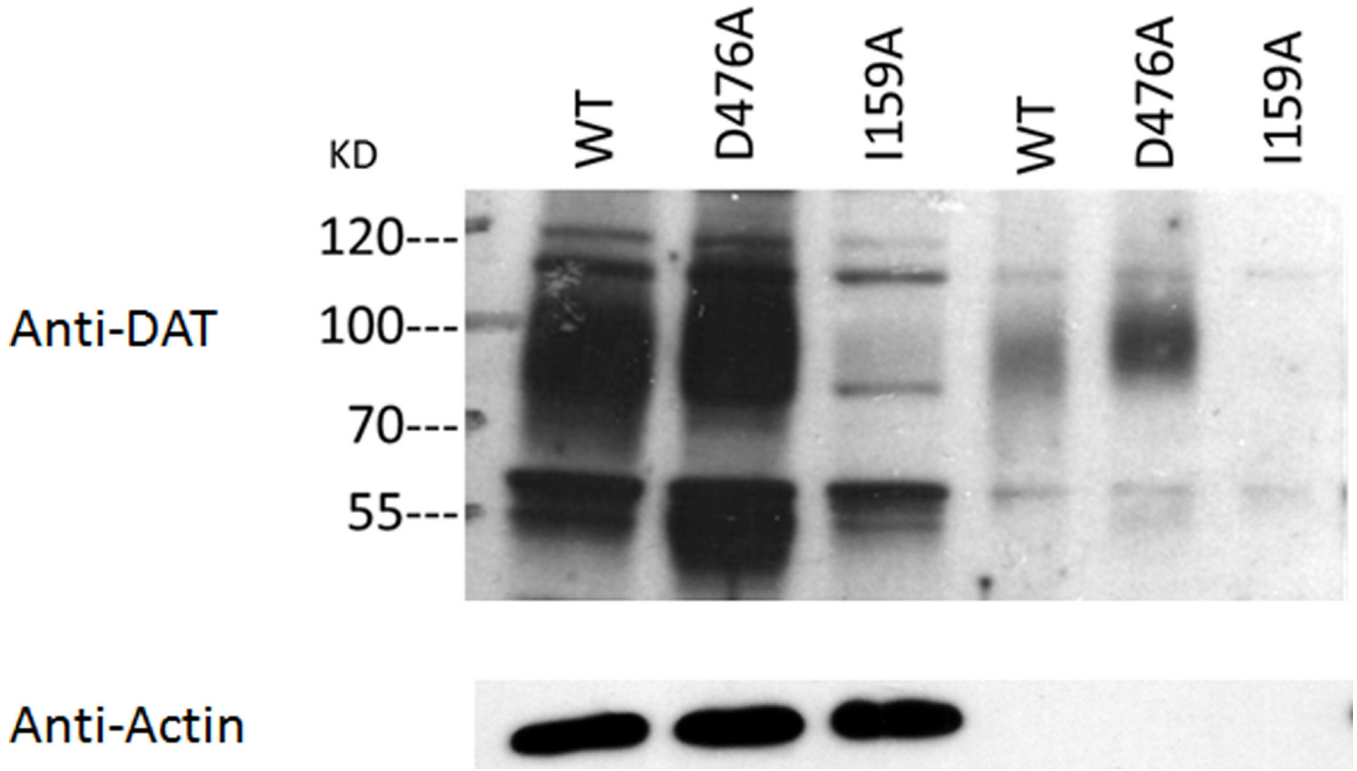

Figure 1.

Quantification of hDAT of total extraction and surface fraction in in LLC-PK1 cells stably expressing hDAT constructs (A) and the representative western blots (B). The integrated-density-value (IDV) for the protein hDAT are expressed as percentage of WT. Note that the bar graphs represent the average and SEM of all obtained results while the gel shown is one typical example.*: P<0.05 vs WT (one-Way ANOVA with post hoc Dunnett's Method)

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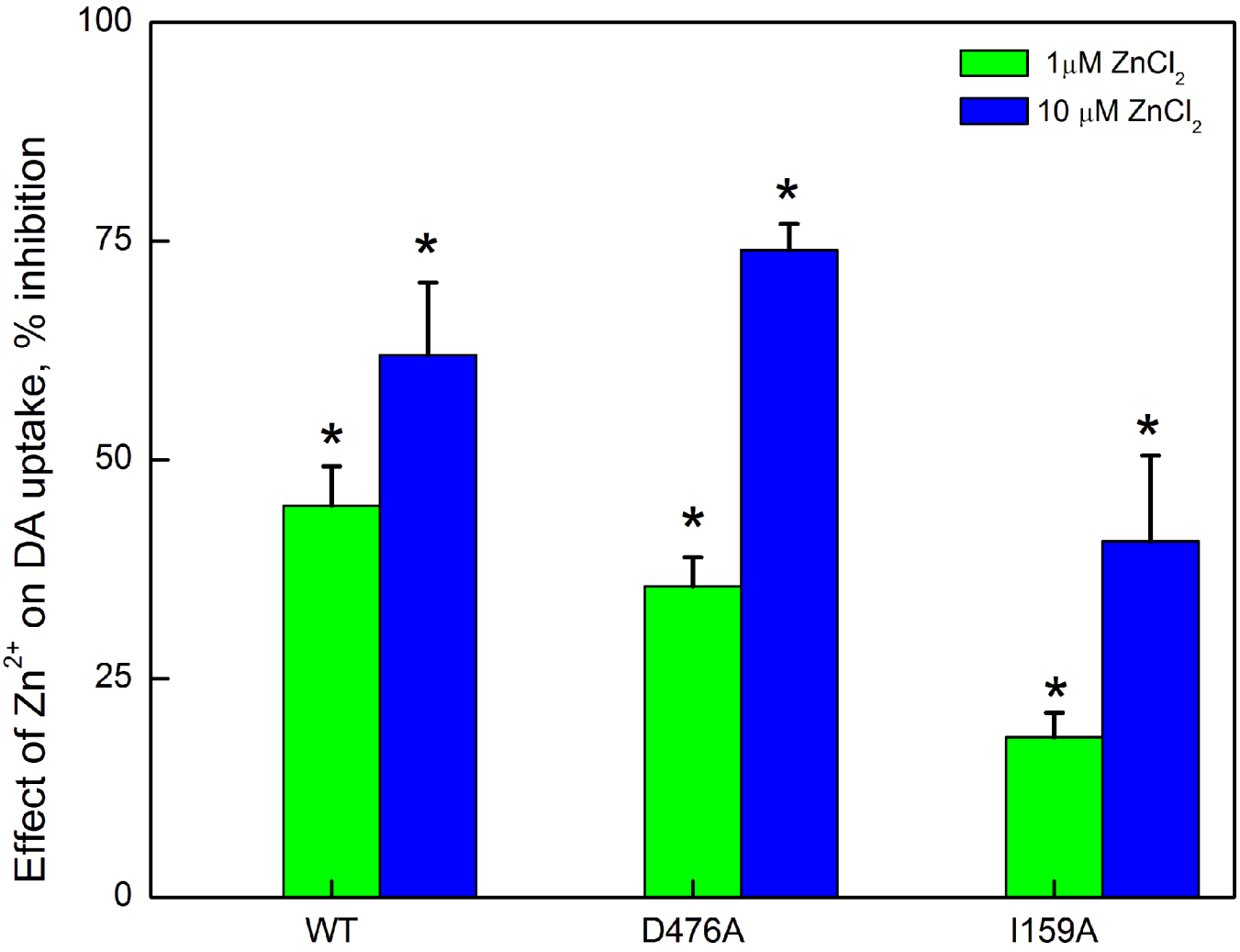

Figure 2.

Inhibitory effect of $ZnCl_2$ on the [3H]DA uptake activity of WT, D476A and I159A. Values are expressed as percent inhibition.*: P<0.05 vs theoretical value of 0% (one sample Student's t-test).

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Table 1

[^{3}H]CFT saturation binding parameters (K_d and B_{max}) and inhibition constant (K_i) of dopamine in competing for the binding of [^{3}H]CFT to hDAT constructs WT, D476A and I159A with (n=4) and without Zn^{2+} treatment (n=3).

Cells	K_d of CFT, nM	B_{max}, pmole/mg	DA K_i, μM
WT	21.5±3.10	2.01±0.22	4.82±0.79
D476A	29.0±7.48	1.11±0.33 *	83.6±3.75 *
I159A	25.1±4.7	0.48±0.08**	26.1±3.88 *
	+ **10 μM $ZnCl_2$**		
WT	6.85±1.01 #	1.28±0.20	67.4±5.49 #
D476A	5.65±1.91 #	1.26±0.02	91.8±21.6
I159A	11.39±1.14 #	0.80±0.21	29.73±5.86

*: P<0.05 vs WT (One-way ANOVA with post-hoc Dunnett's test)

#: P<0.05 vs without Zn^{2+} (Student's t-test)

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Table 2

Kinetic properties of [^{3}H]dopamine uptake for WT, D476A and I159A stably expressed in LLC-PK$_1$ cells (n=3).

Cells	K_m, μM	V_{max},pmole/mg/min	k_{cat}, min^{-1}	k_2, min^{-1}	k_3, min^{-1}
WT	0.76±0.06	4.54±1.18	2.26±0.63	14.3	2.68
D476A	3.27±1.27	0.29±0.10 *	0.26±0.12 *	6.65	0.270
I159A	1.70±0.34	0.33±0.02 *	0.69±0.12 *	10.6	0.738

Additional parameters, DA K_i and [^{3}H]CFT binding B_{max}, needed for these calculations were taken from Table 1.

*: P<0.05 vs WT (One-Way Anova with post hoc Dunnett's Method)