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Latch and trigger role for R445 in DAT transport explains molecular basis of DTDS

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ABSTRACT

A recent study reports on five different mutations as sources of dopamine transporter (DAT) deficiency syndrome (DTDS). One of these mutations, R445C, is believed to be located on the intracellular side of DAT distal to the primary (S1) or secondary (S2) sites to which substrate binding is understood to occur. Thus, the molecular mechanism by which the R445C mutation results in DAT transport deficiency has eluded explanation. However, the recently reported X-ray structures of the endogenous amine transporters for dDAT and hSERT revealed the presence of a putative salt bridge between R445 and E428 suggesting a possible mechanism. To evaluate whether the R445C effect is a result of a salt bridge interaction, the mutants R445E, E428R, and the double mutant E428R/R445E were generated. The single mutants R445E and E428R displayed loss of binding and transport properties of the substrate [^{3}H]DA and inhibitor [^{3}H]CFT at the cell surface while the double mutant E428R/R445E, although nonfunctional, restored [^{3}H]DA and [^{3}H]CFT binding affinity to that of WT. Structure based analyses of these results led to a model wherein R445 plays a dual role in normal DAT function. R445 acts as a component of a *latch* in its formation of a salt bridge with E428 which holds the primary substrate binding site (S1) in place and helps enforce the inward closed protein state. When this salt bridge is broken, R445 acts as a *trigger* which disrupts a local polar network and leads to the release of the N-terminus from its position inducing the inward closed state to one allowing the inward open state. In this manner, both the loss of binding and transport properties of the R445C variant are explained.

Introduction

A key protein in the control of optimal dopamine levels, the dopamine transporter protein (DAT), operates through reuptake of extracellular dopamine. DAT is a Na^+/Cl^- dependent neurotransmitter sodium symporter comprised of 12 transmembrane spanning helices connected by extracellular and intracellular (ECL and ICL) loops. The primary binding site of the substrate is centrally located. This primary binding site (S1) was detailed by the first X-ray structures of the bacterial homolog of DAT, LeuT.[1,2] Computational studies of the migration of substrate through LeuT suggested a number of more weakly bound "stopover" sites on the EC side of the protein.[3,4] One of these sites more proximal to S1, the S2 site, was found to be occupied by tricyclic antidepressants in other X-ray structures of LeuT.[2,4] These models have now been validated by an X-ray structure of hSERT which showed that two molecules of the SSRI S-citalopram (SCIT) were bound to the protein, specifically, one at each of the S1 and S2 sites. Computational studies using the X-ray structure of hSERT supported a similar pathway for the endogenous substrate 5-HT migration through SERT[5] including the presence of the S2 stopover site, also referred to as the vestibule. Based on these findings and others, the emerging model for the overall protein conformations associated with substrate migration considers 3 protein conformational states; 1) outward open (inward closed) 2) outward open and ligand occluded (inward closed) and 3) outward closed (inward open).[1,4,6–13]

Defects in the binding and transport of DA have been shown to have severe effects as evidenced by patients diagnosed with dopamine transporter deficiency syndrome (DTDS).[14–16] The DTDS symptomology is characterized by hyperkinetic movement disorder which progresses to parkinsonism-dystonia. In our recent study, we reported on a cohort of patients whose onset of DTDS

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ranges from infancy to adolescence and whose DAT deficiency is attributed to various identified mutations in DAT.[16] In order to better understand the molecular basis of DTDS, we had embarked on structural investigations of DAT and related transporter proteins. Using comparative modeling methods based on the X-ray crystal structure of LeuT[14–16], we examined the location of five independent mutations[16] heretofore ascribed to DTDS patients. Four of these mutations were in the extracellular vestibule (S2) region of DAT suggesting an adverse effect on initial substrate binding at this site. A fifth mutation, R445C, was located well towards the intracellular side of the protein distal to the S1 primary and S2 secondary substrate binding sites and thus confounding rationalization of its role. Subsequent X-ray structures of dDAT and hSERT[17–20] indicated a possible mechanism which led us to investigate the molecular basis of R445C DAT deficiency as well as to reassess the four previously examined mutations.

Results and discussion

Mutations in the extracellular a.k.a. vestibule region of DAT

In the Ng et al. study[16], we used a homology model for DAT based on the X-ray structure of LeuT to examine the 4 extracellular region mutants associated heretofore with DTDS, i.e., A314V, G386R, Y470S and R85L. Here we reassessed these variants using the subsequently published X-ray structures of dDAT and hSERT which inherently provide more relevant structures. Fig. 1 shows an X-ray structure of dDAT (PDB entry 4XP1[20]) highlighting the various residues associated with DTDS occurrence. For visual reference purposes we show the position of the SCIT molecule occupying the S2 position in SERT (Fig. 1B and D) based on a superposition of the X-ray structures of dDAT and hSERT. In support of our previous findings, the mutant variants are located in the upper, vestibule region near the S2 site suggesting interference with substrate binding. For instance, A314 sits on the outer surface of dDAT pointing away from the S2 pocket which is consistent with the relatively smaller effect of the A314V mutant on DA function and, consequently, the extent of DTDS phenotype. Conversely, R85, which is fairly close to A314 points directly into the vestibule and overlaps with the position at which SCIT binds to SERT. This is consistent with the more deleterious effect of the R85L mutation on DA binding (vida infra) and thus the severity of disease.

The R445C mutation

In agreement with our earlier report, R445 is located on the intracellular side of TM9 distal from the S2 and S1 sites (Fig. 1). It has thus been unclear as to how the R445C mutation linked to cases of DTDS can have the reported adverse effects on DA binding and uptake. Inspection of the dDAT X-ray structure reveals that R445 forms a salt bridge with E428 on TM8. While E428 is not in contact with DA at S1, we note that in the EC direction, TM8 is one of the helices surrounding DA at S1 (Fig. 1C). We therefore postulated that this salt bridge between R445 and E428 holds TM8 in a position optimal for DA binding at S1 and may therefore play a role in DA binding and/or transport, thereby explaining the effect of the R445C mutation. This would suggest that mutation at E428 which precludes the salt bridge would also have a similar effect as the R445C mutant. Furthermore, replacing the E428-R445 salt bridge with a similar salt bridge at these sites, e.g., by interchanging the residues at these positions, may restore DA binding and/or transport. To test these hypotheses we have made the following mutants R445E, E428R and the double mutant E428R/R445E, predicted to re-establish the salt bridge interaction, and report herein the effects of these mutations on substrate binding as compared to the native WT protein.

Substrate binding and transport

Transient expression of the DAT constructs was somewhat suppressed compared with WT, except for that of E428R, both in total protein and in cell surface protein (Fig. 2 left panel). In stably expressing LLCK-PK1 cells, total and surface DAT expression appeared somewhat decreased for the double mutant (Fig. 2 right panel). The latter pattern was mimicked by the apparent average B_{max} values of cocaine analog [^{3}H]CFT binding in stable cell lines (Table 1), although the only statistically significant B_{max} effect was an increase in E428R. More important than the modest changes in DAT expression along with changes in [^{3}H]CFT B_{max} for the constructs were the observed changes in CFT and DA recognition (Table 1). The K_d value for [^{3}H]CFT binding was increased 3- to 5-fold in the single mutants R445E and E428R, and restored to WT level in the double mutant R445E/E428R. The same phenomenon occurred for DA binding as measured via its inhibitory effect on [^{3}H]CFT binding. DA K_i was increased (i.e., affinity decreased) ~6-fold in the single mutants and restored back to normal in the double mutant. The fact that both the CFT K_d and DA K_i are restored in the double mutant, suggests the residues E and R can be in either position (428 or 445) for enabling the formation of the salt bridge. If DA affinity (K_i) would be the only property that is changing in the single mutants, a 6-fold increase in DA K_i would be expected to cause a ~6-fold decrease in DA uptake measured at [DA] far below its K_m. Indeed, the ability of the single constructs to take up [^{3}H]DA was virtually collapsed with uptake levels at only 2–4% of WT uptake (Fig. 3). This indicates that additional factors, other than DA recognition, underlie the greatly reduced DA uptake of the single mutants. In support, while the R445E/E428R double mutant exhibited restored CFT and DA affinity/recognition, DAT function/DA uptake was still notably debilitated. Thus, the specific residues are likely critical for the conformational transitions in DAT that allow for DA transport.

Structural basis for binding and transport effects of R445 and ancillary mutations

Our previous studies have shown that the R445C mutation linked to DTDS adversely affects both binding and transport of DA when compared with WT. These findings mirror the quantitative effects observed with the R445E mutant as presented above which is consistent with the hypothesis that the intact E428-R445 salt bridge is needed for efficacious substrate binding and transport. Similarly, the E428R mutant undergoes an analogous loss of DA binding and transport. Thus, while R445 is distal from the substrate binding site, it's role in forming the salt bridge with E428 on TM8 which lines the DA S1 binding site can be important in maintaining the structure of the S1 site required for effective DA binding. This would explain the loss of DA affinity, and in turn transport, upon disruption of this salt bridge by any of the mutations above. Indeed, evidence to support a role of the native salt bridge in maintaining the correct structure at S1 was shown with the finding that restoration of DA and CFT binding occurred with the E428R/R445E double mutant. However, the inability of the double mutant to maintain substrate transport suggests a more complex mechanism. Based on available structural and mechanistic information, binding and transport of substrate involves dynamic shifts between inward and outward facing states of DAT. Precluding substrate egress from DAT, the transporter is in the outward open or outward open occluded state while the intracellular gates are closed (inward closed). To achieve final substrate egress and concomitant transport into the intracellular milieu, DAT