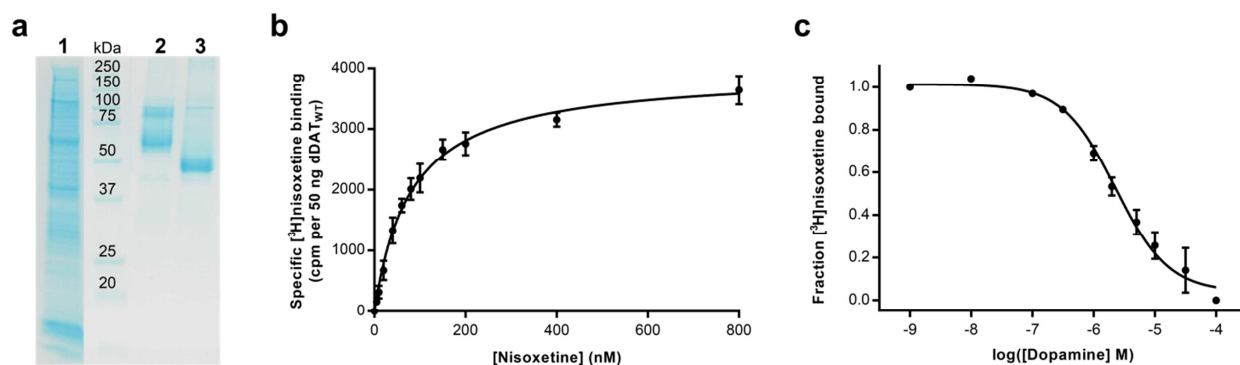


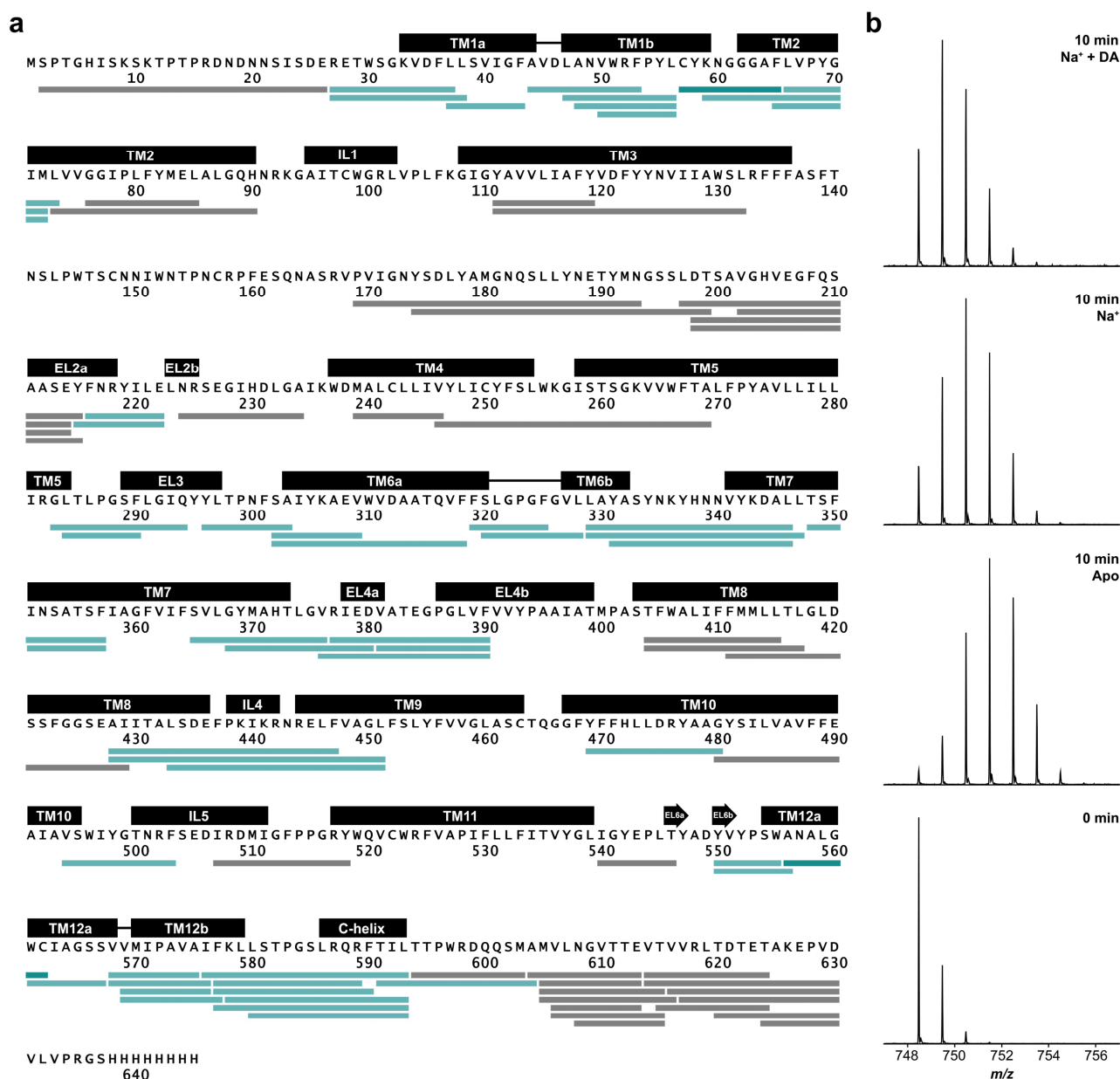
SUPPLEMENTARY INFORMATION for:

Substrate-induced conformational dynamics of the dopamine transporter

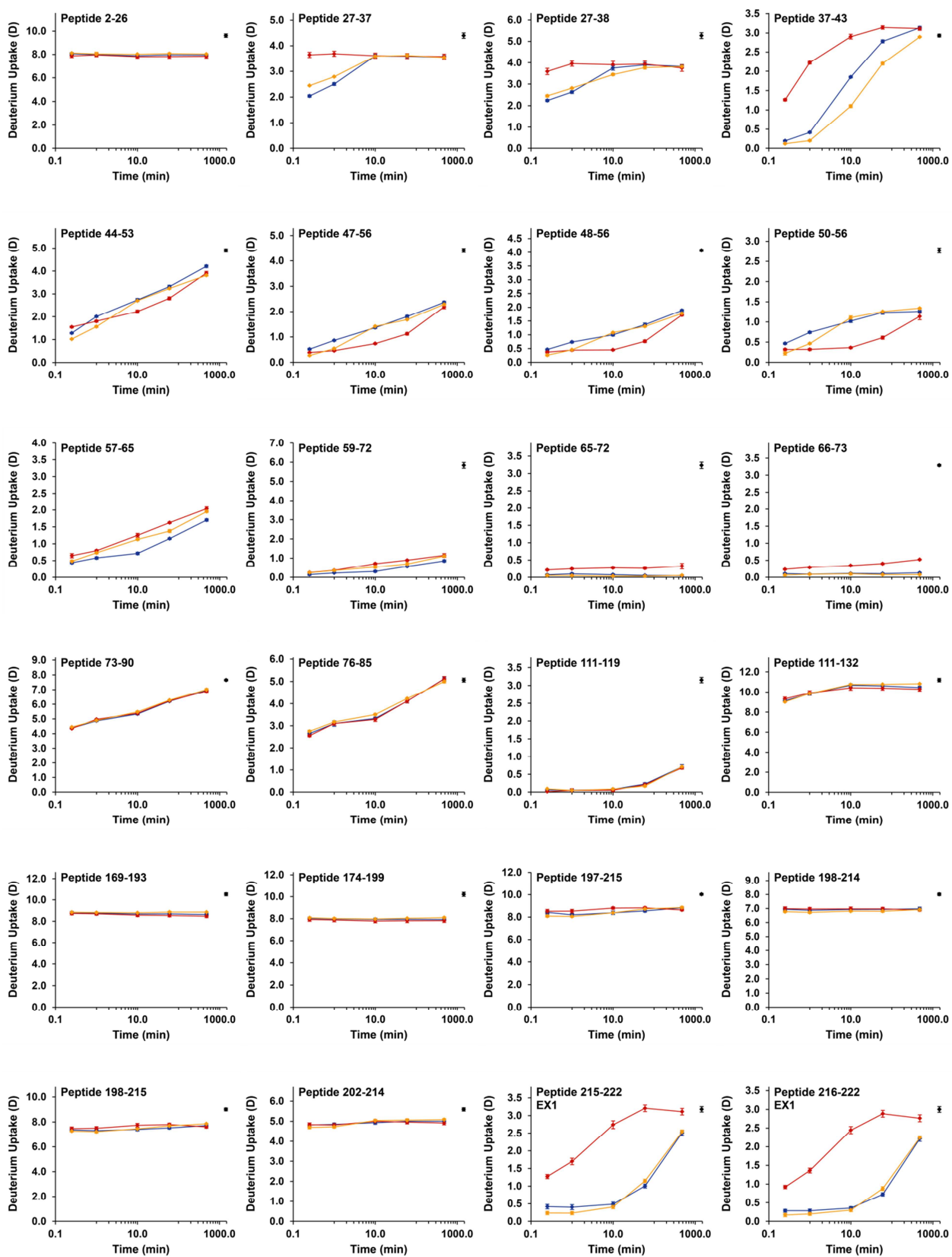
Nielsen et al.

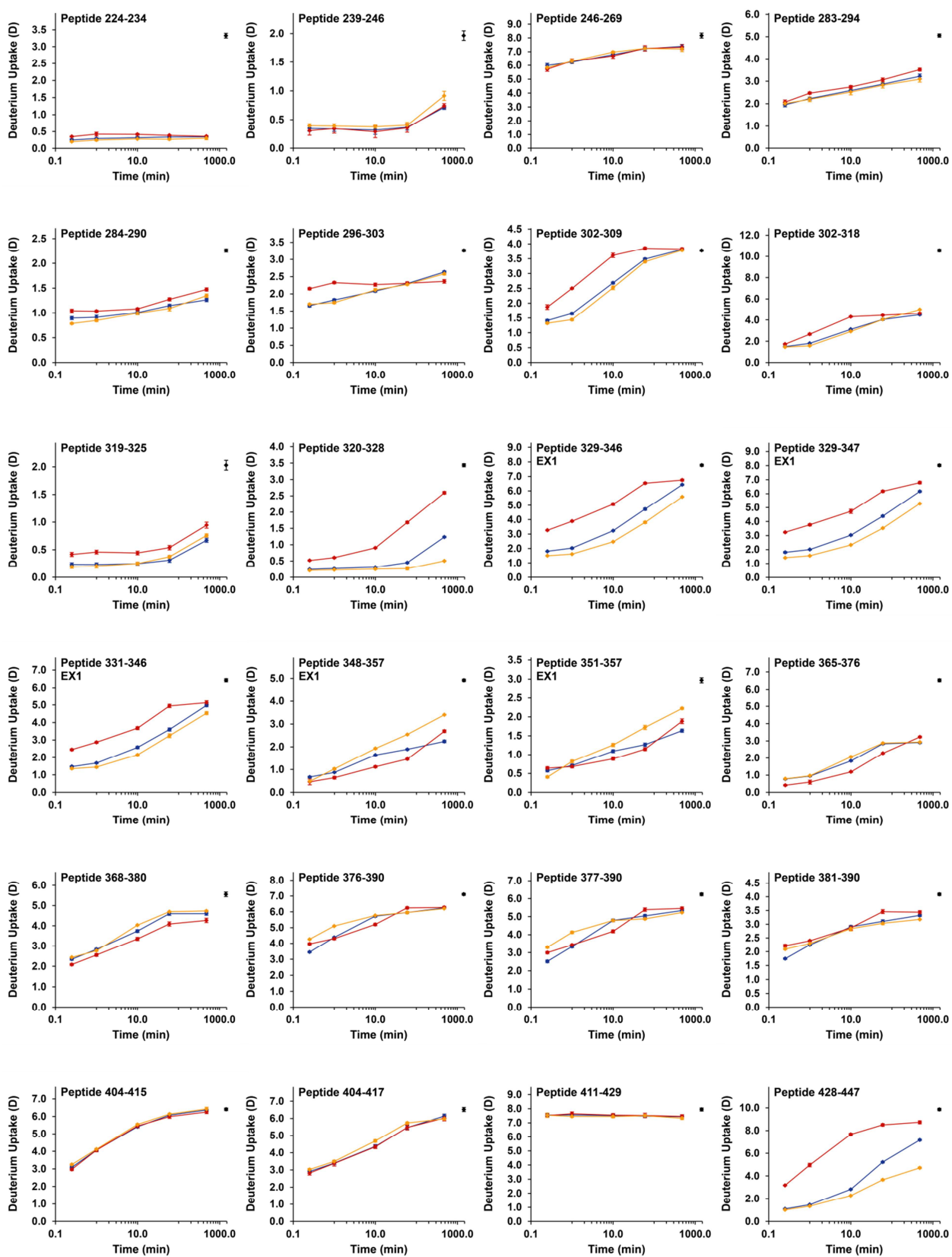


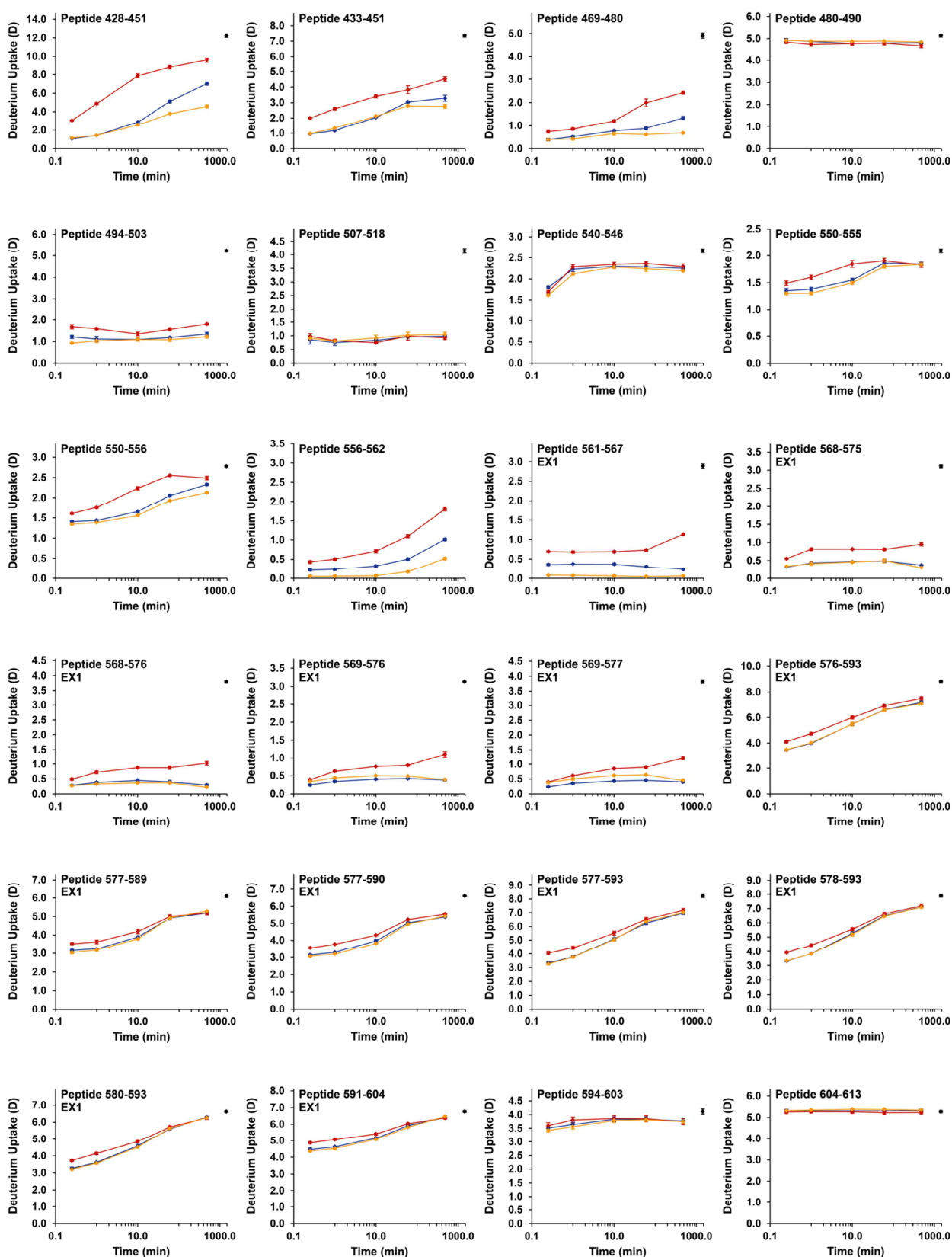
Supplementary Figure 1. Purification and functional characterization of dDAT. **a** Representative SDS-PAGE showing purity of dDAT eluted from nickel immobilized-metal affinity chromatography. Lane 1, solubilized material before incubation with Ni-NTA resin. Lane 2 and 3 show the purity of dDAT eluted from Ni-NTA resin. The two major bands in lane 2 are caused by heterogeneous glycosylation of dDAT as evident from lane 3, where dDAT appears as one single band after the same sample has been deglycosylated with PNGaseF. However, for the following binding and HDX-MS experiments, dDAT was left glycosylated to retain as wild-type like functionality as possible. **b** Saturation binding of [^3H]nisoxetine to purified dDAT with a dissociation constant (K_d) of 80 ± 6 nM. Error bars, s.e.m. of triplicates ($n = 3$). **c** Competitive inhibition of [^3H]nisoxetine binding to purified dDAT by unlabeled DA with an inhibition constant (K_i) of 1.7 ± 0.2 μM . Error bars, s.e.m. of triplicates ($n = 3$). Source data are provided as a Source Data file.

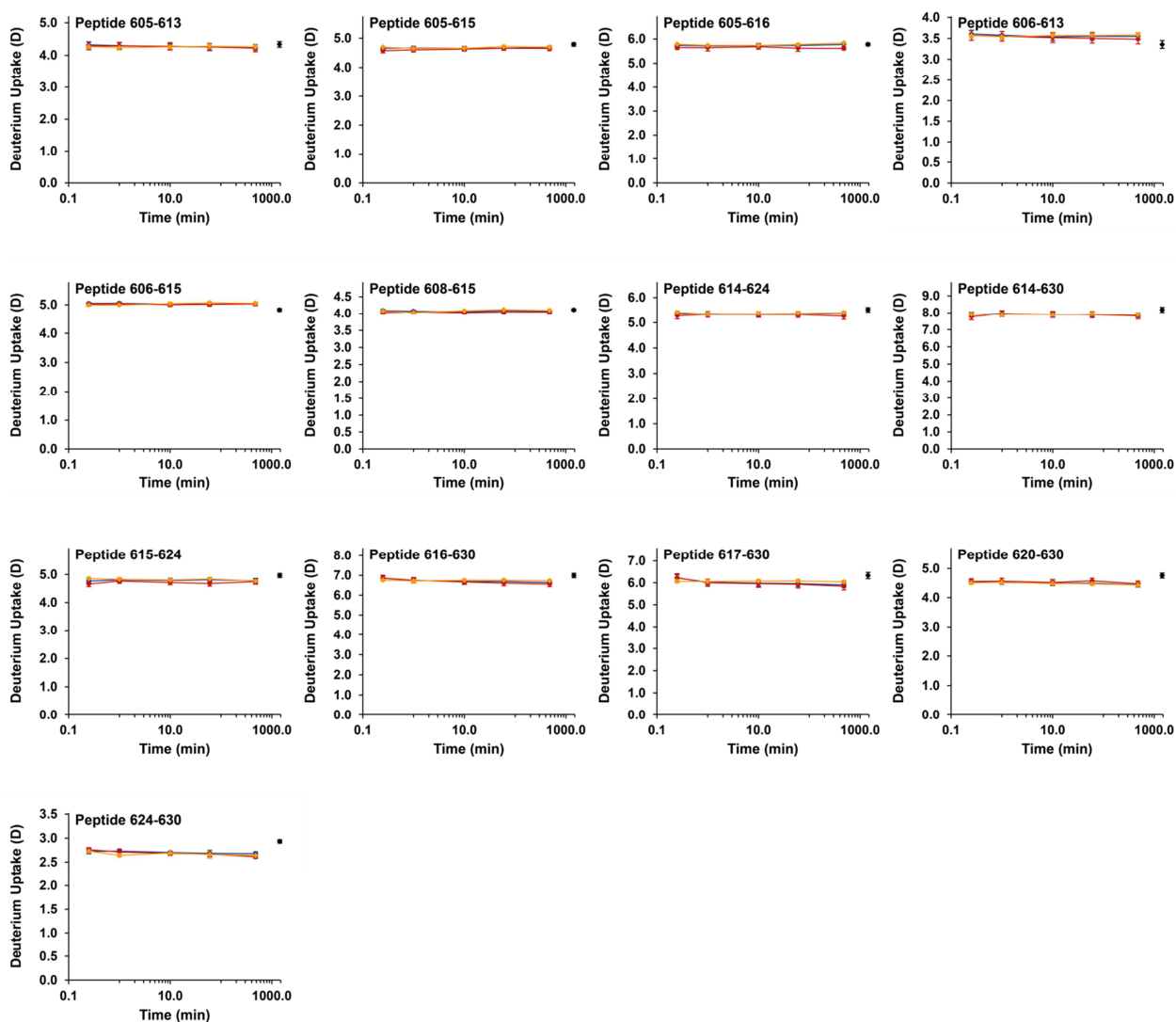


Supplementary Figure 2. Sequence coverage of dDAT. **a** 85 peptides covering 75.5% of the protein sequence (77.2% of the dDAT sequence) were identified following digestion with immobilized pepsin. Individual structural motifs in dDAT are indicated above the protein sequence. Peptides showing a divergence in deuterium uptake of at least one of the sampled states are colored cyan. Peptides, where no signal for the maximum-labeled control was detected, are colored dark cyan (peptide 57 – 65 and peptide 556 - 562). **b** Representative mass spectra for peptide 37-43 from dDAT for undeuterated (0 min) and after 10 min of deuterium exchange. The isotopic envelope shifts to different m/z values as a function of added Na⁺ and DA due to the respective degree of deuterium incorporation.

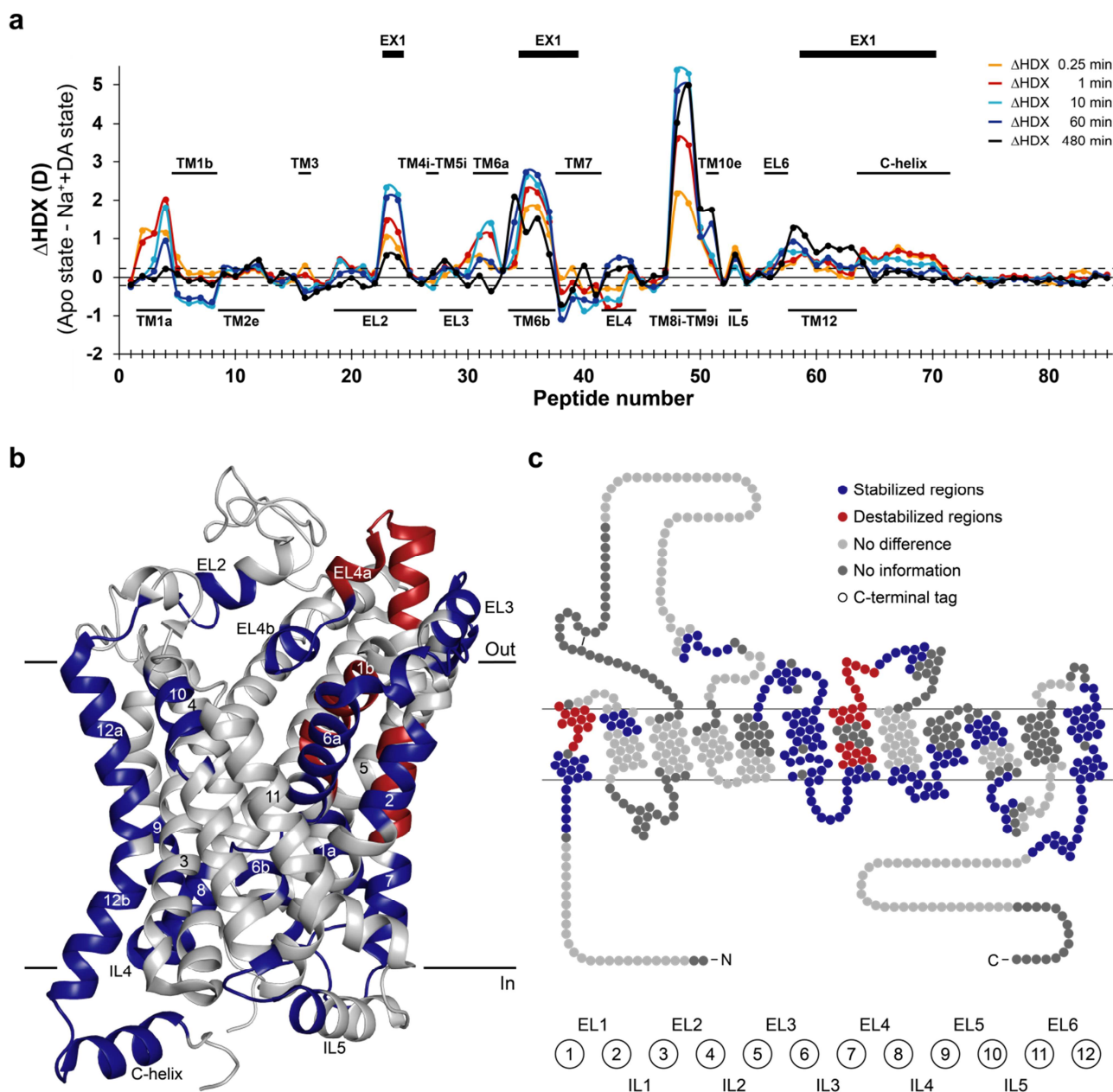






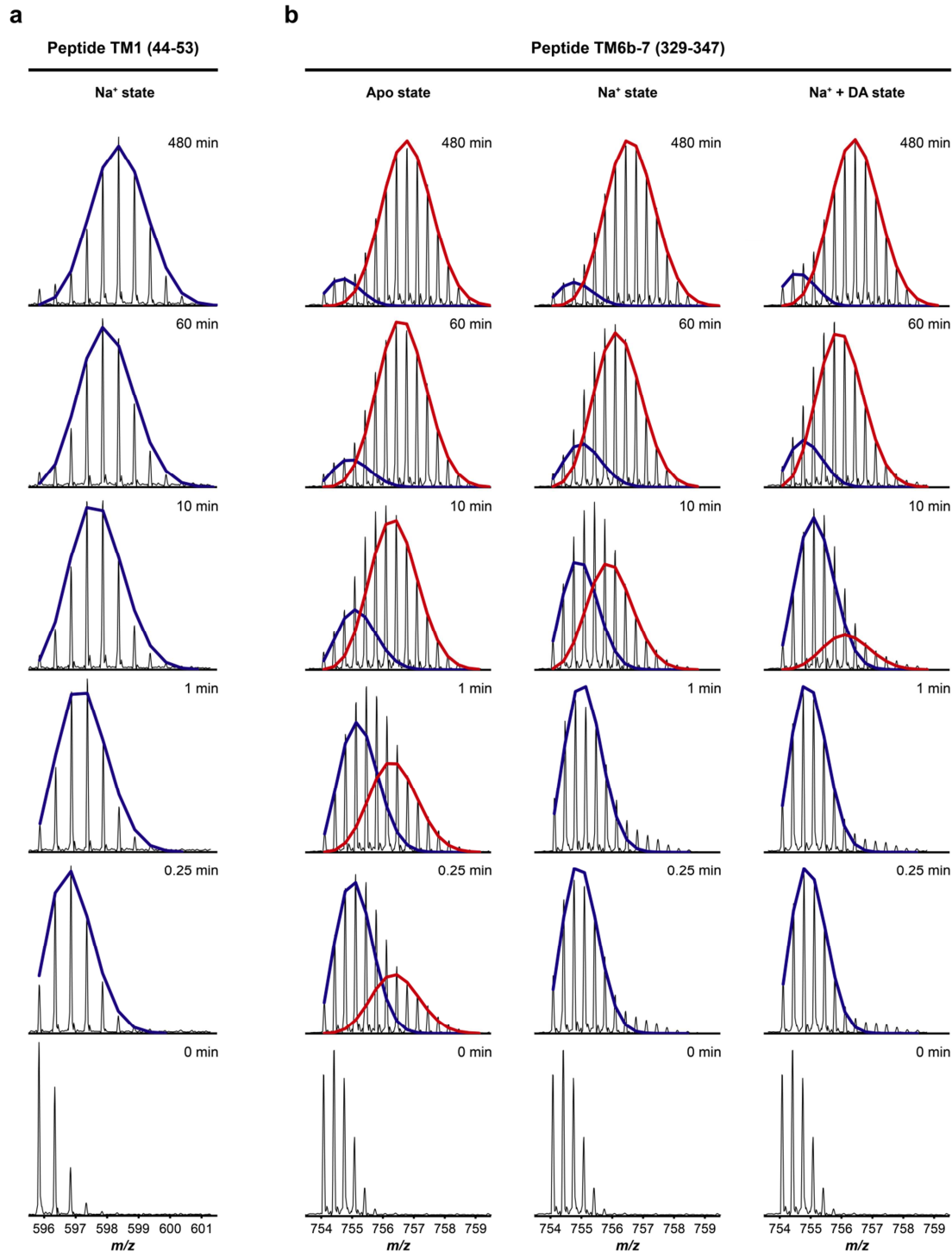


Supplementary Figure 3. Deuterium uptake plots for dDAT in three functional states. The deuterium uptake is plotted as a function of labeling time (*i.e.* 0.25 – 480 min) for all 85 identified dDAT peptides. Red, blue and orange curves represent the apo state, Na⁺-bound state, and Na⁺- and DA-bound state, respectively. Maximum-labeled control samples are shown as black circles at 1440 min. ($n = 3$ for all time points for the apo state and the Na⁺- and DA-bound state, $n = 6$ for all time points for the Na⁺-bound state, and $n = 10$ for the maximum-labeled control). Standard deviations are plotted as error bars but are in most instances too small to be visible. Peptides that showed correlated exchange kinetics are marked 'EX1'. Source data are provided as a Source Data file.



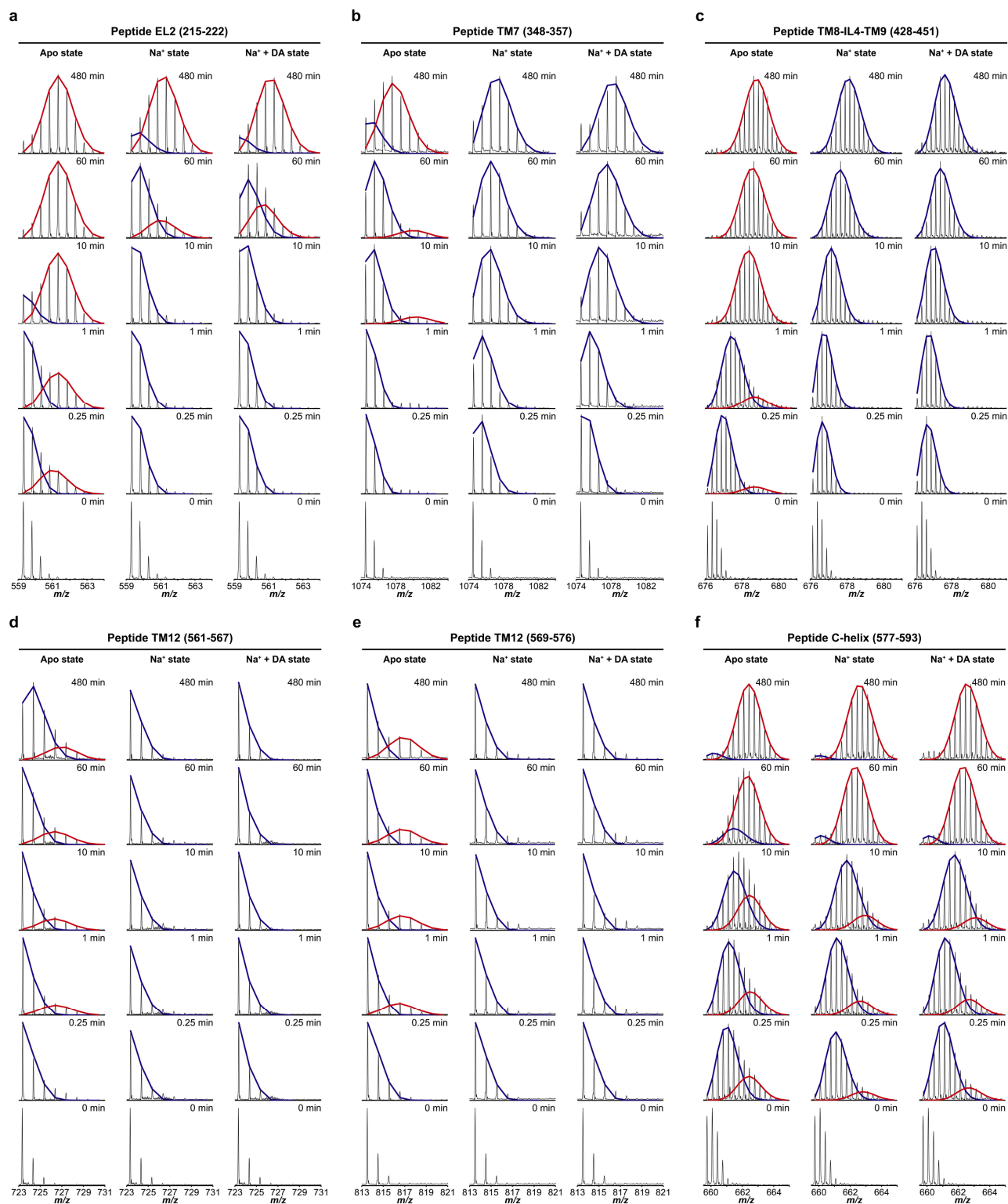
Supplementary Figure 4. Effect of Na^+ and dopamine on the HDX of dDAT compared to the apo state. **a** Chart showing differences in the average deuterium uptake (ΔHDX) between the apo state and the Na^+ - and DA-bound state for the 85 identified peptides at the five sampled time points (orange - 0.25 min; red - 1 min; cyan - 10 min; blue - 1 hour; black - 8 hours). The individual dDAT peptides are arranged along the x-axis starting from the N-terminal and ending on the C-terminal. The peptide number refers to Supplementary Table 2. Positive and negative values indicate reduced and increased HDX, respectively, upon binding of Na^+ and DA. Values represent means of three independent measurements. Structural motifs in dDAT are marked along the x-axis together with regions showing correlated

exchange kinetics (EX1) in at least one of the two states. The dotted lines (± 0.23 D) mark a threshold value for significant differences in HDX corresponding to the 95% confidence interval, calculated from the pooled standard deviations for all time points. **b, c** Regions showing significant differences (Student's *t*-test *p*-value < 0.01) in deuterium uptake between the apo state and the Na⁺- and DA-bound state for at least two consecutive time points are mapped onto the crystal structure (**b**) (PDB ID: 4XP1) and snake diagram (**c**) of dDAT. Regions are colored red and blue to indicate dDAT segments becoming destabilized (increased HDX) or stabilized (decreased HDX), respectively, upon binding of ions and DA. Regions colored light grey displayed unchanged HDX while regions in dark grey were uncovered by peptide sequences. Wild-type dDAT regions including part of EL2 (residue 162-202) and the N- and C-termini (residue 1-24 and 601-645, respectively) are only marked on the snake diagram in (**c**) as they were not resolved in the crystal structure (**b**) or were truncated in the construct used for crystallization. Source data are provided as a Source Data file.



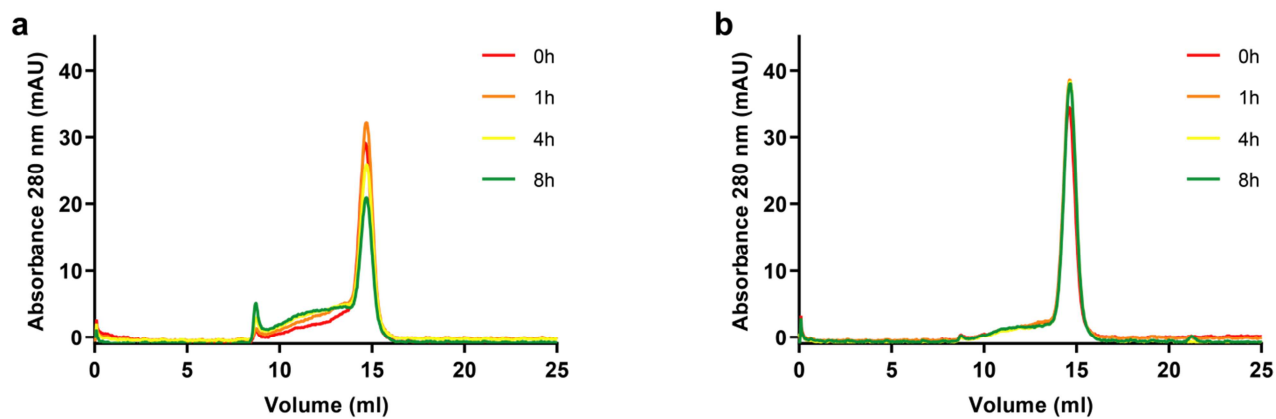
Supplementary Figure 5. Exchange kinetics in dDAT. a Representative mass spectra for peptide 44-53 in the Na⁺-bound state at the five sampled time points showing a typical isotopic pattern seen for regions that exchange through the EX2 kinetic regime due to fast local fluctuations that lead to gradual deuteration. One binomial distribution produced the best fit to the spectra (blue). **b** Representative mass spectra for peptide 329-347, which covers TM6b-TM7, are shown for the three states at all sampled time points. The isotopic pattern reveals EX1/EXX exchange

kinetics due to slow cooperative fluctuations that lead to exchange of all backbone amide hydrogens participating in the motion. Two binomial isotopic envelopes gave the best fit to the spectra – yielding a low- (blue) and high-mass (red) population. The rate of translation from the low-mass population to the high-mass population was reduced upon binding of Na^+ relative to the apo state – and even more so by binding of Na^+ and DA combined.



Supplementary Figure 6. EX1/EXX kinetics of regions in dDAT. Representative mass spectra for the three states at all sampled time points for peptides 215-222 (a), 348-357 (b), 428-451 (c), 561-567 (d), 569-576 (e), and 577-593 (f),

which cover EL2, the middle part of TM7, TM8-IL4-TM9, TM12a, TM12b, and the C-helix, respectively. Two binomial isotopic envelopes produced the best fit to the spectra – a low- (blue) and high-mass (red) population.



Supplementary Figure 7. Stability of dDAT under sampled conditions. a, b Size-exclusion chromatography (SEC) profiles of purified dDAT incubated at 25°C for the indicated time intervals in buffer A containing 200 mM CsCl (**a**) or 200 mM NaCl (**b**). Source data are provided as a Source Data file.

Supplementary Table 1. HDX data summary table (see Supplementary Dataset 1 for full-scale excel version).

Data Set	Na ⁺	Apo (Cs ⁺)	Na ⁺ + DA
HDX reaction details	40 mM Tris, 5% glycerol, 1 mM DDM, 0.2 mM CHS, 14 μM lipids (POPC:POPE:POPG; weight ratio 3:1:1), 200 mM NaCl. Percent deuterium: 75.2%, pD _{read} = 8.036, 25°C.	40 mM Tris, 5% glycerol, 1 mM DDM, 0.2 mM CHS, 14 μM lipids (POPC:POPE:POPG; weight ratio 3:1:1), 200 mM CsCl. Percent deuterium: 75.2%, pD _{read} = 8.045, 25°C.	40 mM Tris, 5% glycerol, 1 mM DDM, 0.2 mM CHS, 14 μM lipids (POPC:POPE:POPG; weight ratio 3:1:1), 200 mM NaCl, 40 μM DA. Percent deuterium: 75.2%, pD _{read} = 8.036, 25°C.
HDX time course	0.25 min, 1 min, 10 min, 60 min, 480 min	0.25 min, 1 min, 10 min, 60 min, 480 min	0.25 min, 1 min, 10 min, 60 min, 480 min
HDX control samples	Maximum labeled control using predigested dDAT and the same HDX reaction details as for the Na ⁺ state.		
Back-exchange (mean / IQR)	32.1% / 9.55%		
# of Peptides	85	85	85
Sequence coverage	75.5% (77.2% of the dDAT sequence)	75.5% (77.2% of the dDAT sequence)	75.5% (77.2% of the dDAT sequence)
Average peptide length / Redundancy	12.44 / 2.17	12.44 / 2.17	12.44 / 2.17
Replicates (biological or technical)	6 (technical)	3 (technical)	3 (technical)
Repeatability	0.0642 (average standard deviation)	0.0807 (average standard deviation)	0.0467 (average standard deviation)
Significant differences in HDX (delta HDX > X D)	Reference state	95% CI: 0.26 D	95% CI: 0.20 D (0.23 D relative to Apo state)

Supplementary Table 2. HDX data table (see Supplementary Dataset 1 for full-scale excel version).

[illegible]