Neurotransmitter/sodium symporters (NSSs) are responsible for Na\(^+\)-dependent reuptake of neurotransmitters and represent key targets for antidepressants and psychostimulants. LeuT, a prokaryotic NSS protein, constitutes a primary structural model for these transporters. Here we show that K\(^+\) inhibits Na\(^+\)-dependent binding of substrate to LeuT, promotes an outward-closed/inward-facing conformation of the transporter and increases uptake. To assess K\(^+\)-induced conformational dynamics we measured fluorescence resonance energy transfer (FRET) between fluorescein site-specifically attached to inserted cysteines and Ni\(^{2+}\) bound to engineered di-histidine motifs (transition metal ion FRET). The measurements supported K\(^+\)-induced closure of the transporter to the outside, which was counteracted by Na\(^+\) and substrate. Promoting an outward-open conformation of LeuT by mutation abolished the K\(^+\)-effect. The K\(^+\)-effect depended on an intact Na\(^+\) site and mutating the Na2 site potentiated K\(^+\) binding by facilitating transition to the inward-facing state. The data reveal an unrecognized ability of K\(^+\) to regulate the LeuT transport cycle.
Neurotransmitter/sodium symporters (NSSs) play an essential role in terminating neurotransmitter action in the central nervous system and operate by utilizing the energy stored in the transmembrane Na\(^+\)-gradient as driving force for substrate transport. Key members of the family include transporters of the neurotransmitters dopamine (DAT), norepinephrine and serotonin (SERT). As a consequence of NSSs involvement in controlling synaptic signaling, the transporters have been established as targets for many important therapeutics and are targeted by illicit drugs such as cocaine, amphetamines and cathinone-derivatives (‘bath salts’). In addition to co-transport of Na\(^+\), all mammalian NSSs are dependent on co-transport of Cl\(^-\) (ref. 4). Importantly, counter-transport of K\(^+\) was reported to stimulate the rate of serotonin (5-HT) uptake by human SERT. Furthermore, it was shown that H\(^+\) can substitute for K\(^+\) as counter-transported cation in SERT, and it was proposed that co-transport of Cl\(^-\) or counter-transport of H\(^+\)/K\(^+\) is a common feature of charge-balance in NSSs. However, to our knowledge, there is no evidence for K\(^+\)-counter-transport in other NSSs that SERT and the molecular details behind its function are largely unknown.

High-resolution X-ray crystal structures of the leucine transporter (LeuT) from *Aquifex aeolicus*, the multi-hydrophobic amino acid transporter from *Bacillus halodurans*, DAT from *drosophila melanogaster* and the human SERT have provided important insight into the molecular structure of this class of proteins. NSSs possess 11 or 12 transmembrane (TM) segments with a substrate-binding site and one or two adjacent Na\(^+\)-binding sites (Na1 and Na2) buried approximately half-way across the membrane bilayer. The conventional model for ion-coupled substrate-transport has been inferred from crystal structures of ‘canonical’ transport-cycle intermediates (outward-facing, substrate-bound occluded and inward-facing). Indeed transport is widely assumed to be governed by a simple allosteric mechanism, where access to and from the substrate-binding site is tightly regulated by intra- and extracellular gating domains.

LeuT has been used as the principal model system for studying NSS structure–function relationships and has served as template for homology models that have guided, for example, binding site mapping in mammalian NSSs. In addition, dynamic inferences about conformational transitions in the NSS transport cycle have been obtained using LeuT as model in studies using biophysical techniques such as double electron–electron resonance, single molecule fluorescence resonance energy transfer (smFRET) and site-directed fluorescence quenching spectroscopy. Nonetheless, despite a growing mechanistic understanding of the translocation process, none of the studies has suggested a putative role of K\(^+\) and thus addressed the question whether the counter-transport of K\(^+\) proposed for SERT is indicative of a general regulatory role of K\(^+\) in other NSS proteins.

In the present study, we provide structural evidence for a role of K\(^+\) in regulating conformational transitions in LeuT. We demonstrate that K\(^+\) interacts with LeuT and competitively inhibits Na\(^+\)-dependent binding of substrate. Moreover, we find that internal K\(^+\) stimulates \(^3^H\)alanine uptake by LeuT in proteoliposomes. Next, we use transition metal ion fluorescence resonance energy transfer (tmFRET), as a highly sensitive method for direct measurements of conformational changes in response to K\(^+\) and show that K\(^+\) inhibits Na\(^+\) and substrate binding possibly by binding an outward-closed conformation of the transporter. The effect of K\(^+\) requires an intact Na1 site and is abolished by mutation of Arg30 that promotes the outward-open conformation of LeuT. Finally, K\(^+\) potency is increased upon mutational disruption of conserved interactions associated with the Na2 site thereby biasing the inward-open conformation.

Our data suggest altogether that K\(^+\) could play a canonical role in regulating the function of NSS proteins. Specifically, we propose a model in which internal K\(^+\) facilitates substrate turnover by inhibiting substrate rebinding in the outward-closed/inward-facing conformation. This will inhibit substrate efflux and result in an increased concentrative capacity of the transporter and, thus, substrate transport against a larger chemical gradient.

**Results**

K\(^+\) competitively inhibits Na\(^+\) binding to LeuT. K\(^+\)-counter-transport by SERT was reported almost four decades ago. However, it has not been addressed whether the role of K\(^+\) in SERT is indicative of a general feature in the NSS transport mechanism. To address this question, we set out to investigate whether K\(^+\) interacts with LeuT. We purified LeuT from *Escherichia coli* and confirmed binding of \(^3^H\)leucine in 200 mM Na\(^+\) or Li\(^+\) using the scintillation proximity assay (SPA) on detergent-solubilized protein as previously reported.

Note that all subsequent experiments were performed on LeuT in detergent (dodecyl-\(\beta\)-D-maltoside, DDM) unless otherwise stated. We observed no \(^3^H\)leucine binding in 200 mM K\(^+\) or other tested monovalent cations (Fig. 1a). To assess the effect of K\(^+\) on LeuT, we performed Na\(^+\)-dependent \(^3^H\)leucine binding where Na\(^+\) was substituted with either K\(^+\) or choline (Ch\(^+\)) to maintain a total ionic concentration of 200 mM. Remarkably, we observed a 3-fold increase of the EC\(_{50}\) for Na\(^+\) when substituted with K\(^+\) compared with Ch\(^+\) (Fig. 1b). Likewise, we observed a 3-fold increase in EC\(_{50}\) for Li\(^+\) substituted with K\(^+\) compared with Ch\(^+\) (Fig. 1b).

We proceeded by investigating Na\(^+\)-dependence of \(^3^H\)leucine binding in the presence of fixed K\(^+\) concentrations (Fig. 1c) and performed a Schild analysis of these data showing the shifts in EC\(_{50}\) as a function of [K\(^+\)]. The Schild analysis provides information whether or not antagonism is competitive in nature: if the regression in a Schild plot is linear with a slope of 1, then the antagonism is competitive. When competitive antagonism is observed, it also allows for determination of the affinity (K\(_A\)) of the antagonist. Here, the Schild plot revealed a linear correlation with a slope of 0.9 [0.8;1.0] (mean [95% confidence interval]) and an equilibrium constant (K\(_A\)) for K\(^+\) of 176 [153;203] mM (Fig. 1d). This suggests that K\(^+\) competitively inhibits Na\(^+\) binding to LeuT WT.

To test whether K\(^+\) interaction with LeuT affected transport, we reconstituted LeuT into proteoliposomes containing buffer with 200 mM KCl or CsCl. \(^3^H\)alanine uptake by the proteoliposomes was measured for up to 45 min in external buffer containing 200 mM NaCl. Interestingly, uptake was faster in K\(^+\) compared with Cs\(^+\) and reached a more than 2-fold higher maximum (Fig. 1e). Previously, Na\(^+\)/substrate symport-coupled H\(^+\) antiport was suggested for prokaryotic NSS proteins and for the homologous NSS protein Tyt1, where it was shown that substrate transport elicits H\(^+\) efflux. To test whether H\(^+\) could substitute for K\(^+\), proteoliposomes were formed in a CsCl buffer (200 mM) pH 6.5 or pH 8 and \(^3^H\)alanine uptake were assessed as in Fig. 1d at pH 8. However, the H\(^+\) gradient appeared to have no effect on uptake: In proteoliposomes formed at pH 8, \(^3^H\)alanine uptake was 106 ± 6% relative to uptake at pH 6.5 (means ± s.e.m., n = 3), suggesting that protons cannot substitute for K\(^+\) as well as that a proton gradient does not stimulate uptake, at least not under the conditions used in the present investigation. Another possible interpretation of the result would be that internal K\(^+\) facilitates transport by binding to LeuT in a way that inhibits rebinding of Na\(^+\) and, accordingly, also substrate. This should reduce substrate efflux and result in an increased concentrative capacity.
of the transporter, that is, substrate can be transported against a larger chemical gradient. To address this question, we performed an efflux experiment. Proteoliposomes were preincubated with $^{3}$H]alanine (50 nM) for 10 min and efflux measurements were initiated by diluting the proteoliposomes five times into a Na$^+$ buffer with a high concentration (500 nM) of unlabelled alanine to prevent any reuptake of released $^{3}$H]alanine. The amount of $^{3}$H]alanine efflux was assessed by comparing the remaining internal $^{3}$H]alanine after 30 min to the initial amount. In agreement with this hypothesis, $^{3}$H]alanine efflux was significantly lower for K$^+$-containing proteoliposomes compared with control (Fig. 1f).

Application of tmFRET in LeuT. To have means for direct investigation of the putative effect of K$^+$ on LeuT conformation, we implemented tmFRET, which is a powerful tool for direct assessment of changes in intramolecular distances on, for example, transitions between conformational states. The method takes advantage of the ability of transition metals to act as a fluorescent quencher of the donor fluorophore: fluorescence from a Cys-conjugated fluorophore (fluorescein, FL) is quenched by a coloured transition metal ion (for example, Ni$^{2+}$, chelated in an engineered metal-binding site (for example,
**Figure 2** | K⁺ induces a different tmFRET state than other tested cations. (a) Chemical structures of fluorescein-5-maleimide (orange) conjugated to a cysteine, and a nickel ion (green) coordinated by the imidazole moieties of two histidines inserted in an alpha-helical motif one helix-turn apart (His-X₃-His). (b) Expected dynamic range (grey area) of the EL4:TM10 tmFRET pair-based LeuT-crystal structures and the FL-Ni²⁺ Förster distance ($R_0 = 12$ Å). (c) Cartoon of LeuT with EL4:TM10 tmFRET pair shown in outward open and outward closed states depicting the principle behind changes in tmFRET as a result of conformational changes. The tmFRET pair reports distance-dependent quenching of fluorescence from FL conjugated to an inserted cysteine at the top of TM10 (orange sphere, K398C) by Ni²⁺ coordinated by a His-X₃-His motif in EL4 (green sphere, A313H-A317H). (d–f) Detecting distance-dependent fluorescence quenching as a function of Ni²⁺ in LeuT with EL4:TM10 tmFRET pair (LeuT A313H-A317H-K398CFL; see Supplementary Fig. 1 for tmFRET proof-of-principle applied to LeuT). (d) tmFRET intensity as a function of [Ni²⁺] performed in 200 mM of the indicated salts yielded saturable tmFRET response. Dotted line represents tmFRET in ChCl (grey) for comparison. (e) Maximal tmFRET values from the experiments performed in d compared with experiments performed in 800 mM of the indicated salts. K⁺ significantly and dose-dependently increases tmFRET values, consistent with a decrease in the TM10-EL4 mean distance. The addition of leucine (100 μM) to Na⁺ or K⁺ does not change tmFRET response relative to the ion alone. (f) Ni²⁺ affinities ($pE_{50}$) obtained from experiments shown in d, indicate that Na⁺, Li⁺, K⁺ and Rb⁺ (compared with Ch⁺) induce a change in the EL4 secondary structure that stabilizes a configuration with increased Ni²⁺ affinity. Data points are means ± s.e.m., n = 3–6. *P < 0.05; **P < 0.01; ***P < 0.0005; ****P < 0.0001; denote significance level from a one-way analysis of variance with post hoc Bonferroni’s multiple comparison test relative to the Ch⁺ condition in the same experimental setup.
His-X₃-His of an α-helix, Fig. 2a and Supplementary Fig. 1a–e). Importantly, tmFRET between FL and Ni²⁺ occurs at a short range and is highly distance dependent, having a Förster distance ($R₀$) of $\sim$12 Å (refs 39–42; Fig. 2b). Accordingly, tmFRET provides an ideal sensitivity range ($\sim$8–18 Å) for detecting changes in intramolecular distances predicted to take place in a transport protein like LeuT during transport (Fig. 2c)16. See the ‘Methods’ section and Supplementary Fig. 1 for a detailed implementation of tmFRET in LeuT.

K⁺ binding induces closure of extracellular domains.

Comparing the crystal structures of LeuT in the outward-open conformation (Na⁺-bound, PDB 3TT1; ref. 17) with the outward-occluded (Na⁺ and leucine bound, PDB 2A65; ref. 11) and inward-open (apo, PDB 3TT3; ref. 17) states reveals a major shift in the positioning of extracellular loop 4 (EL4) that acts like a lid sealing the extracellular gate11,17. As a consequence, EL4 moves closer to the top of TM10 and to EL2 (Fig. 2c). To probe for conformational changes on the extracellular face of LeuT, induced by ion- and substrate binding, we generated a tmFRET pair by inserting a His-X₃-His site on EL4 and related its movement to FL conjugated to the top of TM10 (A313H-A317H-R398C, Fig. 2c–f). The purified and FL-labelled LeuT tmFRET variant retained WT-like [³H]leucine binding (Supplementary Table 1 and Supplementary Fig. 2a–d). Also, the LeuT variant showed no significant change in the EC₅₀ for Na⁺-stimulated binding of [³H]leucine, when measured by K⁺ substitution, indicating that interaction with K⁺ was unaffected in the generated tmFRET pair (Supplementary Table 1 and Supplementary Fig. 2e). We proceeded by investigating the effect of cations on tmFRET between TM10 and EL4 (LeuT A313H-A317H-K398CFL1). The experiments were performed by increasing the concentration of Ni²⁺ in buffers containing 200 mM of cations with varying ionic radii (Li⁺; Na⁺; K⁺; Rb⁺; Cs⁺; Choline (Ch⁺); N-methyl-D-glucamine (NMDG⁺); tri(hydroxymethyl)aminomethane (Tris⁺); Fig. 2d). Consistent with Ni²⁺ quenching of FL bound to K398C, Ni²⁺ caused a dose-dependent increase in the tmFRET value ($1 − F/F_{0\text{ no site}}$) for all cations used (Fig. 2d). Note that, by plotting $1 − F/F_{0\text{ no site}}$ we correct for dilution, collisional quenching and the inner-filter effect of Ni²⁺ as the fluorescence from the tmFRET construct (F) is normalized to the fluorescence from the control construct devoid of the His-X₃-His site ($F_{0\text{ no site}}$; see also Supplementary Fig. 1). Remarkably, K⁺ yielded a significantly higher maximum tmFRET value compared with all other cations used including both Ch⁺ and Na⁺ (Fig. 2d,e and Supplementary Table 2). This suggests that K⁺, but not any of the other ions tested, promotes a decrease in the distance between TM10 and EL4. We next repeated the experiment in 800 mM of the cations, and again observed a substantially higher tmFRET in K⁺ as compared with Ch⁺, Cs⁺ and Na⁺ (Fig. 2e).

A feature of tmFRET is that the Ni²⁺ affinity measured by titrating the quenching response may report transitions in the secondary structure supporting the His-X₃-His site40,43. Interestingly, the apparent affinities for the Ni²⁺ response were significantly increased in Li⁺, Na⁺, Rb⁺ and K⁺, as compared with the large ions (Ch⁺, Cs⁺, NMDG⁺ and Tris/ MES; reflected in increased pEC₅₀ values, Fig. 2f). The shift in the pEC₅₀ values likely reports binding of Li⁺, Na⁺, K⁺ and Rb⁺ to LeuT; however, the effect must be uncoupled from the putative distance changes between EL4 and TM10 as there was no significant difference in Ni²⁺ affinity between the application of K⁺, Na⁺ and Rb⁺.

To further evaluate possible movements of EL4, we investigated tmFRET between EL2 and EL4 (LeuT R142CFL-A313H-A317H, Fig. 3a). Corroborating the observations for LeuT A313H-A317H-K398CFL, we observed again significantly higher tmFRET in 200 mM K⁺ compared with Ch⁺, whereas the tmFRET signal was not significantly different in 200 mM Na⁺ or Cs⁺ compared with Ch⁺ (Fig. 3a and Supplementary Table 2). Here, the application of leucine (in Na⁺) did change the tmFRET signal significantly, suggesting a conformational transition in response to leucine binding between EL2 and EL4. The pEC₅₀ for Ni²⁺ was for this construct also significantly increased in Na⁺ and K⁺ as compared with Ch⁺ and Cs⁺ (Fig. 3a, bottom panel). Finally, we generated a third tmFRET pair between two proposed static domains for reference (TM10–EL2, LeuT K145H-Y149H-K398CFL, Fig. 3b)17,29,44. As expected, we observed no significant differences in tmFRET for the different tested ions (Fig. 3b, Supplementary Table 2).
bottom panel). Similar to the EL4-TM10 tmFRET pair, the EL2-EL4 and TM10-EL2 variants retained WT-like $[^3]$H]leucine binding and showed no significant change in the EC$_{50}$ for Na$^+$-stimulated binding of $[^3]$H]leucine (Supplementary Table 1 and Supplementary Fig. 2c–e).

Taken together, the tmFRET measurements corroborate our observations from radiotracer binding by suggesting that K$^+$ can interact with LeuT. The data also suggest that binding of K$^+$ promotes a movement of EL4 towards both TM10 and EL2. Notably, tmFRET between EL4 and TM10 measured in high K$^+$ (800 mM) approximately corresponded to the expected tmFRET value, calculated from distances between coordinates in the inward-facing crystal structure (Fig. 2e and Supplementary Fig. 3). In contrast, we observed no effect of Na$^+$ on tmFRET between EL4 and TM10, relative to that seen in the presence of non-binding cations.

Na$^+$ competitively inhibits the conformational response to K$^+$. To obtain a dose-response curve for the K$^+$-induced change seen in A313H-A317H-K398CFL, which measures tmFRET between TM10 and EL4 and from now and onwards will be named WT$^{tmFRET}$, we added increasing K$^+$ concentrations (substitution with Ch$^+$) in the presence of a saturating (750 μM) Ni$^{2+}$ concentration (Fig. 4). K$^+$ produced ~22% increase in tmFRET with an EC$_{50}$ for K$^+$ of ~260 mM (Fig. 4a and Table 1). Note that these numbers are only rough estimates because we were unable to obtain full saturation owing to limitations in maximum attainable K$^+$ concentration in the assay (800 mM). The K$^+$ response was specific as no increase in FL quenching was observed when the experiment was carried out on LeuT K398CFL (Fig. 1) lacking the Ni$^{2+}$ site, or when Ni$^{2+}$ was replaced with Zn$^{2+}$ (Supplementary Fig. 4a,b). In accordance with the experiments in Fig. 2, increasing concentration of Na$^+$ (again substitution with Ch$^+$) produced no conformational response in WT$^{tmFRET}$ (Fig. 4a). For LeuT R142CFL-A313H-A317H, measuring tmFRET between EL2 and EL4, we also observed increasing tmFRET values in response to increasing K$^+$ concentrations, reaching a ~16% increase in the presence of 800 mM K$^+$ (Supplementary Fig. 4c). For LeuT K145H-Y149H-K398CFL, measuring tmFRET between EL2 and TM10, we observed no response (Supplementary Fig. 4d).

We proceeded by investigating the ability of Na$^+$ and leucine to modulate the K$^+$-induced tmFRET response between TM10 and EL4 (WT$^{tmFRET}$ construct). Notably, the addition of 100 mM Na$^+$ markedly shifted the dose-response curve to the right resulting in an estimated increase in EC$_{50}$ for K$^+$ by ~3-fold (Fig. 4b). Thus, even though Na$^+$ had no effect on tmFRET by itself, it was able to block the K$^+$-dose-response curve, suggesting that Na$^+$ is able to inhibit the K$^+$-induced conformational change detected by tmFRET. Further addition of leucine (50 μM) completely blocked the K$^+$-response (Fig. 4b). The effect by leucine, however, was Na$^+$-dependent since substitution with 100 mM Cs$^+$ (which does not support leucine binding, Fig. 1a) completely removed the blockade (Fig. 4b).

To further investigate the ability of Na$^+$ to compete for the K$^+$-bound conformation, we performed a tmFRET ‘back titration’ experiment (Fig. 4c). By keeping K$^+$ constant (600 mM), Na$^+$ yielded a FL de-quenching response that was fitted by a variable slope function with an EC$_{50}$ for Na$^+$ of 94.0 [80.9;109] mM (mean [s.e.m. interval]) and a $\triangle$H$_{NI}^{0}$ of 1.13 ± 0.08 (mean ± s.e.m.), suggesting competition of Na$^+$ with one binding site (Fig. 4d and Table 1). Further addition of leucine dose-dependently potentiated the ability of Na$^+$ to compete the K$^+$-bound conformation with a 25-fold decrease in EC$_{50}$ for Na$^+$ in the presence of 50 μM leucine (Fig. 4d and Table 1). The addition of 50 μM alanine, a lower affinity ligand, which is also a better substrate for transport$^{34}$, yielded an EC$_{50}$ for Na$^+$ of 23.0 [21.4;24.7] mM (Supplementary Fig. 4e and Table 1). In contrast, Li$^+$ was less potent against K$^+$ (EC$_{50}$ > 400 mM), and the addition of 50 μM leucine or alanine resulted in more modest potentiation of the Li$^+$ response (Supplementary Fig. 4f and Table 1). Taken together, our data suggest that K$^+$ promotes a more outward-closed state in LeuT that is inhibited by the coupled binding of Na$^+$ and substrate.

**LeuT in the outward-open conformation blocks K$^+$ binding.** Disruption of a conserved salt bridge between Arg30 (TM1) and Asp404 (TM10), located in the extracellular permeation pathway, has previously been shown to trap LeuT in an outward facing conformation$^{17,32}$. We hypothesized that if K$^+$

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**Figure 4 | K$^+$-induced conformation competes with the coupled binding conformation of Na$^+$ and leucine.** (a) tmFRET between EL4 and TM10 as a function of K$^+$ (black) and Na$^+$ (blue) concentrations. K$^+$ produces a tmFRET-specific increase that fitted by the Hill equation indicates a single binding site ($n_{H_{i}} = 1.06 ± 0.15$) having an EC$_{50}$ = 260 [137;495] mM. Na$^+$ showed no conformational effect. (b) The conformational effect by K$^+$ is attenuated by Na$^+$ and further blocked with leucine. Addition of Na$^+$ (100 mM) right-shifted the K$^+$ generated tmFRET response (blue, open squares) relative to K$^+$ alone from a (dotted line). Further addition of leucine (50 μM) completely inhibited the K$^+$ response (blue closed squares). The effect by leucine was Na$^+$-dependent since substitution with 100 mM Cs$^+$ completely removed the blockade (filled pink squares). Note that Cs$^+$ does not support binding of leucine. (c) Reaction scheme for tmFRET change in response to ions, Na$^+$ binding may be assayed by competitively inhibiting K$^+$-induced conformational change. Dotted arrow indicates low tmFRET, that is, high fluorescence; solid arrow indicates high tmFRET. (d) Normalized change in tmFRET between EL4 and TM10 (F$_{0}$/F) as a function of [Na$^+$]. LeuT A313H-A317H-K398CFL (WT$^{tmFRET}$) is stabilized in the K$^+$-bound conformation with 600 mM K$^+$ and ‘back titrated’ with Na$^+$, in the presence of 0.05 or 50 μM Leu, which results in an EC$_{50}$ (in mM) for Na$^+$ of 94.0 [80.9;109] 26.6 [23.9;29.6] and 3.52 [3.29;3.76], respectively. Ch$^+$ was substituted for Na$^+$ or K$^+$ to maintain ionic strength. All the data are performed in 750 μM Na$^+$ efflux. Data are means ± s.e.m. EC$_{50}$ is given as mean with 95% confidence interval, n = 3–8.
binds an outward-closed conformation, then disruption of the Arg30–Asp404 salt bridge should inhibit the K⁺ response in LeuT. To disrupt the salt bridge, we mutated Arg30 to Ala in WTtmFRET, followed by purification and fluorescent labelling of the resulting construct (R30A tmFRET). Importantly, and further supporting that our tmFRET measurements most likely detects the resulting construct (R30A tmFRET). This suggests an increased distance between TM10 and EL4, in agreement with a more outward open conformation of the mutated transporter (Fig. 5a and Supplementary Fig 3d). The tmFRET was slightly increased in Na⁺ and increased to a greater extend by further addition of leucine. However, K⁺ induced no significant change in tmFRET relative to Cs⁺ (Fig. 5a and Supplementary Table 2). To estimate the effect of R30A on K⁺ potency, we performed a Schild analysis, which determined that the effect of K⁺ (Kb) on Na⁺ dependent [³H]leucine binding was essentially lost (Fig. 5b,c). Thus, disrupting the Arg30–Asp404 salt bridge abolishes the effect of K⁺ binding was essentially lost (Fig. 5b,c). Thus, disrupting the Arg30–Asp404 salt bridge abolishes the effect of K⁺ binding, presumably by biasing LeuT towards the outward-open state. This observation lends further support to the assumption that K⁺ binding to LeuT involves a conformation that is different from the Na⁺ and substrate bound conformation.

The K⁺-bound conformation requires an intact Na⁺ site. Next, we assessed the consequence of mutations in the two Na⁺-binding sites on the K⁺ effect. To investigate the role of the Na1 site, we mutated Thr254 to Val (T254V) in the WTtmFRET background (T254V tmFRET). According to the high-resolution structures, Thr254 is one of the key coordinating residues of Na⁺ in the Na1 site. As would be expected, the Kb for [³H]leucine binding (in 800 mM Na⁺) was increased by over three orders of magnitude as Na1 is required for coordination of the substrate carboxy moiety (Fig. 6a and Supplementary Table 1). Unfortunately, this poor leucine affinity prevented a reliable Schild analysis of the K⁺-effect on [³H]leucine binding. However, tmFRET measurements were possible. 800 mM of Ch⁻ or Cs⁻ yielded similar tmFRET values as observed for the background construct WTtmFRET (Fig. 6b and Supplementary Table 2), suggesting that the introduction of the T254V mutation did not change the distance measured between EL4 and TM10 (Fig. 6b). Furthermore, the substitution with 800 mM Na⁺ or the addition of 100 μM leucine (with Na⁺) did not significantly change the tmFRET values compared with Ch⁻ (Fig. 6b). In contrast, 800 mM K⁺ produced a significant increase in tmFRET; however, the magnitude of the tmFRET change was significantly reduced compared with WTtmFRET. Thus, the T254V mutation impairs the K⁺-response, which could be the result of either a disruption of K⁺-coordination or a destabilization of the K⁺-bound conformation.

### Table 1 | Ion binding constants.

| Ion | EC₅₀ (mM) | nHill |
|-----|----------|-------|
| Na⁺ | 94.0 [80.9;109] | 1.13 ± 0.08 |
| 500 mM Leu | 26.6 [23.9;29.6] | 1.42 ± 0.09 |
| 50 μM Leu | 3.52 [3.29;3.76] | 1.23 ± 0.05 |
| 50 μM Ala | 23.0 [21.4;24.7] | 1.35 ± 0.12 |
| Li⁺ | 497 [411;600] | ND |
| 50 μM Leu | 22.5 [20.1;25.2] | 1.21 ± 0.07 |
| 50 μM Ala | 174 [163;187] | 1.58 ± 0.08 |
| K⁺ | 260 [137;495] | 1.06 ± 0.15 |
| ( + T354D) | 198 [131;299] | 1.01 ± 1.00 |

Values were obtained experimentally by tmFRET measurements with LeuT A313H-A317H-T354D (WTtmFRET). Data are shown as means ± s.e.m. or [s.e.m. interval], n = 3–4.

### Figure 5 | K⁺-binding is abolished in LeuT R30A.

(a) tmFRET values between EL4 and TM10 obtained by tmFRET experiments with R30A tmFRET are consistent with an outward-open state for the Cs⁺ condition (0.079 ± 0.007; means ± s.e.m., n = 3). Na⁺ alone and together with leucine significantly increased tmFRET (0.108 ± 0.004 and 0.148 ± 0.004; means ± s.e.m., n = 4 and 7, respectively). No significant change in K⁺-binding was observed in 800 μM of the indicated ions and 100 μM leucine (Na⁺/Leu condition). *P<0.05; ****P<0.0001; denote significance level from a one-way analysis of variance with post hoc Bonferroni’s multiple test relative to the Cs⁺ condition.

(b) Na⁺-dependence of [³H]leucine binding to R30A tmFRET in the presence of the indicated K⁺ concentrations. (c) Schild plot of the data shown in b show that K⁺-inhibition of [³H]leucine binding to R30A tmFRET is severely compromised (Kb >1,000 mM) with no significant effect of up to 800 mM K⁺.
Figure 6 | The K⁺-induced conformational change requires an intact Na1 site. (a) [³H]leucine saturation binding for T354VtmFRET (LeuT T354V-A313H-A317H-K398C; K₀ = 95.0 ± 2.7 μM) performed in the presence of 800 mM Na⁺. The dashed line is the nonlinear regression on similar experiment performed on LeuT WT (see Supplementary Fig. 2c) shown here for comparison. LeuT WT K₀ for leucine is 20.1 ± 4.2 nM (mean ± s.e.m., n = 4, see Supplementary Table 1). (b) FL-quenching values obtained by tmFRET experiments with T254YtmFRET (blue bars) or WTtmFRET (black bars) in 800 mM of the indicated ions, and 100 μM leucine (Na⁺/Leu condition). Data points are means ± s.e.m., n = 3–7. **P<0.01; denotes significance level from a one-way analysis of variance with post hoc Bonferroni test relative to the Ch⁻⁺ condition.

Figure 7 | Disruption of the Na2 site potentiates K⁺ binding. (a) Saturation binding of [³H]leucine measured by the scintillation proximity assay, for the Na2-site mutant T354VtmFRET (orange symbols, K₀ = 5.45 ± 0.40 μM) in the presence of 800 mM Na⁺. The dashed line is the nonlinear regression on similar experiment performed on LeuT WT (see Supplementary Fig. 2c) shown here for comparison. LeuT WT K₀ for leucine is 20.1 ± 4.2 nM (mean ± s.e.m., n = 4, see Supplementary Table 1). (b) TmFRET values obtained with T354VtmFRET (orange bars) compared with the WTtmFRET background (black bars), all in 800 mM of the indicated ions, and 100 μM leucine (Na⁺/Leu condition). T354V induces a K⁺-/like conformation even in the presence of Ch⁺ (0.43 ± 0.02), Cs⁺ (0.46 ± 0.02) or Na⁺ (0.44 ± 0.004). Only 100 μM leucine in Na⁺ reduced the tmFRET value (0.33 ± 0.01) relative to K⁺ (0.44 ± 0.004). (c) Na⁺-dependent [³H]leucine binding to T354VtmFRET in the presence of indicated K⁺ concentrations (substituting with Ch⁺). (d) Schild plot of the EC₅₀ values obtained in e reveal the inhibition constant for K⁺ in T354VtmFRET (orange squares; Kᵦ = 45.0 [312.59.7] mM). To investigate whether the increased K⁺ affinity could be because the T354V bias LeuT towards inward facing, we performed a similar experiment with the inward facing mutant R5D (blue squares), which possessed a similar Kᵦ = 47.3 [36.759.6] mM. LeuT WT is shown for reference (dashed line; see Fig. 1d). Data points and tmFRET data are means ± s.e.m., n = 3–8. Kᵦ values are shown as mean [95% confidence interval]. ****P<0.0001 based on one-way analysis of variance with post hoc Bonferroni’s multiple test relative to the Ch⁺ condition.

Discussion
In this study, we provide, to our knowledge, the first evidence for a role of K⁺ in regulating the function of LeuT; the most commonly used model system for NSSs. We demonstrate that K⁺ inhibits Na⁺-/dependent binding of [³H]leucine to LeuT by an apparent competitive mechanism. We also show that K⁺ is important for LeuT function as [³H]alanine uptake in LeuT proteoliposomes was markedly stimulated when internal Cs⁺ was substituted with K⁺. Moreover, by application of tmFRET, we show that K⁺ binding is linked to the outward-closed/inward facing state of the transport protein. Of particular interest, K⁺ counter-transport has previously been proposed to occur in human SERT based on analyses of plasma membrane vesicles isolated from blood platelets. However, a role for K⁺ in the NSS transport mechanism outside of SERT has, to our knowledge, not been reported.

Visualization of atomic-scale motions is essential for elucidating the mechanistic basis for protein function. In this study, we perform FRET measurements on purified LeuT between cysteine-conjugated fluorescein and Ni²⁺ bound to His-X₃-His motifs. The tmFRET method, which offers several advantages compared with classical FRET between two fluorophores, has been used before in several proteins for assessing conformational dynamics but almost exclusively in soluble proteins. In LeuT, we first validated the application
of tmFRET by generating a series of tmFRET-pairs in the water-exposed EL2. Our results revealed an apparent $R_0 \sim 12$ Å for FL and Ni$^{2+}$, which is in agreement with previous reports and supports the applicability of the method to assess shorter intramolecular distances.$^{39,40}$ Next, we constructed three tmFRET-pairs to triangulate the movement of EL4, which moves substantially between the outward- and inward-facing conformations of LeuT.$^{23,24}$ To our surprise, we observed that in the presence of K$^+$, compared with all other ions tested, tmFRET was markedly higher between EL4 and TM10, as well as between EL4 and EL2, indicating that the diameter of the extracellular permeation pathway became reduced in the presence of K$^+$. Notably, tmFRET measured in high K$^+$ (800 mM) approximately corresponded to the theoretical tmFRET value, calculated from coordinates in the outward-closed/inward-facing crystal structure (compare Fig. 2e with Supplementary Fig. 3d).

On the basis of previous EPR$^{44,46}$, cysteine accessibility$^{47}$ or smFRET$^{32,33,47}$ studies on LeuT, it has been suggested that Na$^+$ and Li$^+$ promotes an outward-open/inward-closed state of the transporter. Our tmFRET measurements, however, did not reveal any effect of Na$^+$ nor Li$^+$ (that have smaller radii than K$^+$) in comparison with the larger ions (Rb$^+$, Cs$^+$, Ch$^+$, NMDG$^+$, Tris$^+$) that do not support substrate binding. It is therefore interesting to note that K$^+$ was used as control for Na$^+$ in the previous cysteine accessibility$^{47}$ or smFRET$^{32,33,47}$ studies, indicating that the ability of K$^+$ to promote a more outward-closed conformation might have been missed. Indeed, we observed that the change in tmFRET, and thus the conformational change in response to K$^+$, was attenuated by Na$^+$ and fully blocked by leucine in combination with Na$^+$. This could reflect that the K$^+$-induced, presumably outward-closed conformation is coupled to reorganization of the Na$^+$ sites as well as the substrate site. Importantly, the crystal structure of inward-facing LeuT also displayed large distortions of the substrate and Na$^+$ sites (PDB: 3TT3). We found moreover that Na$^+$ (and Li$^+$ with a lower potency) could return LeuT to the outward-open state, starting from a transporter population in an outward-closed state promoted by K$^+$. The addition of substrate potentiated the ability of Na$^+$ to return LeuT to the outward-open state.

Although the measurements did not reveal an effect of Na$^+$ on the maximum tmFRET values, Na$^+$ and Li$^+$ (as well as K$^+$ and Rb$^+$) did appear to increase Ni$^{2+}$ affinity for the EL4 His-X$_7$-His site, suggesting that these ions upon binding cause a change in the secondary structure of EL4. Indeed, Na$^+$-induced changes in EL4 have been reported with EPR spectroscopy$^{29}$, and tmFRET has proven eligible for probing changes in $\alpha$-helical secondary structure.$^{40,43}$ When interpreting our data, we should also note that tmFRET between EL4 and TM10 obtained in Na$^+$±leucine appeared to deviate towards a more closed conformation for LeuT WTtmFRET compared with tmFRET values expected from crystal structures (Supplementary Fig. 3). However, tmFRET is based on ensemble measurements of a dynamic protein, which might reflect an average of conformations with a mean distance that differ from the measurements based on crystal structures. Finally, we should note that in the presence of Na$^+$, our tmFRET measurements indicated an apparent decrease in the EL2–EL4 distance on addition of leucine but an equivalent decrease was not seen between EL2 and TM10 (Figs 2e and 3a). According to the available LeuT crystal structures$^{11,17}$, a decrease could be expected in both directions, that is, in the presence of both Na$^+$ and leucine, the transporter should assume a more outward-closed configuration involving a decrease in the two distances. At this stage, we do not have an immediate explanation for this apparent discrepancy; however, crystal structures are static and may not reflect the entire ensemble of movements taking place in the protein in response to binding of ions and substrate.

To probe K$^+$ binding when LeuT is stabilized in the outward-open state, we mutated Arg30 (R30AtmFRET) to disrupt the conserved Arg30–Asp404 salt bridge$^{32}$. The tmFRET measurements confirmed that R30AtmFRET in the absence of binding ions was more open to the outside compared with WTtmFRET. We observed no tmFRET-response to K$^+$ for R30AtmFRET. Intriguingly, tmFRET values for R30A tmFRET between TM10 and EL4 were in the expected range when the Arg30–Asp404 salt bridge was disrupted (Fig. 5 and Supplementary Fig. 3). The data suggest that K$^+$ binding to the outward-facing conformation of LeuT is highly improbable under the investigated concentrations of Na$^+$ and substrate. Another possibility would be that Arg30 participates directly in K$^+$ binding; however, we find this less likely. Given our strong evidence that K$^+$ preferentially binds that outward-closed/inward-facing conformation, it is striking that when biasing the transport towards the outward-open/inward-closed conformation by mutating Arg30, the effect of K$^+$ is essentially gone (as would be expected). Moreover, as internal K$^+$ stimulates the accumulation of substrate, K$^+$ is likely to bind from the inside making it less likely that K$^+$ binds in the extracellular vestibule$^{17}$.

To probe the possible role of the Na sites in K$^+$ binding, we mutated the Na1 and Na2 site in LeuT, respectively. Thr254 was substituted with Val (T254V tmFRET) to disrupt the Na1 site and, as expected, [H]$^+$leucine affinity was greatly reduced in this mutant, because Na1 is required for coordination of the substrate carboxy-moiety.$^{11}$ The mean tmFRET between TM10 and EL4, however, was unchanged for T254VtmFRET compared with WTtmFRET (in Cs$^+$, Ch$^+$, Na$^+$). In 800 mM K$^+$, we observed a significantly higher, tmFRET compared with Ch$^+$ but the increase was markedly smaller than for WTtmFRET and so small that we could not reliably determine the EC$_{50}$ for the K$^+$ effect. Thus, it is possible that an intact Na1 site is required for K$^+$ binding but because of the functional perturbation of LeuT by the T254V mutation, it is not possible to conclude whether T254V disrupts coordination of K$^+$, or perturbs the conformational change required for K$^+$ binding. Indeed, the mutation did not ablate the K$^+$ effect completely.

To disrupt the Na2 site, we mutated Thr354 (T354V tmFRET) and observed that tmFRET between TM10 and EL4, in its apo-form (Ch$^+$ or Cs$^+$), was identical to the K$^+$-bound conformation observed in WTtmFRET, and approximately corresponding to tmFRET predicted from the inward-facing crystal structure (PDB: 3TT3; ref. 17). Schild plots suggested that T354V potentiated K$^+$ binding. This was also observed in the RSD mutation, where the intracellular interaction network has been destabilized$^{8,32}$. Thus, K$^+$ binding to LeuT appears to be favoured when the Na2 site and/or inner gate are disrupted. Taken together, this suggests that Thr354 is involved in stabilizing the outward-open conformation, possibly by forming a low-barrier hydrogen-bond with the backbone carbonyl oxygen of Gly20 (compare PDB: 2A65 and 3TT1)$^{11,17}$. We observed a similar tmFRET profile and EC$_{50}$ for K$^+$ in LeuT T354DtmFRET, suggesting that a similar role of the Na2 site may be plausible in mammalian NSS.

Collectively, our data support a model where internal K$^+$ interacts with an NSS protein in an outward-closed/inward-facing conformation. We propose that internal K$^+$ facilitates transport by binding to LeuT in a way that inhibits rebinding of Na$^+$ and, accordingly, also substrate. This will minimize substrate efflux and result in an increased concentrative capacity of the transporter, that is, substrate can be transported against a larger chemical gradient. Unbinding of K$^+$ from LeuT will then favour...
an outward-open conformation of the transporter that in turn is further stabilized by Na\(^{+}\) and by the engineered His-142C, with a final concentration of lipid 200 mg ml\(^{-1}\). The lipid suspension was sonicated for a total of six cycles (one cycle of 15 s on/45 s off on ice), flash frozen and slowly thawed at room temperature three times. Liposomes were then extruded 11 times with a mini extruder (Avanti) over a filter of pore size 100 nm. Liposomes were then destabilized stepwise with 0.2% (w/v) sodium dodecyl sulfate (SDS) and 120,000 g supernatant. The detergent was removed using four cycles of stepwise Bio-Beads (Bio-Rad) addition (in total 160 mg ml\(^{-1}\)) and centrifugation at 120,000 g for 30 min at room temperature. After each cycle, the detergent density was confirmed by ultraviolet–visible spectroscopy as \(\epsilon_{\text{meas}} = 113.300 \, \text{M}^{-1} \cdot \text{cm}^{-1}\) corrected for FL absorbance at 280 nm as per the manufacturer’s instructions. The FL labelling efficiencies were quantified as \(A_{\text{FL}} = (\epsilon_{\text{FL}} = 83.000 \, \text{M}^{-1} \cdot \text{cm}^{-1})\). SDS–PAGE analysis verified labelling specificity and sample purity (see Supplementary Fig. S3). The samples were stored at \(-80^\circ\) C for future use.

### Protein reconstitution

Proteoliposomes were prepared from E. coli total lipid extract (Avanti Polar lipids Inc.) as reported\(^{10}\) from detergent solubilized LeuT WT using protein:lipid (w/v) ratio of 1:100. In brief, lipid was dried under a gentle stream of nitrogen to remove the organic solvent chloroform. Remaining chloroform traces were removed overnight in a rotavapor. The lipid films were hydrated, with internal solution containing 200 mM KCl, CsCl or NaCl and buffered with 7.5 mM HEPES at pH 7.5 (pH was maintained with ammonium hydroxide), with a final concentration of lipid 200 mg ml\(^{-1}\). The lipid suspension was sonicated for a total of six cycles (one cycle of 15 s on/45 s off on ice), flash frozen and slowly thawed at room temperature three times. Liposomes were then extruded 11 times with a mini extruder (Avanti) over a filter of pore size 100 nm. Liposomes were then destabilized stepwise with 0.2% (w/v) sodium dodecyl sulfate (SDS) and 120,000 g supernatant. The detergent was removed using four cycles of stepwise Bio-Beads (Bio-Rad) addition (in total 160 mg ml\(^{-1}\)) and centrifugation at 120,000 g for 30 min at room temperature. After each cycle, the detergent density was confirmed by ultraviolet–visible spectroscopy as \(\epsilon_{\text{meas}} = 113.300 \, \text{M}^{-1} \cdot \text{cm}^{-1}\) corrected for FL absorbance at 280 nm as per the manufacturer’s instructions. The FL labelling efficiencies were quantified as \(A_{\text{FL}} = (\epsilon_{\text{FL}} = 83.000 \, \text{M}^{-1} \cdot \text{cm}^{-1})\). SDS–PAGE analysis verified labelling specificity and sample purity (see Supplementary Fig. S3). The samples were stored at \(-80^\circ\) C for future use.

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fluorescence intensity from the control construct devoid of the His-3-His site (succinate) and incubated for 24 h on shaker and recorded on a 2450 MicroBeta2 microplate counter.

Fluorescence spectroscopy. FL labelled LeuT variants generated for tmFRET experiments were diluted to 0.5 μg ml⁻¹ (8.4 nM) in Fluorescence Buffer (20 mM Tris-HCl (pH 7.50), 0.1 mM tris-(2-carboxyethyl)-phosphine (TCEP) and 0.05 mM DDM (Anatrace)) that was supplemented with NaCl, KCl, LiCl, RbCl, CsCl, MgCl₂, MnCl₂, MgCl₂ as specified. The samples were incubated for 30 min at room temperature before fluorescence intensity was recorded. Competing effects of ions and substrates on the K⁺ response was investigated by performing the KCl titration experiment in buffers containing 100 mM of NaCl, LiCl or CsCl and in the absence or presence of 50 mM alanine.

3-His site. Furthermore, thrombin cleavage of the C-terminal his-tag that binds Ni²⁺ did not change tmFRET recordings for the generated tmFRET variants.

Absorbance spectra. Ultraviolet–visible absorbance spectra of Ni²⁺, Zn²⁺ and Ca²⁺ diluted in Fluorescence Buffer with 200 mM NaCl, were obtained on a NanoQuant Infinite M200 (Tecan). The obtained absorbance values (A) were plotted as a function of the corresponding log [B] values. K⁺ affinity (pKB) values were calculated with the Schid equation:

\[
\log (d_r - 1) = \log [B] - K_B
\]

All the experiments were repeated at least three times unless otherwise stated. Data points are given as means ± s.e.m. or means with 95% confidence intervals. Kinetic and equilibrium constants and tmFRET values were obtained using non-linear regression algorithms in Prism 5.0 or 6.0 (GraphPad Software). Statistical analyses were performed using Student’s t-test or one-way analysis of variance multiple comparison test as appropriate.

Analysis of fluorescence data. Fluorescence measurements were corrected for dilution, the inner-filter effect of Ni²⁺ and collisional quenching as described by normalizing fluorescence intensities from the tmFRET construct (F) to fluorescence intensities from the control construct devoid of the His-X₃-His site (F₀). The corrected FL quenching (1 – F/F₀) plotted as a function of log [Ni²⁺] and fitted to a single-site model yielded the apparent tmFRET efficiency (E). Theoretical values of E were calculated using the Forster equation:

\[
E = 1 - \frac{1}{R_0^6} \text{where} R_0 = 12 \text{ Å (ref. 39) and donor-acceptor distances \( R \) were determined from published X-ray crystal structures of LeuT (PDB code: 2A6S; 3T1; 3T33); 11,17.}

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions
C.B.B., U.G. and C.J.L. designed the study. C.B.B. generated and purified LeuT variants with support from J.S.M. C.B.B. established protocols for tmFRET and radiotracer binding experiments and performed the majority of experiments with support from J.S.M. A.S. and S.G.S. performed reconstitution and uptake experiments with collated binding controls. C.B.B., U.G. and C.J.L. designed the experiments and interpreted the data with support from L.S., J.S.M., H.H.S. and A.S. C.B.B., U.G. and C.J.L. wrote the manuscript and all the authors commented.

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