The Molecular Mechanism of Intestinal Levodopa Absorption and Its Possible Implications for the Treatment of Parkinson’s Disease

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ABSTRACT

Levodopa (L-DOPA) is the naturally occurring precursor amino acid for dopamine and the main therapeutic agent for neurologic disorders due to dopamine depletion, such as Parkinson’s disease. L-DOPA absorption in small intestine has been suggested to be mediated by the large neutral amino acids transport machinery, but the identity of the involved transporters is unknown. Clinically, coadministration of L-DOPA and dietary amino acids is avoided to decrease competition for transport in intestine and at the blood-brain barrier. L-DOPA is routinely coadministered with levodopa metabolism inhibitors (dopa-decarboxylase and cathechol-O-methyl transferase inhibitors) that share structural similarity with levodopa. In this systematic study involving Xenopus laevis oocytes and Madin-Darby canine kidney epithelia expression systems and ex vivo preparations from wild-type and knockout mice, we identified the neutral and dibasic amino acids exchanger (antiporter) b0,+AT-rBAT (SLC7A9-SLC3A1) as the luminal intestinal L-DOPA transporter. The major luminal cotransporter (symporter) b0,+AT1 (SLC6A19) was not involved in levodopa transport. L-Leucine and L-arginine competed with levodopa across the luminal enterocyte membrane as expected for b0,+AT-rBAT substrates, whereas dopa-decarboxylase and cathechol-O-methyl transferase inhibitors had no effect. The presence of amino acids in the basolateral compartment mimicking the postprandial phase increased transepithelial levodopa efflux from intestinal enterocytes. These results identify the molecular mechanisms mediating small intestinal levodopa absorption and suggest strategies for optimization of delivery and absorption of this important prodrug.

Introduction

Parkinson’s disease (PD) is a neurodegenerative disorder mainly caused by dopamine depletion in the substantia nigra, clinically manifested by symptoms including its hallmark, the trias of bradykinnesia, resting tremor, and rigidity (Nyholm and Lennernas, 2008; Ahlskog, 2011; Hickey and Stacy, 2011). Since its introduction in 1968, levodopa (L-dihydroxyphenylalanine, L-DOPA) is the major therapeutic agent in treating PD (Poewe et al., 2010). After passing the blood-brain barrier (BBB), levodopa is converted into dopamine by the dopa-decarboxylase (DDC) (Nyholm and Lennernas, 2008). To prevent levodopa metabolism prior to its transport across the BBB, orally administered levodopa is given in combination with a DDC inhibitor (e.g., carbidopa or benserazide) (Hollingworth et al., 2011). It may be additionally combined with a catechol-O-methyltransferase (COMT) inhibitor (such as entacapone) to avoid levodopa methylation into 3-O-methyldopa.

Levodopa is a large neutral amino acid (AA) structurally very similar to the aromatic AAs L-phenylalanine (Phe) and L-tyrosine (Tyr). Levodopa is believed to compete with other neutral AAs for its active transport across the BBB, as well as across small intestine enterocytes. In the brain, levodopa has been suggested to be transported by the neutral AA heterodimeric transporter LAT1-4F2hc (SLC7A5-SLC3A2) (Uchino et al., 2002; Morimoto et al., 2008; Ahlskog, 2011). Intestinal levodopa transport has also been suggested to be mediated by neutral AA transporters (Lennernas et al., 1993), but the identity of the transporters involved in its intestinal absorption is still unknown. Several AA and peptide transporters located at the luminal and basolateral enterocyte membrane have been shown to be responsible for neutral AA absorption (Broer, 2008; Verrey et al., 2009; Broer and Palacin, 2011). b0,+AT1 (SLC6A19) is the major transporter for neutral AAs in the apical enterocyte membrane (Broer, 2008; Verrey et al., 2009; Broer and Palacin, 2011). b0,+AT-rBAT (SLC7A9-SLC3A1) is the

ABBREVIATIONS: AA, amino acid; BBB, blood-brain barrier; COMT, catechol-O-methyl transferase; DDC, dopa-decarboxylase; L-DOPA, levodopa; PCR, polymerase chain reaction; PD, Parkinson’s disease.
transporter responsible for cystine reabsorption in the kidney, but transports cationic as well as neutral AAs across the luminal enterocyte membrane (Bertran et al., 1992; Palacin et al., 1998, 2004; Feliubadalo et al., 1999; Pfeiffer et al., 1999; Dave et al., 2004). Among the basolateral enterocyte transporters, the AA exchangers y′LAT1-4F2hc and LAT2-4F2hc (SLC7A8-SLC3A2) (Pfeiffer et al., 1999; Rossier et al., 1999; Sperandeo et al., 2007) and the aromatic AA uniporter TAT1 (SLC16A10) (Kim et al., 2001; Quinones et al., 2004; Ramadan et al., 2006, 2007; Mariotta et al., 2012) were shown to promote accumulation or efflux of AA from the enterocytes to the extracellular space. In addition, the functional interaction between different transporters can affect the net flux of AAs (Nguyen et al., 2007; Ramadan et al., 2007; Verrey et al., 2009).

In the present study, we first investigated the identity of the levodopa transporter(s) on the luminal membrane of small intestinal enterocytes. Several candidate transporters were identified by structural homology of transported substrates to levodopa and tested. Second, the transepithelial transport involving apical and basolateral transporters in cells and along the murine small intestine was assessed using a polarized cell model as well as intestinal everted sacs. Additionally, the impact of luminal neutral AAs (reflecting digested dietary proteins) and routinely coadministered DDC and COMT inhibitors on intestinal levodopa transport was evaluated, as well as the impact of gender and circadian rhythm on intestinal transport expression. Specifically, the role of apical b⁰,⁰-AT1-rBAT and basolateral TAT1 in intestinal levodopa transport was elucidated.

**Materials and Methods**

**Oocytes.** Expression studies and influx assays using radiolabeled AA tracers were performed in *Xenopus laevis* oocytes. The oocytes were injected with cRNA-encoding transporters known or suggested to be expressed in the apical membrane of small intestinal enterocytes. B⁰AT1-ACE2 (SLC6A19-ace2), SIT1-ACE2 (SLC6A20-ace2), PAT1 (SLC36A1), ASC2 (SLC3A2), and b⁰⁰-AT-rBAT (SLC7A8-SLC3A1, fusion protein) cRNA were therefore respectively injected. The transporter expression. Specifically, the role of apical b⁰,⁰-AT1-rBAT and basolateral TAT1 in intestinal levodopa transport was elucidated.

**Materials and Methods**

**Levodopa Transport in Intestine**

Human b⁰⁰-AT was amplified by polymerase chain reaction (PCR) adding with XmaI at 5′ and NsiI at 3′ (primer: sense, 5′-CAAC AAC CCC GGG ATG GGT GAT ACT GGT CTC AGA AAG-3′; antisense, 5′-CCG GAG GAA GAC CCT GAG GGT GCT CCT GAT GGT GCT CCT GGA TGC ATT CAA-3′). The encoding fragment was introduced in frame into the vector containing hrBAT and linker previously linearized using SmaI (compatible with XmaI) and NsiI. For expression in *Xenopus* oocytes, the human b⁰⁰-AT-rBAT psb-plasmid was linearized with HindIII (Promega, Madison, WI) and used as a template for RNA synthesis from the T7 promoter (mMESSAGE mMACHINE; Ambion, Austin, TX).

**Cells.** Madin-Darby canine kidney cells (strain II) were cultured at 37°C in Dulbecco’s modified Eagle’s medium (Invitrogen, Basel, Switzerland) with 2 mM L-glutamine, 1% nonessential AAs (Invitrogen), and 10% fetal calf serum. Cell lines previously described by Bauch et al. (2003) were used. A further transduction on these cells was performed, as previously described, to introduce the basolateral transporter TAT1 (SLC16A10). Four different cell lines were used, as follows: 1) wild-type cells; 2) cells expressing the apical transporter b⁰⁰-AT-rBAT alone; 3) cells expressing apical b⁰⁰-AT-rBAT in the presence of the basolateral transporters LAT2-4F2hc (Sle7a8-Sle3a2) and y′LAT1-4F2hc (Sle7a7-Sle3a2); and 4) cells additionally (to cell line 3) expressing the basolateral uniporter TAT1. The wild-type and stably transfected cell lines were plated at density of 1.6 × 10⁶ cells/24 mm Corning Costar Transwell filters (Corning, Tewksbury, MA) and cultivated for 7 days. The b⁰⁰-AT-rBAT expression was induced 24 h prior to the experiment with 1 μM dexamethasone. Integrity of the monolayers was checked by resistance measurements using the Millicell device (Millipore, Bedford, MA). On the day of the experiment, the cells were incubated for 30 minutes with the DDC inhibitor benserazide (50 μM benserazide, 100 μM citric acid) in 150 mM NaCl, 10 mM HEPES (pH 7.4), 1 mM CaCl₂, 5 mM KCl, 1 mM MgCl₂, and 10 mM glucose at 37°C. After this period, the uptake solution (100 μM l-DOPA, 50 μM benserazide, and 100 μM citric acid) was added to both compartments. The filter compartment, facing the apical membrane of the cellular monolayer, was supplemented with 0.5 μCi/ml [³H]-DOPA. The concentration of levodopa used on these experiments is based on the apparent affinity of the apical transporter b⁰⁰-AT-rBAT for levodopa assayed in *X. laevis* oocytes and differs from the maximal concentration delivered to the patients’ small intestine lumen, which can reach low millimolar range (Quinones et al., 2004).

In the competition experiments, l-tyrosine or L-lysine (1 mM) was additionally added to the apical compartment. To analyze the effect of basolateral AAs on transepithelial levodopa transport (mimicking postabsorptive states with high plasma AA levels), L-leucine or L-lysine (1 mM) was added to the basolateral compartment.

After 30 minutes, apical and basolateral solutions were collected. Transwells were washed in both compartments with Na⁺-free buffer at 4°C. Cells were disrupted by incubation with Na⁺-deoxycholate and rocking for 30 minutes. After neutralizing with 0.1 M HCl, cells were added to vials and supplemented with a scintillation solution (PerkinElmer, Waltham, MA). The solution facing the cells in the filter compartment and the solution in the basolateral compartment were measured in the same way. Pooled data are shown as mean ± S.E.M., with n representing the number of filters used. For each experiment, the values for wild-type cells were subtracted from the overexpressing cells and data were expressed in nmol/h per cm².

**Animals.** Wild-type and tat1 knockout mice were housed in standard conditions and fed a standard diet. A tat1 knockout mouse model was produced by the ENU (N-ethyl-N-nitosourea) mutagenesis (Ingenuity Pharmaceuticals, Planegg, Germany). After 10 backcrossings in a C57Bl/6J background, the animals were used on the described experiments (Mariotta et al., 2012). For all the experiments, 4- to 5-month-old mice from both genders were used. All procedures for mouse handling were performed according to the Swiss Animal Welfare laws (Zürich, Switzerland).

**Intestinal Ring Uptake.** Uptake of radiolabeled AAs was performed, as previously described (Inigo et al., 2006; Camargo et al., 2009), on duodenal, jejunal, and ileal segments. Briefly, everted small...
intestinal segments were incubated in oxygenated (Oxycarbon) Krebs-Tris buffer (pH 7.4) containing 100 μM levodopa (0.05 μCi [3H]-DOPA/ml, 50 μM benserazide, and 100 μM citric acid) for 5 minutes at 37°C or 4°C. After washing the rings with ice cold buffer, the segments were dried at 55°C overnight on cellulose (Sartorius, Göttingen, Germany) and weighed. Segments were then lysed in 0.75 N NaOH for 6 hours and neutralized with 10 N HCl, and the radioactivity was determined by liquid scintillation. AA transport was expressed as pmol/mg per minute of dry tissue weight. Values obtained at 4°C were subtracted from the values of samples assayed at 37°C.

**Everted Intestinal Sacs.** To analyze transepithelial levodopa transport ex vivo, everted jejunal sacs from wild-type or tat1 knockout animals were used. Everted sacs were built by constraining the extremities of approximately 2-cm everted segments with a suture. The sacs were filled with Krebs-Tris buffer (pH 7.4) and incubated in a solution containing levodopa (100 μM L-DOPA, 0.05 μCi [3H]-L-DOPA/ml) for 10 minutes. After washing with ice-cold solution, the liquid content of the sacs (serosa) was collected, and the tissue (mucosa) was weighed and processed as the intestinal rings. The data are shown as mean ± S.E.M., with n representing the number of sacs. The transport is expressed as nmol/10 min per mg dry tissue. Values obtained at 4°C were subtracted from measurements performed at 37°C.

**Brush Border Membrane Vesicles and Western Blot Analysis.** Brush border membrane vesicles were prepared from small intestine mucosal cells using the Mg2+-precipitation technique, as described elsewhere (Biber et al., 2007). Western blotting was performed, as previously described (Bauch et al., 2003). Primary antibodies used were as follows: rabbit affinity-purified anti-mouse b0,+AT1 (diluted 1:500) and monoclonal anti-mouse actin (1:10,000) (Sigma-Aldrich, St. Louis, MO). Secondary antibodies used were as follows: donkey anti-rabbit IgG horseradish peroxidase–conjugated (Amersham Biosciences, Piscataway, NJ) or anti-mouse IgG alkaline phosphatase conjugate (Promega) diluted 1:5000. Antibody binding was detected with Immobilon Western chemiluminescent horseradish peroxidase or alkaline phosphatase substrate (Millipore), and chemiluminescence was visualized with a DIANA III camera (Raytest, Dietikon, Switzerland).

**Quantitative Real-Time PCR.** RNA was extracted from small intestine scraped mucosa, and real-time quantitative reverse-transcription PCR was performed, as previously described (Romeo et al., 2006). The abundance of the target mRNAs was calculated relative to the housekeeping gene hypoxanthine guanine phosphoribosyltransferase. Relative expression ratios were calculated as

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r = 2^{(C_t(\text{reference}) – C_t(\text{test}))},
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with C<sub>t</sub> representing cycle number at the threshold and test representing tested mRNAs. Primers and probes used have been previously described (Dave et al., 2004; Romeo et al., 2006; Moret et al., 2007).

**Data Analysis.** Data are shown as mean ± S.E.M. All assessed data were unpaired and parametric. Differences between one control group and two or more treatment groups were assessed using an analysis of variance with post hoc Dunnett’s multiple comparison test, and differences between three or more groups (executing all possible comparisons between groups) were measured utilizing an analysis of variance with post hoc Bonferroni’s multiple comparison test. For single pairwise comparisons, a Student’s two-tailed t test was performed. The P values of less than 5% were considered significant. Graphs, descriptive statistics, and calculations were performed using the software GraphPad Prism version 4.0 (GraphPad, San Diego, CA).

**Results**

**Levodopa Is Transported by the Luminal Enterocyte Transporter b0,+AT-rBAT but Not by the Major Neutral Amino Acid Transporter b0,AT1-ACE2.** To determine the apical AA transporter(s) responsible for levodopa transport across the luminal enterocyte membrane, candidate transporters were expressed in *X. laevis* oocytes. The uptake of levodopa was compared with known substrates (control AAs) for each given transporter. As depicted in Fig. 1A, oocytes expressing the AA transporters ASCT2 (SLC1A5), SIT1 (SLC6A20), and PAT1 (SLC36A1) did not transport levodopa. Oocytes expressing the AA exchanger b0,+AT-rBAT (SLC7A9-SLC3A1) transported levodopa as efficiently as the control AA (l-leucine) used. The major neutral luminal AA transporter b0,AT1-ACE2 (SLC6A19-ace2) did not transport levodopa. The current generated by the cotransport of sodium (Fig. 1B) and the accumulation of radiolabeled levodopa (Fig. 1C) were negligible, despite structural homology of levodopa and b0,AT1-ACE2 substrates L-phenylalanine and L-tyrosine. The lower pH encountered in the uptake solution was not the cause of the absence of levodopa uptake by b0,AT1, because electrophysiological measurements were conducted without the supplementation of citric acid. b0,+AT-rBAT hence is the only known luminal levodopa transporter of small intestine.

**The Transport of Levodopa by b0,+AT-rBAT Can Be Inhibited by the Presence of Luminal Neutral Amino Acids, but Not by Carbidopa, Entacapone, or Benserazide.** To test whether levodopa and the neutral AA l-leucine compete for b0,+AT1-rBAT–mediated transport, radiolabeled levodopa influx experiments in *X. laevis* oocytes were performed in the presence or absence of l-leucine and vice versa. Both radiolabeled levodopa and l-leucine uptake were abolished by the presence of excess of unlabeled l-leucine and levodopa, respectively (Fig. 2A).

Because DDC inhibitors benserazide and carbidopa and the COMT inhibitor entacapone display a similar structure to levodopa, a potential effect of these substrates on b0,+AT1-rBAT accumulated levodopa intra- and transepithelial levodopa transport, respectively (Fig. 3A, white bars). Cells additionally expressing the antiporter LAT2-4F2hc in the basolateral membrane showed a 3- and 2-fold increase in intracellular levodopa accumulation and transepithelial levodopa transport, respectively (Fig. 3A, gray bars). Maximal transepithelial transport was observed in cells additionally overexpressing the facilitated diffusion protein TAT1 in their basolateral membrane. In these cells,
a fivefold increase in basolateral and threefold decrease in intracellular accumulation were observed (Fig. 3A, black bars).

To mimic the absorptive (presence of high AA concentrations in the lumen) or postabsorptive (presence of high AA concentrations in the extracellular space) state, excess concentrations of L-leucine or L-lysine were respectively added to the apical or basolateral compartments. The presence of excess L-leucine (Fig. 3B) or L-lysine (Fig. 3C) in the apical compartment reduced intracellular levodopa accumulation, as well as the transepithelial transport in all cell lines used. These results suggest that both L-lysine and L-leucine compete with levodopa, as expected for transport via $b_0^{+}\alpha T-rBAT$.

Adding excess L-leucine to the basolateral compartment increased transepithelial transport of levodopa in cells expressing LAT2-4F2hc (Fig. 3D, gray bars). This result suggests that basolateral L-leucine stimulates the antiporter LAT2-4F2hc to export intracellularly accumulated levodopa to the basolateral compartment. To test whether $y^{+}LAT1-4F2hc$ could also be involved in basolateral levodopa efflux, we analyzed the influence of the $y^{+}LAT1-4F2hc$ substrate lysine. The presence of excess L-lysine in the basolateral compartment had no effect on levodopa transport (Fig. 3E), suggesting that it does not transstimulate the efflux of intracellularly accumulated levodopa via antiporter $y^{+}LAT1-4F2hc$. Based on these results, we suggest that LAT2-4F2hc, but not $y^{+}LAT1-4F2hc$, participates in basolateral levodopa efflux.

**Neutral and Cationic Amino Acids Compete with Levodopa for Its Absorption in the Murine Small Intestine.**

Levodopa accumulation in intestinal enterocytes was tested using mouse everted rings of three different small intestinal segments (duodenum, jejunum, and ileum). Cellular uptake was similar in all intestinal segments in the condition tested (Fig. 4A), and uptake rates were therefore pooled for the competition experiments, as depicted in Fig. 4B. Adding excess

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**Fig. 1.** Levodopa is transported by the luminal enterocyte transporter $b_0^{+}\alpha T-rBAT$, but not by the major neutral amino acid transporter $B_0^\text{AT1-ACE2}$. (A) Oocytes expressing $b_0^{+}\alpha T-rBAT$ transport levodopa as efficiently as L-leucine. Oocytes were injected with cRNA-encoding mouse PAT1 (20 ng), human SIT1 (5 ng), and human accessory protein ACE2 (20 ng); human $B_0^\text{AT1}$ (10 ng) and human accessory protein ACE2 (10 ng); human ASCT2 (10 ng); or human $b_0^{+}\alpha T-rBAT$ fusion protein (10 ng). Levodopa uptake rates (100 µM; white bars) were compared with uptake rates of 100 µM glycine (PAT1), 100 µM L-proline (SIT1-ACE2), 100 µM L-leucine ($B_0^\text{AT1-ACE2}$ and $b_0^{+}\alpha T-rBAT$), and 100 µM L-alanine (ASCT2) (dark gray bars), respectively. The uptakes were performed at 25°C for 10 minutes. Results are expressed in pmol/oocyte per hour, and values of noninjected oocytes were subtracted. Uptake results of control AAs were normalized to 1. Bars represent means ± S.E.M., n = 4–9 injected oocytes per group. **P < 0.01; ***P < 0.001; ns, not significant (unpaired two-tailed t tests). (B and C) Oocytes expressing $B_0^\text{AT1-ACE2}$ do not transport levodopa. (B) Levodopa does not induce current in oocytes expressing $B_0^\text{AT1-ACE2}$: oocytes expressing $B_0^\text{AT1-ACE2}$ were superfused with L-leucine, L-phenylalanine, L-tyrosine, or levodopa (1 mM each). Results are expressed as current generated by the transport at a membrane potential of −50 mV and are represented as means ± S.E.M., n = 4 oocytes per group. *P < 0.05 (analysis of variance with post hoc Dunnett’s multiple comparison test). (C) Levodopa is not accumulated in oocytes expressing $B_0^\text{AT1-ACE2}$. The transport of L-leucine, L-phenylalanine, L-tyrosine, and levodopa was assayed by measuring radiolabeled compound accumulation (1 mM, 30 minutes). Results are expressed in pmol/oocyte per hour, and values of the noninjected oocytes were subtracted. Results are given as means ± S.E.M., n = 5–6 oocytes per group. *P < 0.05 (analysis of variance with post hoc Dunnett’s multiple comparison test).
Basolateral Uniporter TAT1 Affects Transepithelial Levodopa Transport In Vivo. To analyze the role of the basolateral AA uniporter TAT1 on levodopa efflux from mucosal cells into the serosal compartment and on its transepithelial transport, levodopa accumulation was measured using everted intestinal sacs. Intracellular levodopa accumulation in tat1 (slc16a10) knockout mice did not differ from wild-type animals (Fig. 4C), whereas transepithelial levodopa transport was significantly reduced in preparations made from knockout animals when compared with wild-type mice (Fig. 4D). These results suggest that the aromatic AA transporter TAT1 plays a major role in mediating levodopa efflux from intestinal enterocytes.

Circadian Rhythm and Gender Influence on Transporters Involved in Levodopa Absorption. Absorption of levodopa in humans follows a circadian rhythm with faster absorption during day and delayed absorption during night (Nyholm et al., 2010). Furthermore, gender differences in levodopa bioavailability and in clinical presentation were observed (Kompoliti et al., 2002; Martinelli et al., 2003). We analyzed the expression of transporters involved in levodopa transport in the small intestine of male and female rodents during the active (related to daytime in humans) and nonactive phase (related to night-time in humans). Intestinal gene expression of transporters involved in levodopa absorption (b0,AT-rBAT, LAT2-4F2hc, and TAT1) was not different along the small intestine between male and female mice and during active and nonactive phases (Fig. 5A). The RNA expression of b0,AT-rBAT was not different between males and females or between the different activity periods (Fig. 5A). However, the protein expression of b0,AT showed a tendency to higher levels during the active phase in male as compared with female rodents, but did not differ among groups during the nonactive period (Fig. 5B).

Discussion

For more than 20 years, it was suggested that levodopa absorption would take place by the same active transporters as large neutral AAs, but the identity of the transporter(s) was not known (Frankel et al., 1989). This study identified the intestinal players involved in the absorption of levodopa. Using several experimental models, we demonstrated that b0,AT-rBAT (apical), LAT2-4F2hc, and TAT1 (basolateral) are responsible for small intestinal levodopa absorption.

Levodopa Is Transported by the Luminal Entercyte Transporter b0,AT-rBAT but Not by the Major Neutral Amino Acid Transporter B0AT1-ACE2. Several different AA transporters for neutral AAs are expressed in the apical membrane of small intestinal enterocytes (Verrey et al., 2009) and might therefore transport levodopa. The RNA expression of b0,AT-rBAT was not different between males and females or between the different activity periods (Fig. 5A). However, the protein expression of b0,AT showed a tendency to higher levels during the active phase in male as compared with female rodents, but did not differ among groups during the nonactive period (Fig. 5B).

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Fig. 3. Transepithelial transport of levodopa depends on b₀⁺AT-rBAT expression in the apical and LAT2-4F2hc and TAT1 in the basolateral membrane. Madin-Darby canine kidney cells expressing the apical transporter b₀⁺AT-rBAT (white bars), the apical transporter b₀⁺AT-rBAT, and basolateral transporters LAT2-4F2hc and γ⁺LAT1-4F2hc (gray bars) and (additionally to apical b₀⁺AT-rBAT and basolateral LAT2-4F2hc and γ⁺LAT1-4F2hc) TAT1 (black bars) were assayed in five different conditions (A–E). (A) Radiolabeled levodopa was added to the apical compartment. (B) Radiolabeled levodopa and L-leucine (1 mM) were added to the apical compartment. (C) Radiolabeled levodopa and L-lysine (1 mM) were added to the apical compartment. (D) Radiolabeled levodopa in the apical compartment and L-leucine (1 mM) in the basolateral compartment. (E) Radiolabeled levodopa in the apical compartment and L-lysine (1 mM) in the basolateral compartment.
AA transporter B₀AT₁-ACE₂ transports Tyr and Phe, levodopa is not transported. Levodopa differs from Tyr and Phe only by the number of hydroxyl groups on the aromatic ring. The hydroxyl group substitution seems to reduce B₀AT₁-ACE₂ affinity for this substrate, but has no effect on b₀,₁AT-rBAT-mediated transport. b₀,₁AT-rBAT transports Phe, Leu, Tyr, as well as L-methionine, L-glutamine, L-histidine, L-ornithine, cationic AAs, and the dipeptide cystine (Bertran et al., 1992; Wells and Hediger, 1992; Pfeiffer et al., 1999). Furthermore, the transporter b₀,₁AT-rBAT was also previously shown to be involved in intestinal drug (gabapentin) absorption in rodents (Nguyen et al., 2007) and renal levodopa reabsorption (Quinones et al., 2004). Its broad substrate selectivity makes luminal intestinal and proximal tubule transporter b₀,₁AT-rBAT an interesting pharmacological target. A broad selectivity was also observed for the basolateral antipporter LAT₂-4F₂hc and for the BBB levodopa transporter LAT₁-4F₂hc. These two system L antiporters were indeed shown to transport a number of metabolites, hormones, and drugs in addition to proteinogenic neutral AAs (Morimoto et al., 2008).

In human small intestine, the coadministration of Leu was shown to decrease the absorption of levodopa (Lennernäs et al., 1993), an effect that may be explained by competition of these AAs for transepithelial transport. In our study, we elucidate the molecular mechanism of this competition in the small intestine. By using oocytes and cell cultures overexpressing b₀,₁AT-rBAT, we could indeed demonstrate that Leu or cationic AAs inhibit levodopa transport. Furthermore, Leu and cationic AAs competed for levodopa uptake along the mouse small intestine, similarly to the observations reported in humans. Despite the differential b₀,₁AT-rBAT mRNA and protein expression along mouse intestine (Dave et al., 2004), levodopa entered the apical compartment, and L-lysine (1 mM) in the basolateral compartment. In the conditions in A–E, both compartments contained solutions supplemented with 100 μM levodopa, 50 μM benserazide, and 100 μM ascorbic acid. The intracellular (middle panels) and the basolateral accumulation (right panels) of levodopa were measured and subtracted from the wild-type cell values. Results are expressed in nmol/h per cm² and given as mean ± S.E.M., n = 5–6 filters per experiment. *P < 0.05 (analysis of variance with post hoc Bonferroni’s multiple comparison test); **P < 0.01; ***P < 0.0001. ns, not significant.

Fig. 4. Levodopa transport takes place all along the small intestine and can be inhibited by addition of luminal L-leucine and L-arginine or by ablation of basolateral transporter TAT₁. (A) Levodopa accumulates similarly in all segments of the small intestine. Everted rings from duodenum, jejunum, and ileum were incubated with levodopa (100 μM) at 37°C (white bars) or 4°C (cross-hatched bar). Results are expressed in pmol/mg dry tissue per 5 minutes and given as mean ± S.E.M., n = 6–8 experiments per group. No significant differences between different intestinal localizations were seen (analysis of variance with post hoc Bonferroni’s multiple comparison test; *P > 0.05). (B) Neutral and cationic amino acids compete with levodopa transport. Excess of L-leucine (1 mM, gray bar) and L-arginine (1 mM, black bar) was added to the uptake solution. Everted rings from all three small intestine segments were used in this experiment. Values obtained at 4°C were subtracted from uptakes performed at 37°C. Results are expressed in pmol/mg dry tissue per 5 minutes and given as mean ± S.E.M., n = 5–7 experiments per group. *P < 0.05; **P < 0.01 (analysis of variance with post hoc Dunnett’s multiple comparison test). (C and D) The ablation of TAT₁ decreases transepithelial levodopa transport. Everted jejunal sacs of wild-type (black bars) and TAT₁ knockout animals (gray bars) were assayed for levodopa transport. The accumulation in the tissue (mucosa) (C) and the levodopa accumulation in the sacs resulting from the transepithelial transport (serosal) (D) were quantified. Results are expressed in nmol/mg dry tissue per 10 minutes. Values at 4°C were subtracted, and data are given as mean ± S.E.M., n = 11–12 everted sacs per group. ns, not significant; *P < 0.05 (unpaired two-tailed t tests).
uptake was comparable in all segments of mouse small intestine assayed in vitro. Taken together, b0,+AT-rBAT appears to be the only luminal enterocyte transporter for levodopa, and its function is competitively inhibited by luminal neutral and dibasic AAs.

Levodopa is metabolized to dopamine by the DDC in different tissues, including brain, kidney, and intestine (Gomes and Soares-da-Silva, 2002; Quinones et al., 2004). It is usually administered in combination with a DDC inhibitor such as carbidopa or benserazide or the COMT inhibitor entacapone (Hollingworth et al., 2011). Carbidopa is not transported by LAT1-4F2hc or LAT2-4F2hc (Uchino et al., 2002; Morimoto et al., 2008); however, a possible effect of carbidopa, benserazide, or entacapone on b0,+AT-rBAT transport had not yet been assayed. Despite the fact that carbidopa, entacapone, or benserazide has some structural similarity to levodopa, no inhibition of levodopa transport was observed when coadministered. We therefore conclude that levodopa transport across the luminal enterocyte membrane mediated by b0,+AT-rBAT is not affected by DDC and COMT inhibitor coadministration.

Nguyen et al. (2007) showed that peptides transported inside the cell by PEPT1 (Slc15a1) and subsequently metabolized to single AAs could be used by b0,+AT-rBAT as exchanger substrates (referred to as trans-stimulation), resulting in increased accumulation of gabapentin in rat jejunum cells. By increasing the intracellular concentration of the b0,+AT-rBAT substrate L-arginine in X. laevis oocytes, we correspondingly observed a trend of trans-stimulation of levodopa uptake and higher intracellular accumulation (not statistically significant). These results suggest that a preload of cells before the ingestion of levodopa may not be deleterious.  

**Fig. 5.** Intestinal b0,+AT-rBAT expression varies with gender and circadian rhythm at protein but not at RNA level. (A) Expression of mRNAs encoding the transporter b0,+AT-rBAT (Slc7a9-Slc3a1), LAT2-4F2hc (Slc7a8-Slc3a2), TAT1 (Slc16a10), and peptide transporter PEPT1 (Slc15a1) in male and female animals during active and inactive periods was unchanged. The gene expression of animals in the active (4 hours after the dark-phase onset) and nonactive phase (4 hours after the light-phase onset) was analyzed by quantitative real-time PCR. Results are expressed relative to the housekeeping gene hypoxanthine guanine phosphoribosyltransferase and given as mean ± S.E.M., n = 3 female (solid bars) and 3 male (hatched bars) animals. ns, not significant; *P < 0.05 (analysis of variance with post hoc Bonferroni’s multiple comparison test). (B) The protein level of b0,+AT in male animals is higher during the active phase when compared with the inactive phase. (D) Expression of b0,+AT in the duodenum (white bars), jejunum (gray bars), and ileum (black bars) of female (solid bars) and male (hatched bars) mice in the active and inactive phase was analyzed by Western blot analysis of brush border membrane vesicles (50 μg). The Western blots were quantified by densitometry and normalized to β-actin. The results are expressed relative to actin and given as mean ± S.E.M., n = 3 animals each gender. ns, not significant; *P < 0.05 (analysis of variance with post hoc Bonferroni’s multiple comparison test).
levels of some small intestinal transporters have been shown to flow (Pacha and Sumova, 2013). Furthermore, gene expression data suggest that nocturnal delayed gastric emptying, supine position, and betaxolol are faster during the daytime (Nyholm et al., 2010). This differential effect might be due to variations in absorption at the level of the basolateral compartment. In cells additionally expressing TAT1, the same level of labeled levodopa efflux was observed in the absence of basolateral Leu. This may be explained by the fact that TAT1 is a unipporter (facilitated diffusion pathway) and does not depend on the presence of contralateral substrates (Kim et al., 2001; Ramadan et al., 2006). Using this epithelial coexpression system, we thus showed that both AA transporters LAT2-4F2hc and TAT1 can function as basolateral levodopa efflux pathways and consequently participate in its transepithelial transport. Interestingly, a functional interaction of these two basolateral enterocyte transporters was previously shown (Ramadan et al., 2007). Similarly, as observed for PEPT1 and b0,1 AT-RBAT in the apical membrane, TAT1 can stimulate the function of the antiporter LAT2-4F2hc. For instance, aromatic AAs exported by the unipporter TAT1 represent high-affinity extracellular substrates for LAT2-4F2hc that can thus be exchanged by this antiporter with intracellular substrates such as levodopa, thereby increasing transepithelial transport. This functional interaction is postulated to be crucial for the net directional transport of LAT2-4F2hc substrate AAs and may thus influence the net levodopa flux. In vivo experiments using everted gut sacs of tat1 knockout animals confirmed that TAT1 plays a central role as basolateral efflux pathway for levodopa by using experimental conditions without basolateral AAs.

Levodopa Absorption along the Small Intestine, Gender, and Circadian Variation of Transporters. Parkinson patients develop several nonmotor symptoms with a diurnal component, suggesting circadian dysfunction (Kallio et al., 2000; Buijs et al., 2003; Willison et al., 2013). Orally administered levodopa is usually delivered throughout the day in several doses because the absorption of the drug was shown to be faster during the daytime (Nyholm et al., 2010). This difference in circadian pharmacokinetics may be a combination of the nocturnal delayed gastric emptying, supine position, and daily rhythmicity in gastrointestinal enzyme activity and blood flow (Pacha and Sumova, 2013). Furthermore, gene expression levels of some small intestinal transporters have been shown to exhibit circadian rhythms. For instance, sugar transporters, including the apical Na+/glucose cotransporter 1 (SGLT1/Slc5a1) and fructose transporter 5 (GLUT5/Sle2a5) as well as the basolateral hexose transporter GLUT2/Sle2a2, were shown to have rhythmic expressions (Castello et al., 1995; Pan et al., 2004; Pan and Hussain, 2009). Additionally, the expression of the proton-coupled peptide transporter 1 (PEPT1/Slc15a1), a transporter involved in drug absorption, also shows a circadian cycle (Pan et al., 2003, 2004; and Pan and Hussain, 2009; Qandeel et al., 2009). The expression of the gene encoding the PEPT1 transporter, which was previously shown to increase in the active phase (Pan and Hussain, 2009), showed a similar, nonstatistically significant trend in our experiments. The mRNA expression of intestinal transporters b0,1 AT, rBAT, 4F2hc, LAT2, and TAT1 during the active (reflecting daytime expression in humans) versus the nonactive (reflecting nocturnal gene expression in humans) phase of wild-type mice was not significantly different in the present study. In contrast, the protein expression level of the apical transporter b0,1 AT measured in brush border membrane vesicles was significantly increased during the active phase in male animals, but interestingly not in females. Because the changes in RNA levels precede the changes at the protein level, as shown for PEPT1 (Qandeel et al., 2009), we may have missed RNA changes that potentially took place earlier. Differences in the circadian cycle of male and females as observed in our study are not yet well understood (Bailey and Silver, 2014), but importantly, gender differences have also been suggested to play a major role in PD patients (Kompoliti et al., 2002; Steffansen et al., 2004; Miller and Cronin-Golomb, 2010; Pavon et al., 2010; Doi et al., 2012).

Additionally, the effect of the disease and of levodopa treatment on transporter expression and function is as yet uncharacterized. For example, it has been observed that, after sustained periods of treatment with constant levodopa concentrations delivered to intestinal lumen, patients can require adjustments to levodopa dosage (Lundqvist, 2007). This observation suggests that variations in absorption may occur due to either drug treatment or disease progression. Therefore, whereas the identification of the transporters involved in levodopa intestinal absorption and knowledge about their mechanism of action and kinetic properties, such as trans-stimulation or competitive inhibition, is an important first step, a careful analysis of transporter expression and its correlation with levodopa transport pharmacokinetics in Parkinson mouse models and in patients is critical for the further optimization of strategies to enhance delivery and absorption of levodopa.

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Authorship Contributions

Participated in research design: Vuille-dit-Bille, Götzé, Camargo. Conducted experiments: Huggel, Singer, Vuille-dit-Bille, Mariotta, Ramadan, Camargo. Performed data analysis: Vuille-dit-Bille, Camargo. Wrote or contributed to the writing of the manuscript: Vuille-dit-Bille, Camargo, Mariotta, Ramadan, Verrey, Huggel, Singer, Götzé.

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