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Title page: The molecular mechanism of intestinal levodopa absorption and its possible implications for the treatment of Parkinson's disease.

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Running title page: levodopa transport in intestine

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Nonstandard abbreviations used in the paper:

AA – amino acid

BBB - Blood Brain Barrier

BBMV - Brush border membrane vesicles

COMT - cathechol-o-methyl transferase

DDC - dopa decarboxylase

HPRT - hypoxanthine guanine phosphoribosyltransferase

PD - Parkinson's disease

TEVC - two-microelectrode voltage clamp

X. laevis – *Xenopus laevis*

Vh - membrane holding potential

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34 text pages (from title to references)

5 figures

59 references

249 words in the abstract

441 words in the introduction

1377 words in the discussion

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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. Levodopa 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, co-administration of levodopa and dietary amino acids is avoided to decrease competition for transport in intestine and at the blood brain barrier. Levodopa is routinely co-administered 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 MDCK epithelia expression systems and *ex vivo* preparations from wild type and knockout mice, we identified the neutral and dibasic amino acids exchanger (antiporter) $b^{0,+}$AT-rBAT (SLC7A9-SLC3A1) as the luminal intestinal levodopa transporter. The major luminal co-transporter (symporter) B^0AT1 (SLC6A19) was not involved in levodopa transport. L-leucine and L-arginine competed with levodopa across the luminal enterocyte membrane as expected for $b^{0,+}$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 transport by stimulating basolateral efflux via the antiporter LAT2-4F2 (SLC7A8-SLC3A2). Additionally, the aromatic amino acid uniporter TAT1 (SLC16A10) was shown to play a major role in 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 pro-drug.

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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 bradykinesia, 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-decarbocylase (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 (as e. g. carbidopa or benserazide) (Hollingworth et al., 2011). It may be additionally combined with a cathechol-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 blood-brain barrier, 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). B^0AT1 (SLC6A19) is the major transporter for neutral AAs in the apical enterocyte membrane (Broer et al., 2004; Kleta et al., 2004; Camargo et al., 2005; Romeo et al., 2006). The neutral and dibasic AA exchanger (antiporter) $b^{0,+}AT$-rBAT (SLC7A9-SLC3A1) is the 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; Feliubadalo et al., 1999; Pfeiffer et al., 1999; Dave et al., 2004; Palacin et al.,

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2005). 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; Ramadan et al., 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 co-administered DDC and COMT inhibitors on intestinal levodopa transport was evaluated, as well as the impact of gender and circadian rhythm on intestinal transporter expression. Specifically the role of apical $b^{0,+}$AT1-rBAT and basolateral TAT1 in intestinal levodopa transport was elucidated.

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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. B^0AT1-ACE2 (SLC6A19-*ace2*), SIT1-ACE2 (SLC6A20-*ace2*), PAT1 (SLC36A1), ASCT2 (SLC1A5) and $b^{0,+}$AT-rBAT (SLC7A9-SLC3A1, fusion protein) cRNA were therefore respectively injected. The transport rate of levodopa (100 µM, 20 µCu / ml ^{3}H-levodopa, containing 100 µM citric acid in 100 mM NaCl, 10 mM HEPES pH 7.4, 1 mM $CaCl_2$, 2 mM KCl, and 1 mM $MgCl_2$) was measured and compared to specific substrate uptake rates for each transporter (as further described in the Figure legends). Citric acid is used to inhibit levodopa oxidation and was shown to reduce the pH of the buffer by 1.5 pH unit. The levodopa transport was also assayed without citric acid addition to the buffer by electrophysiology, as described below. Data obtained for non-injected oocytes were subtracted, and levodopa uptake rates were normalized to those of the known substrates. Competition experiments of levodopa or leucine (100 µM) transport were conducted in the presence of 5 mM L-leucine or levodopa, respectively. For the DDC and COMT inhibitors, uptake was conducted with 1 µM levodopa (20 µCu / ml ^{3}H-levodopa, 100 µM citric acid, for 1 minute) in the presence of 50 µM benserazide, carbidopa or entacapone, respectively. For the main neutral AA transporter B^0AT1-ACE2, the transports of levodopa, L-leucine, L-phenyalanine, and L-tyrosine (1mM) were further measured using the two-microelectrode voltage clamp (TEVC) technique. Recordings were carried out as previously described (Camargo et al., 2005) at a membrane holding potential (Vh) of −50 mV. Pooled data were shown as mean ± SEM with *n* representing the number of pooled cells.

Construction of the hrBAT-$hb^{0,+}$AT Fusion Protein

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To ensure that $b^{0,+}$AT-rBAT transport analyzed in *X. laevis* oocytes was due to the human protein, a fusion protein of human $b^{0,+}$AT and human rBAT was used. Briefly, we used the previously described fusion protein (Pfeiffer et al., 1999) and substituted mouse $b^{0,+}$AT by the human orthologue. Human $b^{0,+}$AT was amplified by PCR adding with XmaI at 5' and NsiI at 3' (primer: sense': CAA AAC CCC GGG ATG GGG GAT ACT GGC CTG AGA AAG; antisense: CCG GAG GAA GAC CCT GAG GGT GCT GCT CCT GAT GGT GCT CCT GGA TGC ATT CAA). 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 $b^{0,+}$AT-rBAT-pSport plasmid was linearized with Hind III (Promega) and used as template for RNA synthesis from the T7 promoter (mMESSAGE mMACHINE, Ambion, Austin, Tex., USA).

Cells

MDCK (Madine 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% non-essential AAs (Invitrogen) and 10% fetal calf serum. Cell lines previously described by Bauch and collaborators (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: wild type cells (1); cells expressing the apical transporter $b^{0,+}$AT-rBAT alone (2); cells expressing apical $b^{0,+}$AT-rBAT in the presence of the basolateral transporters LAT2-4F2hc (Slc7a8-Slc3a2) and y+LAT1-4F2hc (Slc7a7-Slc3a2) (3); and cells additionally (to cell line 3) expressing the basolateral uniporter TAT1 (4). The wild type and stably transfected cell lines were plated at density of 1.6 $x10^6$ cells / 24 mm Corning Costar Transwell filters and cultivated for 7 days. $b^{0,+}$AT-rBAT expression was induced 24h 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, cells were incubated for 30 minutes with the DDC inhibitor Benserazide (50µM Benserazide, 100 µM citric acid) in 150 mM NaCl, 10 mM HEPES pH

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7.4, 1 mM $CaCl_2$,5 mM KCl, 1 mM $MgCl_2$, 10mM glucose at 37°C. After this period, the uptake solution (100 µM levodopa, 50 µM Benserazide, 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 ^{3}H-levodopa. The concentration of levodopa used on these experiments is based on the apparent affinity of the apical transporter $b^{0,+}$AT-rBAT for levodopa assayed in X. laevis oocytes and differ 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-leucine or L-lysine (1 mM) was additionally added to the apical compartment. To analyze the effect of basolateral AAs on transepithelial levodopa transport (mimicking post-absorptive states with high plasma AA levels), L-leucine or L-lysine (1 mM) were 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 (Perking Elmer). 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 ± SEM 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/hour/cm^2.

<u>Animals</u>

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-nitrosurea) mutagenesis (Ingenium Pharmaceuticals AG, 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 four to five months old mice from both genders were used. All

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procedures for mice handling were performed according to the Swiss Animal Welfare laws Zurich, 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-L-levodopa/mL, 50µM Benserazide, 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 AG, Göttingen, Germany) and weighed. Segments were then lysed in 0.75N NaOH for 6 hours and neutralized with 10N HCl, and the radioactivity was determined by liquid scintillation. AA transport was expressed as pmol/mg/5min of dry tissue weight. Values obtained at 4°C were subtracted from the values of samples assayed at 37°C.

Everted intestinal sacs

In order 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 circa 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 levodopa, 0.05 µCi 3H-levodopa/mL) for 10 minutes. After washing with ice-cold solution, the liquid content of the sacs (serosa) was collected, the tissue (mucosa) was weighed and processed as the intestinal rings. The data are shown as mean ± SEM with *n* representing the number of sacs. The transport is expressed as nmol/10 minutes/mg of dry tissue. Values obtained at 4°C were subtracted from measurements performed at 37°C.

Brush border membrane vesicles (BBMV) and Western blot analysis

BBMV were prepared from small intestine mucosal cells using the Mg^{2+} precipitation technique as described elsewhere (Biber et al., 2007). Western blotting was performed as

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previously described (Bauch et al., 2003). Primary antibodies used were: rabbit affinity purified anti-mouse $b^{0,+}$AT1 (diluted 1:500) and monoclonal anti-mouse actin (1:10'000) (Sigma, St Louis, MO). Secondary antibodies: donkey anti-rabbit IgG horseradish peroxidase conjugated (Amersham Biosciences, Piscataway, NJ) or anti-mouse IgG alkaline phosphatase conjugate (Promega, Madison, WI) were diluted 1:5'000. Antibody binding was detected with Immobilon Western Chemiluminescent HRP or AP substrate (Millipore, Billerica, MA) and chemiluminescence visualized with a DIANA III camera (Raytest, Dietikon, Switzerland).

Quantitative real time PCR (qPCR)

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 (HPRT). Relative expression ratios were calculated as $r = 2^{(Ct(reference)\ -\ Ct(test))}$, whit *Ct* 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 ± SEM. All assessed data were unpaired and parametric. Differences between one 'control group' and two or more 'treatment groups' were assessed using an ANOVA 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 ANOVA with post-hoc Bonferroni's Multiple Comparison test. For single pairwise comparisons, a Student's two-tailed *t*-test was performed. *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, Calif., USA).

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Results

Levodopa is transported by the luminal enterocyte transporter $b^{0,+}$AT-rBAT but not by the major neutral amino acid transporter B^0AT1-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 to known substrates (control AAs) for each given transporter. As depicted in Figure 1A, oocytes expressing the AA transporters ASCT2 (SLC1A5), SIT1 (SLC6A20) and PAT1 (SLC36A1) did not transport levodopa. Oocytes expressing the AA exchanger $b^{0,+}$AT-rBAT (SLC7A9-SLC3A1) transported levodopa as efficiently as the control AA (L-leucine) used. The major neutral luminal AA transporter B^0AT1-ACE2 (SLC6A19-*ace2*) did not transport levodopa. The current generated by the co-transport of sodium (Figure 1B) and the accumulation of radiolabeled levodopa (Figure 1C) were negligible, despite structural homology of levodopa and B^0AT1-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 B^0AT1, since electrophysiology measurements were conducted without the supplementation of citric acid. $b^{0,+}$AT-rBAT hence is the only known luminal levodopa transporter of small intestine.

The transport of levodopa by $b^{0,+}$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 $b^{0,+}$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 was abolished by the presence of excess of non-labeled L-leucine and levodopa, respectively (Figure 2A).

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Since DDC inhibitors benserazide, carbidopa and the COMT inhibitor entacapone display a similar structure to levodopa, a potential effect of these substrates on $b^{0,+}$AT1-rBAT-mediated levodopa transport was tested. Levodopa formulations are presented in 4:1 ratio levodopa to DDC inhibitors and 2:1 to COMT inhibitors. We analyzed the accumulation of levodopa in the presence of a 1:50 ratio of levodopa to inhibitors. The presence of benserazide, carbidopa or entacapone did not affect levodopa transport (Figure 2B).

To analyze the impact of high intracellular neutral AA concentrations (mimicking the post-absorptive state) on AA uptake via the antiporter $b^{0,+}$AT-rBAT, uptake rates of levodopa and L-leucine, were measured after pre-injecting *X. laevis* oocytes with either L-arginine (12.5 mM) or water (negative control). Pre-loading cells with L-arginine resulted in a small but non-significant rise of levodopa and L-leucine uptake (Figure 2C).

Transepithelial transport of levodopa depends on expression of $b^{0,+}$AT-rBAT in the apical- and LAT2-4F2hc and TAT1 in the basolateral membrane

To analyze the interaction of apical and basolateral membrane AA transporters on transepithelial levodopa transport, mammalian MDCK epithelial cell models overexpressing various combinations of heterodimeric AA antiporters (Bauch et al., 2003) and TAT1 uniporter were used. Cells overexpressing only $b^{0,+}$AT-rBAT accumulated levodopa intracellularly, and showed – to some extent – a basolateral levodopa efflux (Figure 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 (Figure 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 5-fold increase in basolateral- and 3-fold decrease in intracellular accumulation was observed (Figure 3A, black bars).

To mimic the absorptive (presence of high AA concentrations in the lumen) or post-absorptive (presence of high AA concentrations in the extracellular space) state, excess

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concentrations of L-leucine or L-lysine were respectively added to the apical or basolateral compartments. The presence of excess L-leucine (Figure 3B) or L-lysine (Figure 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 $b^{0,+}$AT-rBAT.

Adding excess L-leucine to the basolateral compartment increased transepithelial transport of levodopa in cells expressing LAT2-4F2hc (Figure 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 (Figure 3E), suggesting that it does not trans-stimulate 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 (Figure 4A) and uptake-rates were therefore pooled for the competition experiments as depicted in Figure 4B. Adding excess non-labeled cationic AA L-arginine or neutral AA L-leucine to the uptake solution reduced intestinal levodopa uptake (Figure 4B), similarly to the observation in the cell culture (Figure 3B, C) or in *Xenopus laevis* oocytes (Figure 2B) for transport via $b^{0,+}$AT-rBAT.

Basolateral uniporter TAT1 affects transepithelial levodopa transport in vivo

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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 (Figure 4C), whereas transepithelial levodopa transport was significantly reduced in preparations made from knock-out animals when compared to wild type mice (Figure 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 (Martinelli et al., 2003) (Kompoliti et al., 2002). 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 non-active phase (related to nighttime in humans). Intestinal gene expression of transporters involved in levodopa absorption ($b^{0,+}$AT-rBAT, LAT2-4F2hc and TAT1) were not different along the small intestine between male and female mice and during active and non-active phases (Figure 5A). The RNA expression of $b^{0,+}$AT-rBAT was not different between males and females or between the different activity periods (Figure 5A). However, the protein expression of $b^{0,+}$AT showed a tendency to higher levels during the active phase in male as compared to female rodents, but did not differ among groups during the non-active period (Figure 5B).

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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 $b^{0,+}$AT-rBAT (apical), LAT2-4F2hc and TAT1 (basolateral) are responsible for small intestinal levodopa absorption.

Levodopa is transported by the luminal enterocyte transporter $b^{0,+}$AT-rBAT but not by the major neutral amino acid transporter B^0AT1-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. B^0AT1-ACE2 (SLC6A19) transports AAs with close structural homology to levodopa (Phe and Tyr). The IMINO transporter SIT1 (SLC6A20) and the proton-dependent AA transporter PAT1 (SLC36A1) transport neutral AAs to some extent (Miyauchi et al., 2005; Ristic et al., 2006). The AA exchanger ASCT2 (SLC1A5) transports neutral AAs, and has been suggested to be localized apically in rabbits (Avissar et al., 2001) and basolaterally in mouse enterocytes (Broer et al., 2011). We therefore assayed the potential transport of levodopa by these transporters. However, only cells expressing the antiporter $b^{0,+}$AT-rBAT accumulated levodopa. Despite the fact that the main neutral AA transporter B^0AT1-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 B^0AT1-ACE2 affinity for this substrate, but has no effect on $b^{0,+}$AT-rBAT mediated transport. $b^{0,+}$AT-rBAT transports Phe, Leu, Tyr, as well as L-methionine, L-glutamine, L-histidine, L-ornithine, cationic AAs and the di-peptide cystine (Bertran et al., 1992; Wells and Hediger, 1992; Pfeiffer et al., 1999). Furthermore, the transporter $b^{0,+}$AT-rBAT was also previously shown to be involved in intestinal drug (gabapentin) absorption in rodents (Nguyen et al., 2007) and renal levodopa re-absorption (Quinones et al., 2004). Its broad substrate

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selectivity makes luminal intestinal and proximal tubule transporter $b^{0,+}$AT-rBAT an interesting pharmacological target. A broad selectivity was also observed for the basolateral antiporter 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 co-administration of Leu was shown to decrease the absorption of levodopa (Lennernas 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 $b^{0,+}$AT-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 $b^{0,+}$AT-rBAT mRNA and protein expression along mouse intestine (Dave et al., 2004), levodopa uptake was comparable in all segments of mouse small intestine assayed in vitro. Taken together, $b^{0,+}$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 dopamine decarboxylase (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 $b^{0,+}$AT-rBAT transport had not yet been assayed. Despite the fact that carbidopa, entacapone or benserazide have some structural similarity to levodopa, no inhibition of levodopa transport was observed when co-administered. We therefore conclude that levodopa transport across the luminal enterocyte membrane mediated by $b^{0,+}$AT-rBAT is not affected by DDC and COMT inhibitors co-administration.

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Nguyen and colleagues showed that peptides transported inside the cell by PEPT1 (Slc15a1) and subsequently metabolized to single AAs could be used by $b^{0,+}$AT-rBAT as exchanger substrates (referred to as *trans-stimulation)* resulting in increased accumulation of gabapentin in rat jejunum cells (Nguyen et al., 2007). By increasing the intracellular concentration of the $b^{0,+}$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.

Transepithelial transport of levodopa depends on LAT2-4F2hc and TAT1 expression in the basolateral membrane

The basolateral large neutral AA antiporter LAT2-4F2hc (SLC7A8-SLC3A2) and the uniporter TAT1 (SLC16A10) (Kim et al., 2001; Quinones et al., 2004; Soares-Da-Silva et al., 2004) were previously assayed in overexpression systems for their ability to transport levodopa. In our epithelial cell culture model (Bauch et al., 2003), expression of the basolateral antiporter LAT2-4F2hc induced an increase in basolateral efflux of labeled levodopa, which was trans-stimulated by Leu added to 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 uniporter (facilitated diffusion pathway) and does not depend on the presence of contralateral substrates (Kim et al., 2001; Ramadan et al., 2006). Using this epithelial co-expression 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 $b^{0,+}$AT-rBAT in the apical membrane, TAT1 can stimulate the function of the antiporter LAT2-4F2hc. For instance, aromatic AAs exported by the uniporter TAT1 represent high affinity extracellular substrates for LAT2-4F2hc that can thus be exchanged by this antiporter with intracellular substrates such as

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levodopa, thereby increasing trans-epithelial 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 flow. Ex-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 non-motor 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 since 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 co-transporter 1 (SGLT1/Slc5a1) and fructose transporter 5 (GLUT5/Slc2a5) as well as the basolateral hexose transporter GLUT2/Slc2a2 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 expression cycle (Pan et al., 2003; Pan et al., 2004; Pan and Hussain, 2009; Qandeel et al., 2009). The expression of the gene encoding the PEPT1 transporter, that was previously shown to increase in the active phase (Pan and Hussain, 2009), showed a similar non-statistically significant trend in our experiments. The mRNA expression of intestinal transporters $b^{0,+}$AT, rBAT, 4F2hc, LAT2 and TAT1 during the active (reflecting day-time expression in humans) versus the non-active (reflecting nocturnal gene expression in humans) phase of wild-type mice were not significantly different in the present study. In

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contrast, the protein expression level of the apical transporter $b^{0,+}$AT measured in BBMV was significantly increased during the active phase in male animals, but interestingly not in females. Since 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 either due to drug treatment or disease progression. Therefore while 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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Legends for Figures

Figure 1: Levodopa is transported by the luminal enterocyte transporter $b^{0,+}$AT-rBAT, but not by the major neutral amino acid transporter B^0AT1-ACE2. A) Oocytes expressing $b^{0,+}$AT-rBAT transport levodopa as efficiently as L-leucine. Oocytes were injected with cRNA encoding mouse PAT1 (20ng), human SIT1 (5ng) and human accessory protein ACE2 (20 ng), human B^0AT1 (10 ng) and human accessory protein ACE2 (10ng), human ASCT2 (10 ng), or human $b^{0,+}$AT-rBAT fusion protein (10 ng). Levodopa uptake rates (100 µM; white bars) were compared to uptake rates of 100 µM glycine (PAT1), 100 µM L-proline (SIT1-ACE2), 100 µM L-leucine (B^0AT1-ACE2 and $b^{0,+}$AT-rBAT) and 100 µM L-alanine (ASCT2) (dark grey bars), respectively. The uptakes were performed at 25°C for 10 minutes. Results are expressed in pmol/oocyte/hour and values of non-injected oocytes were subtracted. Uptake results of control AAs were normalized to 1. Bars represent means ± SEM, n= 4-9 injected oocytes per group. $^{**}P < 0.01$; $^{***}P < 0.0001$; ns, not significant (unpaired two-tailed *t*-tests). **B and C) Oocytes expressing B^0AT1-ACE2 do not transport levodopa.** B) Levodopa does not induce current in oocytes expressing B^0AT1-ACE2: Oocytes expressing B^0AT1-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 ± SEM, n= 4 oocytes per group. $^{*}P < 0.05$ (ANOVA with post-hoc Dunnett's Multiple Comparison test). C) Levodopa is not accumulated in oocytes expressing B^0AT1-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/hour and values of the non-injected oocytes were subtracted. Results are given as means ± SEM, n= 5-6 oocytes per group. $^{*}P < 0.05$ (ANOVA with post-hoc Dunnett's Multiple Comparison test).

Figure 2: The transport of levodopa by $b^{0,+}$AT-rBAT can be inhibited by excess of neutral amino acids (and vice versa), but not by DDC or COMT inhibitors. Trans-stimulating cells with L-arginine does not significantly increase levodopa uptake. A)

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Competitive inhibition of levodopa and L-leucine transport. Radiolabeled levodopa (50 µM, white bars) and L-leucine (50 µM, black bars) uptake in *X. laevis* oocytes expressing $b^{0,+}$AT-rBAT was assayed in the presence or absence of excess non-labeled L-leucine (5mM) and levodopa (5mM), respectively. Results are expressed in pmol/oocyte/hour. Non-injected oocytes were subtracted. Results are expressed as mean ± SEM, n= 7-8 injected oocytes per group. ** P < 0.01; *** P < 0.0001 (unpaired two-tailed *t*-tests). **B) DDC inhibitors benserazide and carbidopa and the COMT inhibitor entacapone do not compete with $b^{0,+}$AT-rBAT-mediated levodopa transport.** The transport of levodopa (1 µM / minute) was assayed alone (white bar), in the presence of benserazide (50 µM, gray bar), carbidopa (50 µM, crosshatch bar), or entacapone (50 µM, black bar). Results are expressed in pmol/oocyte/hour and values of non-injected cells were subtracted. Data are represented as means ± SEM, n= 7-8 injected oocytes per group. The comparison of the 'treatment' groups with the 'control' group (levodopa alone) yielded no differences (ANOVA with post-hoc Dunnett's Multiple Comparison test). **C) Intracellular L-arginine does not affect levodopa uptake into *X. laevis* oocytes expressing $b^{0,+}$AT-rBAT.** Oocytes were injected with the $b^{0,+}$AT-rBAT antiporter substrate L-arginine (final concentration in the oocyte achieved was 12.5 mM) or water (negative control). After 4 hours, the uptake with L-levodopa (50 µM; white bars) or L-leucine (50 µM; black bars), was performed. Data are represented as means ± SEM, n= 8-9 injected oocytes per experiment. No significant differences were seen (unpaired two-tailed *t*-tests; P > 0.05).

Figure 3: Transepithelial transport of levodopa depends on $b^{0,+}$AT-rBAT expression in the apical- and LAT2-4F2hc and TAT1 in the basolateral membrane. MDCK cells expressing the apical transporter $b^{0,+}$AT-rBAT (white bars), the apical transporter $b^{0,+}$AT-rBAT and basolateral transporters LAT2-4F2hc and y+LAT1-4F2hc (gray bars) and (additionally to apical $b^{0,+}$AT-rBAT and basolateral LAT2-4F2hc and y+LAT1-4F2hc) TAT1 (black bars) were assayed in 5 different conditions (3A-E). **A)** Radiolabeled levodopa was added to the apical compartment. **B)**, Radiolabeled levodopa and L-leucine (1 mM) were added to the apical

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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. In the conditions A-E both compartments contained solutions supplemented with 100 µM levodopa, 50 µM benserazide, and 100 µM of 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/hour/cm^2 and given as mean ± SEM, n= 5-6 filters per experiment. * $P < 0.05$ (ANOVA with post-hoc Bonferroni's Multiple Comparison test).

Figure 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 (crosshatched bar). Results are expressed in pmol/mg dry tissue/ 5min and given as mean ± SEM, n= 6 - 8 experiments per group. No significant differences between different intestinal localizations were seen (ANOVA 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) were 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/ 5min and given as mean ± SEM, n= 5 - 7 experiments per group. * $P < 0.05$; ** $P < 0.01$ (ANOVA 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) (**4C**) and the levodopa accumulation in the sacs resulting from the transepithelial transport (serosal) (**4D**) were quantified. Results are expressed in nmol/mg of

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dry tissue/ 10 minutes. Values at 4°C were subtracted and data are given as mean ± SEM, n= 11-12 everted sacs per group. ns, not significant; * *P* < 0.05 (unpaired two-tailed *t*-tests).

Figure 5: Intestinal $b^{0,+}$AT-rBAT expression varies with gender and circadian rhythm at protein but not at RNA level. A) Expression of mRNA encoding the transporter $b^{0,+}$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 were unchanged. The gene expression of animals in the active (4 hours after the dark phase onset) and non-active phase (4 hours after the light phase onset) were analyzed by quantitative real time PCR. Results are expressed relative to the housekeeping gene HPRT and given as mean ± SEM, n= 3 female (solid bars) and 3 male (hatched bars) animals. ns, not significant; * *P* < 0.05 (ANOVA with post-hoc Bonferroni's Multiple Comparison test). **B) The protein level of $b^{0,+}$AT in male animals is higher during the active phase when compared to the inactive phase.** B) Expression of $b^{0,+}$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 were analyzed by Western blot analysis of brush border membrane vesicles (50 µg). The Western blots were quantified by densitometry and normalized to beta actin. The results are expressed relative to actin and given as mean ± SEM, n= 3 animals each gender. ns, not significant; * *P* < 0.05 (ANOVA with post-hoc Bonferroni's Multiple Comparison test).

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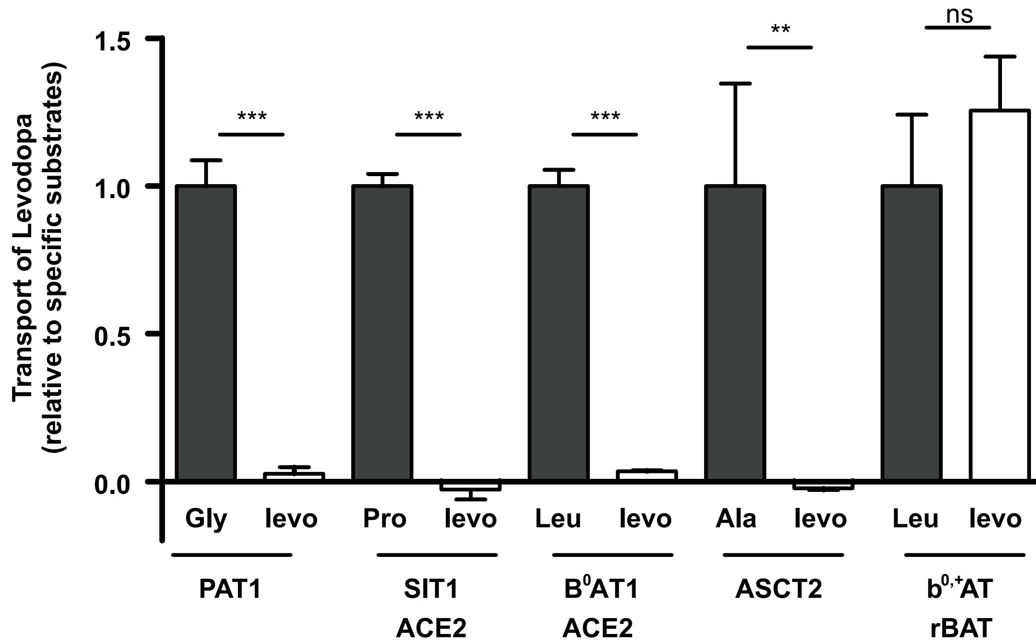

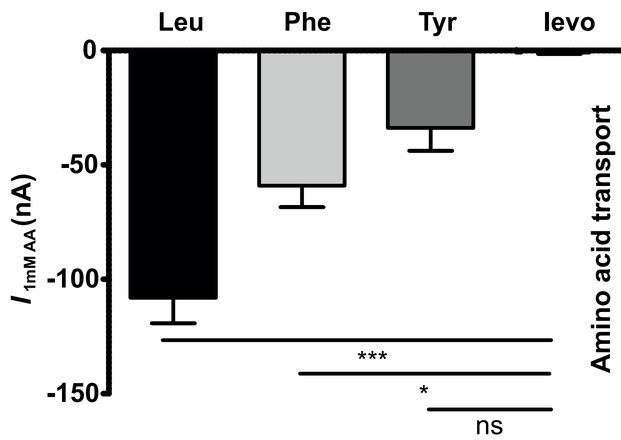

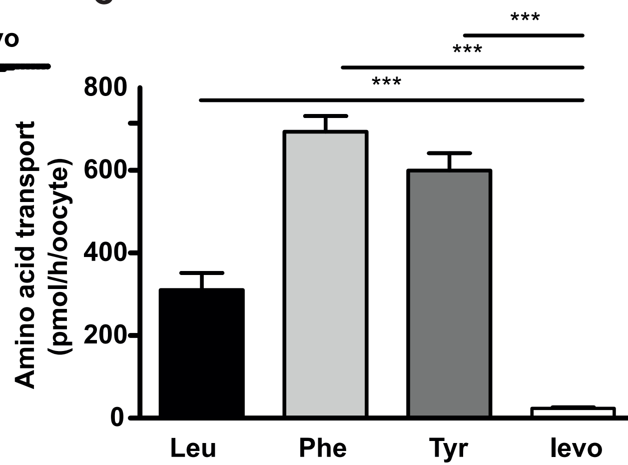

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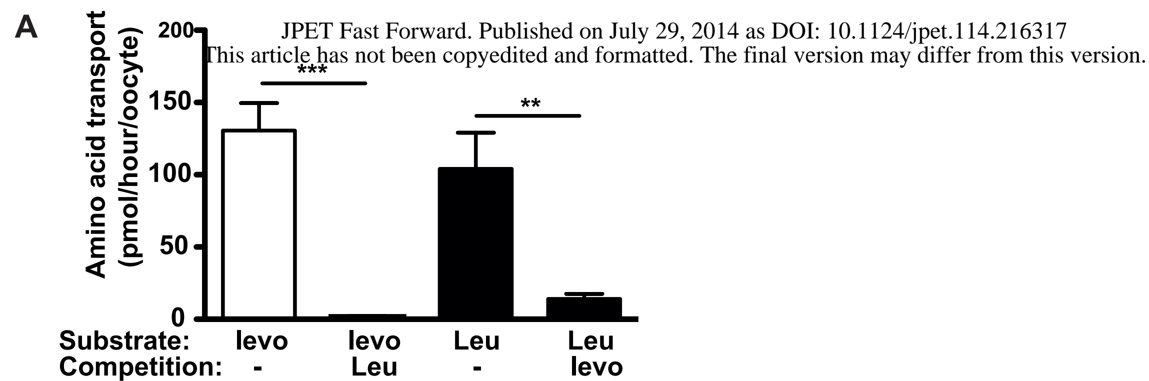

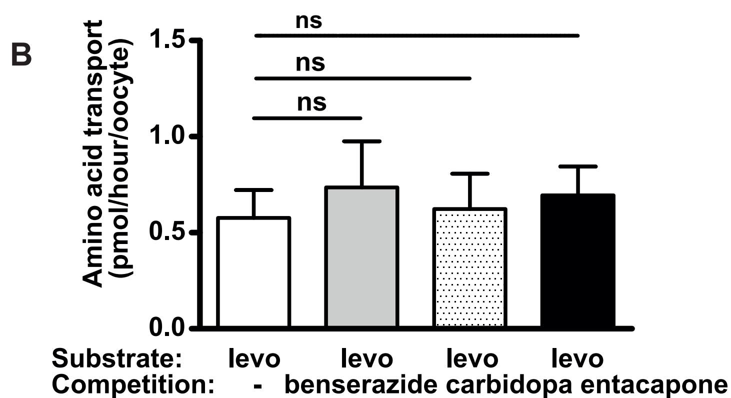

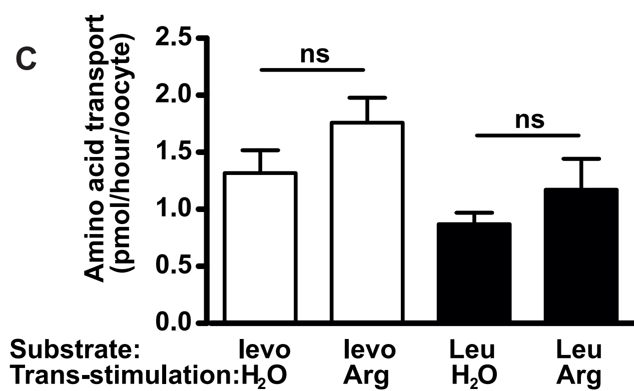

Figure 3

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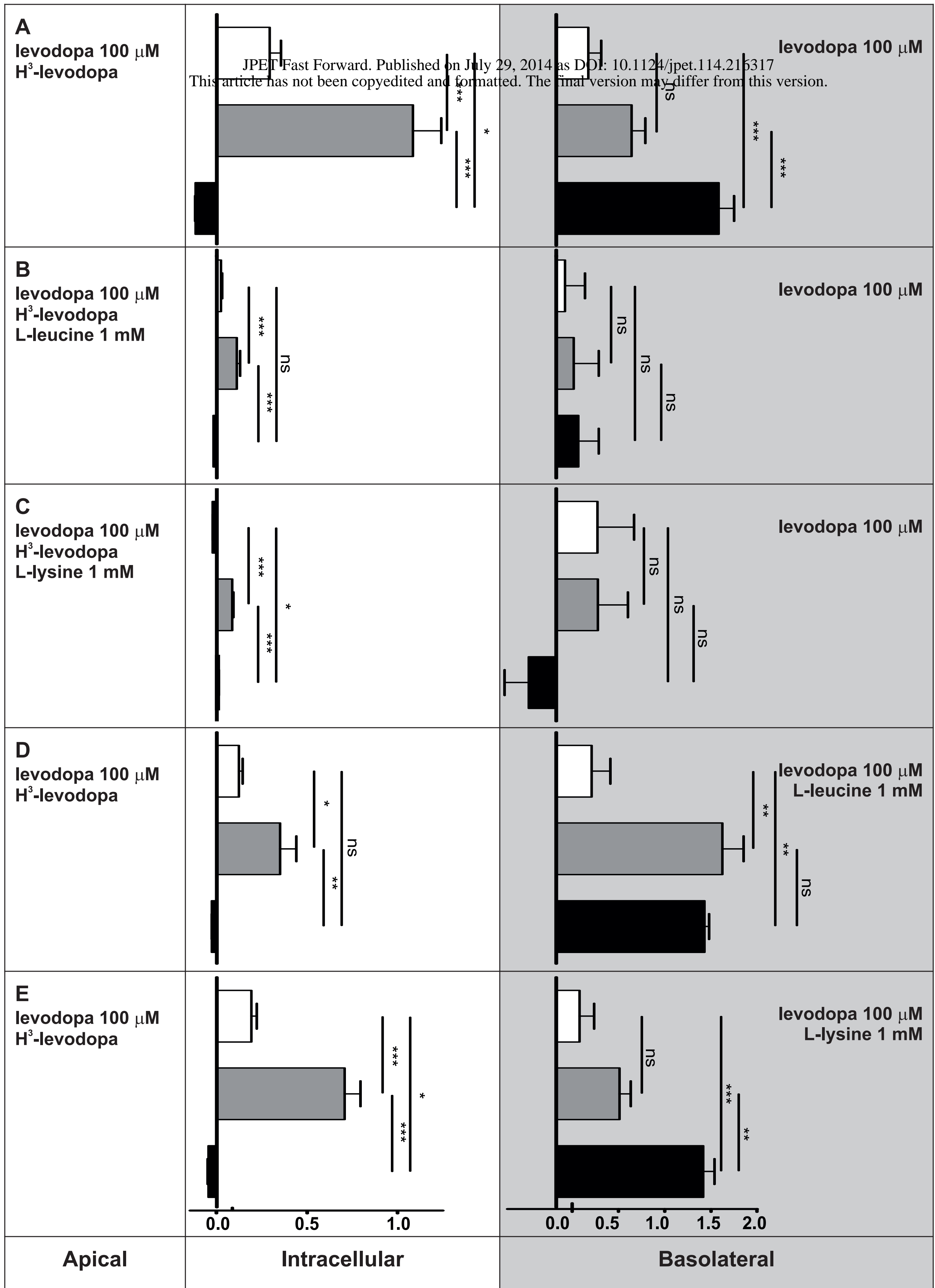

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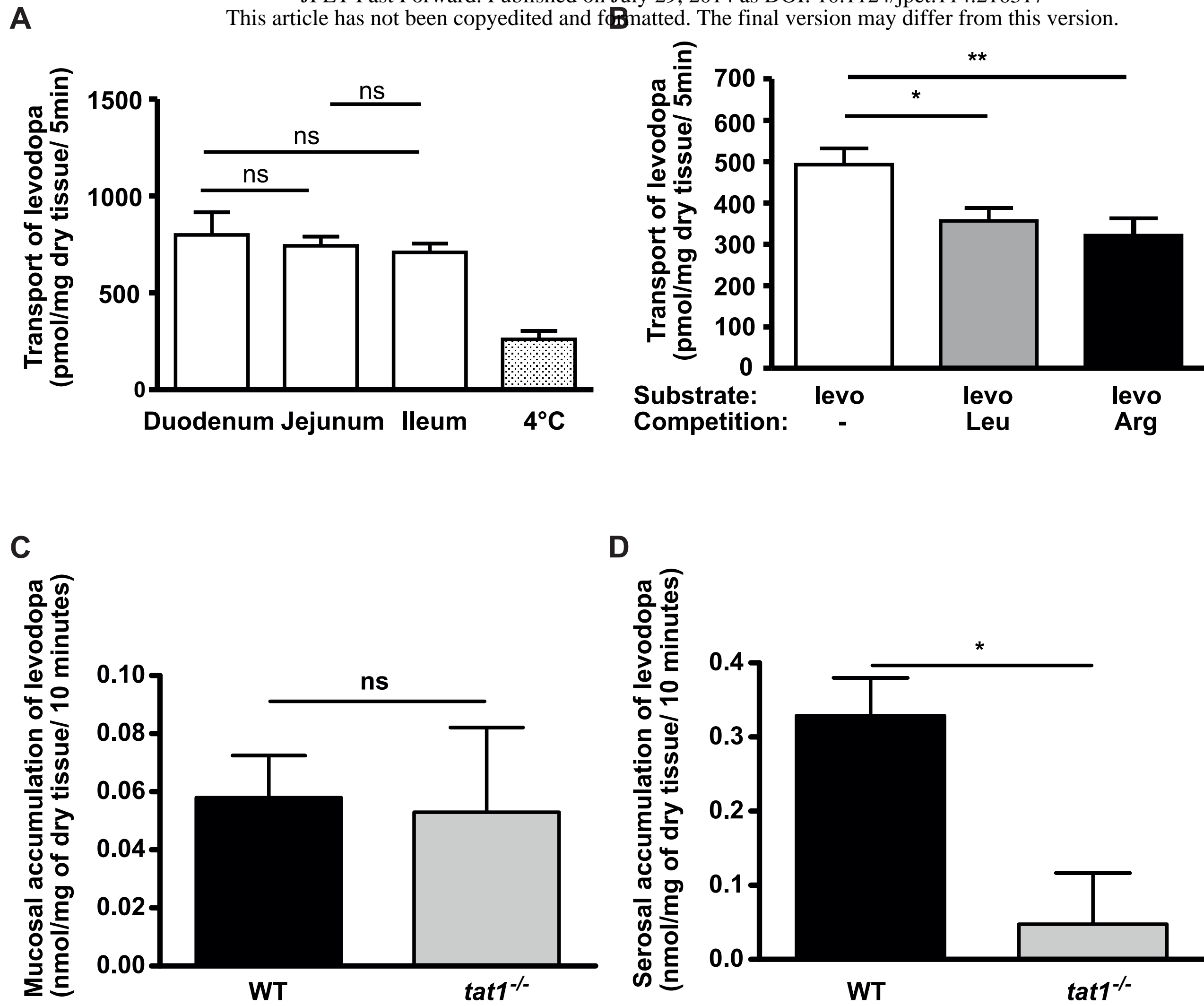

Figure 5

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