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Functional characterization of 5-oxoproline transport via SLC16A1/MCT1*

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*Running title: *H⁺-coupled transporter for amino acid derivative*

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Background: The amino acid derivative 5-oxoproline, which is an endogenous compound in the brain, is a monocarboxylate.

Results: Na⁺-dependent and amino acid transport systems scarcely contributed to 5-oxoproline transport in T98G cells as an astrocyte cell model.

Conclusion: 5-Oxoproline is taken up only by the monocarboxylate transporter SLC16A1.

Significance: 5-Oxoproline transport may be an important physiological function for SLC16A1.

ABSTRACT

Thyrotropin-releasing hormone is a

tri-peptide that consists of 5-oxoproline, histidine and proline. The peptide is rapidly metabolized by various enzymes. 5-Oxoproline is produced by enzymatic hydrolysis in a variety of peptides. Previous studies showed that 5-oxoproline could become a possible biomarker for autism spectrum disorders. Here we demonstrate the involvement of SLC16A1 in the transport of 5-oxoproline. An *SLC16A1* polymorphism (rs1049434) was recently identified. However, there is no information about the effect of the polymorphism on SLC16A1 function. In this study, the polymorphism caused observable change in 5-oxoproline and

lactate transport via SLC16A1. The Michaelis constant (K_m) was increased in an SLC16A1 mutant compared with that in the wild type. In addition, the proton concentration required to produce half-maximal activation of transport activity ($K_{0.5, H^+}$) was increased in the SLC16A1 mutant compared with that in the wild type. Furthermore, we examined the transport of 5-oxoproline in T98G cells as an astrocyte cell model. Despite the fact that 5-oxoproline is an amino acid derivative, Na^+ -dependent and amino acid transport systems scarcely contributed to 5-oxoproline transport. Based on our findings, we conclude that H^+ -coupled 5-oxoproline transport is mediated solely by SLC16A1 in the cells.

According to Takeda Pharmaceutical Company Ltd., TRH (thyrotropin-releasing hormone), also known as protirelin, is a tri-peptide that consists of 5-oxoproline, histidine and proline, and it is used for medical treatment of spinocerebellar ataxia (<http://www.takedamed.com/content/medicine/pdf/060-110223.pdf>). Administration of TRH to normal subjects results in a prompt increase of serum thyrotropin concentration. Stwertka *et al.* reported that TRH might play an important role in memory modulation and have therapeutic value in certain disease states in humans (1).

TRH is rapidly metabolized and its mean half-life is 5 min (2). 5-Oxoproline (also known as pyroglutamic acid, glutimic acid or pyrrolidonecarboxylic acid) is found as an

N-terminal modification in many neuronal peptides and hormones that also include the accumulating peptides in Alzheimer's disease and familial dementia (3). 5-Oxoproline is also generated by γ -glutamyl cyclotransferase, which converts γ -glutamyl amino acids into 5-oxoproline and corresponding free amino acids (4). The γ -glutamyl cycle in the brain influences amino acid transport indirectly through 5-oxoproline to maintain low concentrations of glutamate, aspartate and glycine in the brain (5). Grioli *et al.* reported that 5-oxoproline was effective in improving some verbal memory functions in subjects affected by age-related memory decline (6). A recent study has shown that 5-oxoproline could become a possible biomarker for autism spectrum disorders (ASD) (7).

The present study was undertaken to determine the role of SLC16A1 (also known as a monocarboxylate transporter, MCT1) in the transport of 5-oxoproline. SLC16A1 and SLC16A3 are expressed in astrocytes (8). Despite the fact that 5-oxoproline is an amino acid derivative, it exists predominantly as an anionic monocarboxylate, indicating that 5-oxoproline may be transported by SLC16A1.

Furthermore, an *SLC16A1* polymorphism (rs1049434) has recently been identified (Table 1) (9-15). According to the National Center for Biotechnology Information (NCBI), minor allele frequency (MAF) of this gene polymorphism is extremely high (MAF = 0.3425). However, the influence of *SLC16A1* polymorphism (rs1049434) on the

function of this protein has not been determined. It is necessary to wait for results of scientific and detailed epidemiology analysis of clinical data and to draw a conclusion about the cause-and-effect relationship between *SLC16A1* polymorphism and various diseases. In advance of epidemiology analysis, the impact of this polymorphism on the transport function of SLC16A1 was also investigated in this study.

EXPERIMENTAL PROCEDURES

Materials—L-[¹⁴C]lactate was purchased from American Radiolabeled Chemicals. 5-[³H]oxoproline (also known as [³H]Pyroglutamic acid) was purchased from Moravsek. Other chemicals and solvents were purchased from Sigma-Aldrich.

Site-directed mutagenesis—The cDNA gene of SLC16A1, which was prepared from the human brain, was purchased from DNAFORM and cloned using a strategy similar to that described in a previous report on SLC16A3 (16). The molecular cloning of SLC16A3 was previously described (16). These plasmids were linearized with *Xho*I, and SLC16A1 and SLC16A3 cRNAs were synthesized *in vitro* using T7 RNA polymerase (Cell script) from SLC16A1 and SLC16A3 cDNAs. An SLC16A1 mutant was obtained by using the Quickchange protocol (Agilent). The mutagenic oligonucleotides used were 5'-GAAAGACACAGAAGGAGGGGCCCAA GG-3' for the forward primer and

5'-CCTTGGGCCCCTCCTTCTGTGTCTTTC -3' for the reverse primer, which replace an aspartate residue at position 490 with glutamate (*SLC16A1-T1470A*). A High Pure Plasmid Isolation Kit (Roche) was used to isolate the plasmid DNA. Mutation was confirmed by DNA sequencing using a BigDye Terminator v3.1 Cycle Sequencing Kit[®] from Applied Biosystems.

Transport assay—*Xenopus laevis* oocytes were collected under anesthesia (immersion in a solution of 1 g/l MS-222) from toads. This study was approved by the Committee on Animal Experimentation, Hokkaido University. Stage IV-V oocytes from *Xenopus laevis* were isolated and maintained at 17°C in Barth's solution as previously described (16). Oocytes were injected with 50 ng cRNA in a 50-nl volume and incubated for 3-6 days. The transport function of heterologously expressed SLC16A1 was monitored at 25°C. We measured the uptake of radiolabeled compounds by using a liquid scintillation counter. Uptake of a radiolabeled compound in water-injected and cRNA-injected oocytes was performed, and oocytes were washed with ice-cold transport buffer as previously described (16). All data are given as means±S.E. of three independent experiments, and 12-15 oocytes were used in each of the experiments on uptake of radiolabeled compounds.

Electrophysiological studies were performed with the TEVC technique as described previously (17). SLC5A8-expressing oocytes (kindly

H⁺-coupled transporter for amino acid derivative

provided by Dr. Seiji Miyauchi, Toho University School of Pharmaceutical Sciences) were used as controls. The oocytes were used for electrophysiological studies 3-4 days after cRNA injection. An oocyte was voltage-clamped at -50 mV. The oocyte was superfused with perfusion buffer (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM HEPES/Tris with pH adjusted to 7.5). After the current had stabilized, the oocyte was superfused with the transport buffer. The oocyte was superfused with the same buffer additionally containing 5-oxoproline. The differences between the steady-state currents measured in the presence and absence of 5-oxoproline were considered at the substrate-induced currents. Electrophysiological measurements of substrate-induced currents were repeated at least four times with separate oocytes. Data represent means±S.E.

Immunohistochemistry—The immunohistochemistry procedure used was the same as that previously described (16). SLC16A1 cRNA-injected and water-injected oocytes were prepared for immunohistochemistry. Briefly, the oocytes were fixed in 10% formalin. The fixed oocytes were embedded in paraffin and stained by a rabbit anti-SLC16A1 antibody (Santa Cruz Biotechnology: H-70; Figure 5A, C-20; Figure 7). The prepared oocytes were monitored under a confocal scanning microscope (OLYMPUS).

Cell culture and transport measurements—The human glioblastoma

multiforme tumor-derived T98G cell line (RIKEN) was grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum in plastic flasks or Petri dishes at 37°C in a 5% CO₂ incubator. For transport measurements, the cells were plated onto 24-well plates and were grown to confluence. The cells were washed twice with 0.5 ml of uptake buffer (140 mM NaCl, 3 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM D-glucose, 5 mM Good's buffer) and incubated at 37°C in uptake buffer containing radiolabeled compounds. Radioactivity was determined after solubilization of the cells with 0.5 ml of 0.5 N NaOH by liquid scintillation counting. HEPES was used for pH 8.0-7.0 buffer, and MES was used for pH 7.0-5.5 buffer.

Determination of expression of mRNAs—Expression of mRNAs was determined by RT-PCR. Total RNA was isolated from T98G cells using Isogen II reagent (NIPPON GENE). Total RNA was reverse-transcribed using ReverTra Ace (TOYOBO) and random primers according to the manufacturer's instructions. PCR was then performed using SLC16A1, SLC16A3, SLC5A12 specific primers and KAPATaq Extra (NIPPON Genetics). Primers specific for human actin-beta were also included in each reaction as an internal standard. The primer sequences and expected sizes are shown in Table 6. The PCR method consisted of 25 cycles of denaturing at 95°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 60 s, followed by a final extension

at 72°C for 60 s. The products were size-fractionated on an agarose gel and stained by ethidium bromide.

Statistical analysis—The difference between mean values was assessed using Student's unpaired *t* test, with significance assigned at $p < 0.05$. When multiple comparisons were required, ANOVA was performed and differences were determined using the least significant difference. Significance was assigned at $p < 0.05$.

RESULTS

The chemical structures of TRH and 5-oxoproline are shown in Figure 1. TRH is a tri-peptide that consists of 5-oxoproline, histidine and proline. 5-Oxoproline is a TRH metabolite in which the amino group of glutamic acid cyclized to form a lactam. 5-Oxoproline, which is an uncommon amino acid derivative, has a pK_a value of 3.6 (18). Hence, it exists mostly as a monovalent anion in the living body. On the other hand, histidine and proline are common amino acids and exist principally as multivalent ions at physiological pH.

We investigated the transport of 5-oxoproline *via* SLC16A1 using a heterologous expression system. We expressed SLC16A1 in *Xenopus laevis* oocytes by injection of SLC16A1 cRNA. The transport activity of SLC16A1 was monitored by the uptake of [¹⁴C]lactate as a classical substrate of SLC16A1. The uptake of lactate in SLC16A1-expressing oocytes was 25-fold higher than that in water-injected oocytes

(data not shown). SLC16A1-mediated uptake was calculated by subtracting the uptake in water-injected oocytes from the uptake in cRNA-injected oocytes. The uptake of lactate was linear for at least 20 min. We tested the effects of 5-oxoproline, histidine, proline and lactate on SLC16A1-mediated lactate uptake (Figure 2). 5-Oxoproline (10 mM) showed a strong inhibitory effect on SLC16A1-mediated [¹⁴C]lactate uptake, which was reduced to 10-15% of the control level. This inhibition resembles the inhibition caused by unlabeled lactate (10 mM). Histidine and proline had no effect on SLC16A1-specific lactate uptake ($p > 0.05$).

Therefore, we assessed SLC16A1-mediated 5-oxoproline transport by monitoring the uptake of 5-[³H]oxoproline in the same heterologous expression system. Uptake of 5-oxoproline in SLC16A1 cRNA-injected oocytes was 300-fold higher than that in water-injected oocytes (Figure 3A). This transport activity was enhanced by a proton or protons. The accumulation of 5-oxoproline by SLC16A1-expressing oocytes was markedly reduced by alkalizing the buffer pH. The transport of 5-oxoproline *via* SLC16A1 was inhibited by substrates of the transporter (Figure 3B). Histidine and proline did not suppress the transport process ($p > 0.05$).

We also investigated the effects of SLC16A2 substrates including tri-iodothyronine (T3) and thyroxine (T4) on SLC16A1-mediated 5-oxoproline uptake (Figure 4). When present at 30 μ M, we could

H⁺-coupled transporter for amino acid derivative

not detect interactions of T3 and T4 with SLC16A1. The reason is low identity; pairwise basic local alignment search tool (BLAST) analysis showed that the amino acid identity relative to SLC16A1 was only 22.6% for SLC16A2.

To test the effects of ions on SLC16A1-mediated 5-oxoproline uptake, a sodium-free condition (replacement of N-methyl-D-glucamine (NMDG) in the transport buffer by sodium) and chloride-free condition (replacement of gluconate in the transport buffer by chloride) did not significantly change the uptake (Table 2). On the other hand, SLC16A1 transport activity was greatly reduced by carbonyl cyanide 3-chlorophenyl hydrazine (CCCP) as a protonophore. These results indicate that the transport process is proton/5-oxoproline cotransport. We then examined the transport of 5-oxoproline under the conditions of normal (2 mM) and high (50 mM) extracellular potassium concentrations as the latter condition acts to depolarize the membrane potential (19). SLC16A1-mediated 5-oxoproline uptake was not changed by a high potassium concentration condition. These results suggest that the proton:5-oxoproline stoichiometry of 1:1 shows an electroneutral property of the transport process. Therefore, we used the two-electrode voltage clamp (TEVC) technique to investigate the electroneutral property of 5-oxoproline transport *via* SLC16A1. The well-known electrogenicity of SLC5A8, as an

Na⁺-coupled monocarboxylate transporter, was used for comparison. SLC5A8-expressing oocytes were perfused with 5-oxoproline (5 mM) under voltage-clamp conditions and the currents at pH 7.5 and 5.5 are 103±4 nA and 68±1 nA. Such currents were not detectable in SLC16A1-expressing oocytes.

In the next series of experiments, the effect of the polymorphism (rs1049434) on transport of 5-oxoproline *via* SLC16A1 was tested. We first established that an SLC16A1 mutant (D490E) was properly expressed in *Xenopus laevis* oocytes. The transporter protein localized mainly to the plasma membrane as evidenced by fluorescent laser scanning confocal microscopy (Figure 5A). We then investigated the kinetic characteristics of 5-oxoproline transport *via* SLC16A1-D490E. The transport of 5-oxoproline *via* SLC16A1 wild type (WT) was saturated at high concentrations of 5-oxoproline (Figure 5B). The mutant-mediated 5-oxoproline transport process was also saturable (Figure 5C). The Michaelis constant (K_m) was increased in SLC16A1-D490E compared with that in the wild type (Figure 5D).

Proton-activation kinetics indicated that the relationships between 5-oxoproline uptake *via* SLC16A1-WT and -D490E and proton concentrations were not sigmoidal (Figures 6A and 6B), suggesting involvement of one proton in the activation process. Determination of the Hill coefficient using the Hill equation gave values of 0.9±0.1

(WT) and 1.1 ± 0.1 (D490E). These results indicate that for one molecule of 5-oxoproline transported, one proton is then cotransported. The data were fitted to the Henderson-Hasselbalch equation and pK_a values were calculated. The values were 6.31 ± 0.05 (WT) and 5.96 ± 0.07 (D490E) (Figures 6C and 6D). The proton concentration required to produce half-maximal activation of transport activity ($K_{0.5, H^+}$) was increased in SLC16A1-D490E compared with that in the wild type (Figure 6E). We also investigated the effect of the polymorphism on transport of lactate as a classical substrate *via* SLC16A1 (Table 3). Lactate uptake *via* SLC16A1-D490E was increased compared with the wild type, although lactate uptake of other mutants was comparable to that of SLC16A1-WT ($p > 0.05$). Staining of the other indicated mutants revealed a sharp signal for SLC16A1 in the plasma membrane (Figure 7).

We also examined the transport of 5-oxoproline *in cells*. In this study, we used T98G cells for the experiments as an astrocyte cell model because the cells resemble primary astrocytes biologically (20-22). The uptake of 5-oxoproline by T98G cells was measured in the range of 0-30 min. The time course for the accumulation of 5-oxoproline by T98G cells is shown in Figure 8A. The accumulation of 5-oxoproline was linear up to 5 min after incubation with 5-oxoproline. Therefore, the initial uptake rate was calculated from the slope of the linear portion (Figure 8A; inset) and

determined within 5 min after onset. The relationship between the initial uptake rate and 5-oxoproline concentration is depicted in Figure 8B. The uptake was saturated at high concentrations of 5-oxoproline. This indicates that the uptake process is comprised of a saturable process at a low concentration and a non-saturable process at a high concentration. The inset in Figure 8B shows an Eadie-Hofstee plot of the data after subtraction of non-saturable transport components. The kinetic parameters were calculated by non-linear regression of the Michaelis-Menten equation, and K_m (Michaelis constant), V_{max} (maximal velocity) and K_d (coefficient of passive diffusion) were estimated to be 3.2 ± 0.2 mM, 3.3 ± 0.1 nmol/min/mg protein and 0.04 ± 0.01 μ l/min/mg protein, respectively. The uptake rate of 200 μ M 5-oxoproline under an ice-cold condition was 0.008 ± 0.001 nmol/min/mg protein. These results imply that passive diffusion hardly contributed to the kinetics because the K_d value was very low, indicating that 5-oxoproline uptake is mediated by a carrier protein.

To elucidate the carrier for 5-oxoproline, 5-oxoproline uptake was measured under various conditions (Table 4). First, we checked the dependency of the membrane potential. The addition of valinomycin (50 μ M) to the medium had no effect on 5-oxoproline uptake ($p > 0.05$). Because Na^+ -dependent transporters for monocarboxylic acids have been reported (23), we replaced Na^+ with NMDG in the

medium. However, this condition caused no observable change in 5-oxoproline uptake ($p>0.05$). Based on these findings, we speculate that this 5-oxoproline transport is neither an electrogenic nor Na^+ -dependent process. In contrast, we observed marked pH dependency. When the extracellular H^+ concentration was decreased from 3.2 μM to 0.032 μM , 5-oxoproline uptake decreased greatly. We also examined the effect of proton electrochemical potential energy on 5-oxoproline uptake. Uptake of 5-oxoproline was measured in the presence of 40 μM CCCP. Uptake of 5-oxoproline was significantly suppressed by CCCP. Hence, this transport process is driven by the proton gradient, that is, a proton symport system. Uptake of 5-oxoproline was markedly inhibited by 2.5 mM diethyl pyrocarbonate (DEPC), which modified the histidine residues. DEPC is known to suppress the H^+ acceptor or donor function of histidine residues of proteins (24). In addition, we performed an inhibition experiment using various compounds. As shown in Table 5, the monocarboxylate lactate strongly reduced the uptake of 5-oxoproline to 20% of the control level. On the other hand, the organic anion transporter substrate *p*-aminohyppurate (25), the oligopeptide transporter substrate glycylsarcosine (26), and amino acid transporter substrates histidine and proline (27, 28) had no effect on 5-oxoproline uptake ($p>0.05$). The observations that 5-oxoproline and lactate may be transported through a common carrier in T98G cells and that

5-oxoproline is not taken up by an Na^+ -dependent transport system.

A study by Suzuki *et al.* (8) has shown that monocarboxylate transporters are expressed in astrocytes. To determine whether 5-oxoproline is taken up through monocarboxylate transporters, RT-PCR was performed with specific primers for each of these transporters (Table 6). SLC16A1 and SLC16A3 were expressed in T98G cells (Figure 9). However, SLC5A12 was not expressed in T98G cells but was expressed in BeWo cells used as a positive control (data not shown). Therefore, we speculated from these results that 5-oxoproline is taken up through SLC16A1 and/or SLC16A3.

Hence, we investigated the transport of 5-oxoproline using a *Xenopus laevis* oocyte expression system. The transporters were expressed in oocytes by injection of SLC16A1 or SLC16A3 cRNA. Water-injected oocytes were used as controls. The uptake of 5-oxoproline in SLC16A1 cRNA-injected oocytes was higher than that in water-injected oocytes (Figure 10). However, there was no significant difference in uptake between SLC16A3-expressing oocytes and water-injected oocytes. These results indicate that H^+ -coupled 5-oxoproline transport is mediated solely by SLC16A1.

DISCUSSION

In this study, we demonstrated transport of 5-oxoproline via SLC16A1 and the impact of a polymorphism (rs1049434). The gene polymorphism brought about SLC16A1

functional change-induced breakdown of homeostasis because *SLC16A1* knock-out is lethal (29). Lactate as a classical substrate for *SLC16A1* is a monocarboxylate with no structural resemblance to 5-oxoproline. However, lactate and 5-oxoproline blood levels in ASD patients are decreased compared with those in healthy persons (7), suggesting potential involvement of the same transport system in the lactate transport process. Miyauchi *et al.* reported that the monocarboxylate transporter *SLC5A8* is involved in renal reabsorption of the amino acid derivative 5-oxoproline (30). It is also expressed in neurons but not in astrocytes (23). Therefore, we speculated that the contribution of *SLC16A1* to 5-oxoproline transport in astrocytes is large.

We have shown dramatic activity of *SLC16A1*-mediated 5-oxoproline transport by a heterologous expression system. Expression of *SLC16A1* in *Xenopus laevis* oocytes induced pH-dependent transport of 5-oxoproline, which was suppressed by lactate. The K_m value was 10-fold larger than the value of *SLC5A8* (30). This value is appropriate because the human brain has 100 billion neurons and 10- to 50-fold more glial cells (31). Proton activation of the transport exhibited non-linear kinetics with a Hill coefficient of 0.9 ± 0.1 , indicating that proton:5-oxoproline stoichiometry of 1:1 shows an electroneutral property of the transport process. *SLC16A2*, which belongs to the same family to which *SLC16A1* belongs, is a monocarboxylate transporter for

thyroid hormones (32-37). We also studied the effects of hormones as *SLC16A2* substrates, including T3 and T4, on transport of 5-oxoproline via *SLC16A1*. It is of importance that even mild thyroid failure can have a number of clinical effects such as depression, memory loss, cognitive impairment and a variety of neuromuscular complaints (38, 39). Under the experimental conditions used in our study, we could not detect any interaction of thyroid hormones with *SLC16A1*. Although *SLC16A1* and *SLC16A2* belong to the same family, the amino acid identity relative to *SLC16A1* is only 22.6% for *SLC16A2*. Therefore, we speculated that the recognition mechanisms of these transporters for the core structure except for monocarboxylic acid of the substrate are greatly different.

Next, in order to determine whether *SLC16A1* polymorphisms affect the function of *SLC16A1* protein, we performed site-directed mutagenesis. None of the mutants showed an effect on the correct localization of the protein in the plasma membrane. The *SLC16A1* polymorphism (rs1049434) influences the K_m value of *SLC16A1*-mediated 5-oxoproline transport. However, the mutation site of this polymorphism is Asp490 in the carboxy terminal facing the cytosol. Hence, we speculated that the change of the K_m value is not mediated by change of a direct interaction with 5-oxoproline. A pK_a value of 6.3 strongly indicates that the amino acid residue may be histidine, which is involved in

regulation of the transport activity by a proton. Aspartate is a partner of histidine in a number of enzymes that have an active site motif known as a catalytic triad. The positively charged histidine at low pH may disconnect with negatively charged Asp490, resulting in a change in the conformation of SLC16A1 and activation of the transporter. Therefore, we examined the effect of the *SLC16A1* polymorphism (rs1049434) on the pH dependency of transport activity. The mutation of Asp490 had an effect on the $K_{0.5, H^+}$ value of SLC16A1-mediated 5-oxoproline transport, indicating that the mutation suppresses histidine protonation. Consequently, the K_m value of SLC16A1-mediated 5-oxoproline transport was increased by the *SLC16A1* polymorphism (rs1049434) because the transporter is an electroneutral proton/5-oxoproline cotransporter. More research is needed to clarify the relationship between *SLC16A1* polymorphisms and 5-oxoproline concentration in the human brain and to develop a tool for noninvasive measurement of the intracerebral concentration of 5-oxoproline.

We also studied the transport of 5-oxoproline in an astrocyte cell model. The

transport process in T98G cells was Na⁺-independent, H⁺-dependent and electroneutral. Of interest, a carrier that has these characteristics has not been reported. Furthermore, only one carrier-mediated transport component was detected by analysis of concentration dependency. The K_m value of 5-oxoproline uptake in the cells was similar to the K_m value of 5-oxoproline uptake by SLC16A1-expressing oocytes. These results suggest that SLC16A1 contributes to 5-oxoproline transport in the cells. On the other hand, the uptake of 5-oxoproline in the cells was inhibited by lactate but not by amino acids. Based on these findings, we speculate that several pH-dependent amino acid transport systems scarcely contributed to 5-oxoproline transport in the cells.

Taken together, the results indicate that SLC16A1 is an electroneutral low-affinity transporter for 5-oxoproline. Despite the fact that 5-oxoproline is an amino acid derivative, Na⁺-dependent and amino acid transport systems scarcely contributed to 5-oxoproline transport. Our findings strongly argue against a primary role of SLC16A1 in membrane transport of 5-oxoproline.

REFERENCES

1. Stwertka, S. A., Vincent, G. P., Gamzu, E. R., MacNeil, D. A., and Verderese, A. G. (1992) TRH protection against memory retrieval deficits is independent of endocrine effects. *Pharmacol Biochem Behav.* **41**, 145-52
2. Bassiri, R. M., and Utiger, R. D. (1973) Metabolism and excretion of exogenous

- thyrotropin-releasing hormone in humans. *J Clin Invest.* **52**, 1616-9
3. Kumar, A., and Bachhawat, A. K. (2012) Pyroglutamic acid: throwing light on a lightly studied metabolite. *Current Science.* **102**, 288-97
 4. Lee, W. J., Hawkins, R. A., Peterson, D. R., and Viña, J. R. (1996) Role of oxoproline in the regulation of neutral amino acid transport across the blood-brain barrier. *J Biol Chem.* **271**, 19129-33
 5. Viljoen, J., Bergh, J. J., Mienie, L. J., Kotze, H. F., and Terre'Blanche, G. (2012) Paracetamol prevents hyperglycinemia in vervet monkeys treated with valproate. *Metab Brain Dis.* **27**, 327-35
 6. Grioli, S., Lomeo, C., Quattropiani, M. C., Spignoli, G., and Villardita, C. (1990) Pyroglutamic acid improves the age associated memory impairment. *Fundam Clin Pharmacol.* **4**, 169-73
 7. Kuwabara, H., Yamasue, H., Koike, S., Inoue, H., Kawakubo, Y., Kuroda, M., Takano, Y., Iwashiro, N., Natsubori, T., Aoki, Y., Kano, Y., and Kasai, K. (2013) Altered metabolites in the plasma of autism spectrum disorder: a capillary electrophoresis time-of-flight mass spectroscopy study. *PLoS One.* **8**, e73814
 8. Suzuki, A., Stern, S. A., Bozdagi, O., Huntley, G. W., Walker, R. H., Magistretti, P. J., and Alberini, C. M. (2011) Astrocyte-neuron lactate transport is required for long-term memory formation. *Cell.* **144**, 810-23
 9. Merezinskaya, N., Fishbein, W. N., Davis, J. I., and Foellmer, J. W. (2000) Mutations in MCT1 cDNA in patients with symptomatic deficiency in lactate transport. *Muscle Nerve.* **23**, 90-7
 10. Lean, C. B., and Lee, E. J. (2009) Genetic variations in the MCT1 (SLC16A1) gene in the Chinese population of Singapore. *Drug Metab Pharmacokinet.* **24**, 469-74
 11. Sawczuk, M., Banting, L. K., Cieżczyk, P., Maciejewska-Karłowska, A., Zarębska, A., Leońska-Duniec, A., Jastrzębski, Z., Bishop, D. J., and Eynon, N. (2014) MCT1 A1470T: A novel polymorphism for sprint performance? *J Sci Med Sport.* pii: S1440-2440(13)00525-2
 12. Cupeiro, R., Benito, P. J., Maffulli, N., Calderón, F. J., and González-Lamuño, D. (2010) MCT1 genetic polymorphism influence in high intensity circuit training: a pilot study. *J Sci Med Sport.* **13**, 526-30
 13. Fedotovskaya, O. N., Mustafina, L. J., Popov, D. V., Vinogradova, O. L., and Ahmetov, I. I. (2014) A common polymorphism of the MCT1 gene and athletic performance. *Int J Sports Physiol Perform.* **9**, 173-80
 14. Hildebrand, M. S., Damiano, J. A., Mullen, S. A., Bellows, S. T., Oliver, K. L., Dahl, H. H., Scheffer, I. E., and Berkovic, S. F. (2014) Glucose metabolism transporters and epilepsy: only GLUT1 has an established role. *Epilepsia.* **55**, e18-21

15. Ben-Zaken, S., Eliakim, A., Nemet, D., Rabinovich, M., Kassem, E., and Meckel, Y. (2014) Differences in MCT1 A1470T polymorphism prevalence between runners and swimmers. *Scand J Med Sci Sports*. doi: 10.1111/sms.12226.
16. Sasaki, S., Kobayashi, M., Futagi, Y., Ogura, J., Yamaguchi, H., Takahashi, N., and Iseki, K. (2013) Crucial residue involved in L-lactate recognition by human monocarboxylate transporter 4 (hMCT4). *PLoS One*. **8**, e67690
17. Seki, A., Miyauchi, S., Hayashi, S., Kikukawa, T., Kubo, M., Demura, M., Ganapathy, V., and Kamo, N. (2007) Heterologous expression of Pharaonis halorhodopsin in *Xenopus laevis* oocytes and electrophysiological characterization of its light-driven Cl⁻ pump activity. *Biophys J*. **92**, 2559-69
18. Emmett, M. (2014) Acetaminophen toxicity and 5-oxoproline (pyroglutamic acid): a tale of two cycles, one an ATP-depleting futile cycle and the other a useful cycle. *Clin J Am Soc Nephrol*. **9**, 191-200
19. Pillai, S. M., and Meredith, D. (2011) SLC36A4 (hPAT4) is a high affinity amino acid transporter when expressed in *Xenopus laevis* oocytes. *J Biol Chem*. **286**, 2455-60
20. Mao, X., Moerman-Herzog, A. M., Wang, W., and Barger, S. W. (2006) Differential transcriptional control of the superoxide dismutase-2 kappaB element in neurons and astrocytes. *J Biol Chem*. **281**, 35863-72.
21. Avila-Rodriguez, M., Garcia-Segura, L. M., Cabezas, R., Torrente, D., Capani, F., Gonzalez, J., and Barreto, G. E. (2014) Tibolone protects T98G cells from glucose deprivation. *J Steroid Biochem Mol Biol*. doi: 10.1016/j.jsbmb.2014.07.009.
22. Gasque, P., Chan, P., Mauger, C., Schouft, M. T., Singhrao, S., Dierich, M. P., Morgan, B. P., and Fontaine, M. (1996) Identification and characterization of complement C3 receptors on human astrocytes. *J Immunol*. **156**, 2247-55.
23. Ganapathy, V., Thangaraju, M., Gopal, E., Martin, P. M., Itagaki, S., Miyauchi, S., and Prasad, P. D. (2008) Sodium-coupled monocarboxylate transporters in normal tissues and in cancer. *AAPS J*. **10**, 193-9
24. Weng LX¹, Xu JL, Li Q, Birch RG, Zhang LH. (2003) Identification of the essential histidine residue for high-affinity binding of AlbA protein to albicidin antibiotics. *Microbiology*. **149**, 451-7
25. Takekuma, Y., Kakiuchi, H., Yamazaki, K., Miyauchi, S., Kikukawa, T., Kamo, N., Ganapathy, V., and Sugawara, M. (2007) Difference between pharmacokinetics of mycophenolic acid (MPA) in rats and that in humans is caused by different affinities of MRP2 to a glucuronized form. *J Pharm Pharm Sci*. **10**, 71-85
26. Fujisawa, Y., Kitagawa, T., Miyake, M., Nara, T., Kamo, N., and Miyauchi, S. (2006) Measurement of electric current evoked by substrate transport via bi-directional

- H⁺/oligopeptide transporter over-expressed in HeLa cells: electrogenic efflux and existence of a newly observed channel-like state. *Arch Biochem Biophys.* **445**, 166-73
27. Foltz, M., Boll, M., Raschka, L., Kottra, G., and Daniel, H. (2004) A novel bifunctionality: PAT1 and PAT2 mediate electrogenic proton/amino acid and electroneutral proton/fatty acid symport. *FASEB J.* **18**, 1758-60
28. Fujita, T., Kishida, T., Wada, M., Okada, N., Yamamoto, A., Leibach, F. H., and Ganapathy, V. (2004) Functional characterization of brain peptide transporter in rat cerebral cortex: identification of the high-affinity type H⁺/peptide transporter PEPT2. *Brain Res.* **997**, 52-61
29. Lengacher, S., Nehiri-Sitayeb, T., Steiner, N., Carneiro, L., Favrod, C., Preitner, F., Thorens, B., Stehle, J. C., Dix, L., Pralong, F., Magistretti, P. J., and Pellerin, L. (2013) Resistance to diet-induced obesity and associated metabolic perturbations in haploinsufficient monocarboxylate transporter 1 mice. *PLoS One.* **8**, e82505
30. Miyauchi, S., Gopal, E., Babu, E., Srinivas, S. R., Kubo, Y., Umapathy, N. S., Thakkar, S. V., Ganapathy, V., and Prasad, P. D. (2010) Sodium-coupled electrogenic transport of pyroglutamate (5-oxoproline) via SLC5A8, a monocarboxylate transporter. *Biochim Biophys Acta.* **1798**, 1164-71
31. Herculano-Houzel, S. (2012) The remarkable, yet not extraordinary, human brain as a scaled-up primate brain and its associated cost. *Proc Natl Acad Sci U S A.* **109**, 10661-8
32. de, Vrieze, E., van, de, Wiel, S. M., Zethof, J., Flik, G., Klaren, P. H., and Arjona, F. J. (2014) Knockdown of monocarboxylate transporter 8 (mct8) disturbs brain development and locomotion in zebrafish. *Endocrinology.* en20131962
33. Selmi-Ruby, S., Bouazza, L., Obregon, M. J., Conscience, A., Flamant, F., Samarut, J., Borson-Chazot, F., and Rousset, B. (2014) The targeted inactivation of TR β gene in thyroid follicular cells suggests a new mechanism of regulation of thyroid hormone production. *Endocrinology.* **155**, 635-46
34. Groeneweg, S., Friesema, E. C., Kersseboom, S., Klootwijk, W., Visser, W. E., Peeters, R. P., and Visser, T. J. (2014) The role of Arg445 and Asp498 in the human thyroid hormone transporter MCT8. *Endocrinology.* **155**, 618-26
35. Groeneweg, S., Lima, de, Souza, E. C., Visser, W. E., Peeters, R. P., and Visser, T. J. (2013) Importance of His192 in the human thyroid hormone transporter MCT8 for substrate recognition. *Endocrinology.* **154**, 2525-32
36. Lima, de, Souza, E. C., Groeneweg, S., Visser, W. E., Peeters, R. P., and Visser, T. J. (2013) Importance of cysteine residues in the thyroid hormone transporter MCT8. *Endocrinology.* **154**, 1948-55
37. Arjona, F. J., de, Vrieze, E., Visser, T. J., Flik, G., and Klaren, P. H. (2011) Identification

and functional characterization of zebrafish solute carrier Slc16a2 (Mct8) as a thyroid hormone membrane transporter. *Endocrinology*. **152**, 5065-73

38. Schindler, A. E. (2003) Thyroid function and postmenopause. *Gynecol Endocrinol*. **17**, 79-85
39. Pharoah, P. O., and Connolly, K. J. (1991) Relationship between maternal thyroxine levels during pregnancy and memory function in childhood. *Early Hum Dev*. **25**, 43-51

FOOTNOTE

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FIGURE LEGENDS

FIGURE 1. Chemical structures of TRH and 5-oxoproline.

FIGURE 2. Uptake of [¹⁴C]lactate (0.67 μ M) was monitored with 20-min incubation, and SLC16A1-specific transport of lactate was calculated by subtracting the uptake in water-injected oocytes from the uptake in cRNA-injected oocytes. Uptake measurements were made in the absence or presence of 5-oxoproline, histidine, proline and lactate (10 mM). Uptake measured in the absence of inhibitors was taken as 100% (control), and uptake in the presence of inhibitors is given as a percent of this control value. The experiment was done in triplicate. Data represent means $\pm$ S.E. (^a p <0.05 compared to control, ^b p >0.05 compared to control)

FIGURE 3. (A) SLC16A1 was expressed in *Xenopus laevis* oocytes. Water-injected oocytes served as controls. Uptake of 5-[³H]oxoproline (60 nM) was measured for various periods ([H⁺]: 3.2 or 0.032 μ M). The experiment was carried out in triplicate. The data represent means $\pm$ S.E. (B) Inhibition of SLC16A1-mediated 5-oxoproline uptake by lactate as a classical substrate of SLC16A1. Oocytes were injected with SLC16A1 cRNA or water. Uptake of 5-[³H]oxoproline (60 nM) was measured with 15-min incubation in parallel in water-injected oocytes and cRNA-injected oocytes, and SLC16A1-specific transport was calculated by subtracting the uptake in water-injected oocytes from the uptake in cRNA-injected oocytes. Uptake measurements were made in the absence or presence of 5-oxoproline, histidine, proline and lactate (10 mM). Uptake measured in the absence of inhibitors was taken as 100% (control), and uptake in the presence of inhibitors is given as a percent of this control value. The experiment

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was done in triplicate. Data are given as means±S.E. (^a*p*<0.05 compared to control, ^b*p*>0.05 compared to control)

FIGURE 4. Influence of thyroid hormones on SLC16A1-mediated 5-oxoproline transport. Uptake of 5-[³H]oxoproline (60 nM) was monitored with 15-min incubation, and SLC16A1-specific transport of 5-oxoproline was calculated by subtracting the uptake in water-injected oocytes from the uptake in cRNA-injected oocytes. Uptake measurements were made in the absence or presence of T3 and T4 (30 µM). Uptake measured in the absence of thyroid hormones was taken as 100% (control), and uptake in the presence of thyroid hormones is given as a percent of this control value. The experiment was done in triplicate. Data represent means±S.E. (^b*p*>0.05 compared to control)

FIGURE 5. Detection of SLC16A1 protein (A). Oocytes were sectioned and the location of SLC16A1 was revealed using confocal immunofluorescence microscopy. Saturation kinetics of 5-oxoproline transport via SLC16A1-WT (B) and -D490E (C) in the *Xenopus laevis* oocyte expression system. Uptake of 5-oxoproline ([H⁺]: 3.2 µM) was monitored with 15-min incubation in the presence of increasing concentrations of 5-oxoproline (0.1-20 mM). SLC16A1-specific uptake was calculated by subtracting the uptake in water-injected oocytes from the uptake in cRNA-injected oocytes. Only the SLC16A1-specific uptake was used for kinetic analysis. Inset: Eadie-Hofstee plot. Data represent means±S.E. for three experiments. Effect of polymorphism (rs1049434) on *K_m* of SLC16A1-expressed oocytes (D). Values shown are means±S.E. calculated from the Eadie-Hofstee plot to experimental data of the type shown in Figures 5B and 5C. (^a*p*<0.05 compared to wild type)

FIGURE 6. H⁺-activation kinetics of 5-oxoproline transport via SLC16A1-WT and -D490E. Oocytes were injected with either water or SLC16A1 cRNA. Uptake of 5-[³H]oxoproline (60 nM) was measured with 15-min incubation in the presence of increasing concentrations of H⁺ (0.032-10 µM). SLC16A1-specific uptake was calculated by subtracting the uptake in water-injected oocytes from the uptake in cRNA-injected oocytes. Only the SLC16A1-specific uptake was used for the Hill equation (A: WT, B: D490E) and Henderson-Hasselbalch equation (C: WT, D: D490E). Data represent means±S.E. for three experiments. Effect of polymorphism (rs1049434) on *K_{0.5, H⁺}* of SLC16A1-expressed oocytes (E). Uptake of 5-[³H]oxoproline (60 nM) was performed with H⁺ concentrations of 0.032-10 µM. Values shown are means±S.E. calculated from curve-fitting to experimental data of the type shown in Figures 6A and 6B. *K_{0.5, H⁺}* values refer to the [H⁺] required for half-maximal activation of 60 nM 5-oxoproline transport.

FIGURE 7. Localization of SLC16A1 in oocytes injected with SLC16A1-WT or the indicated mutants. Oocytes were treated with antibodies against SLC16A1.

FIGURE 8. (A) Time dependency of the uptake of 5-oxoproline by T98G cells. The uptake of 5-oxoproline (60 nM) was measured at 37°C in a uptake buffer ([H⁺]: 3.2 μM). Inset: the initial uptake is linear with time. (B) Concentration dependency of the 5-oxoproline uptake rate by T98G cells. Initial uptake rate was determined by linear regression analysis of the linear portion of 5-oxoproline uptake vs. time. The inset shows an Eadie-Hofstee plot of 5-oxoproline uptake. Each point represents the mean±S.E. of three experiments.

FIGURE 9. RT-PCR analysis of mRNA expression of SLC16A1, SLC16A3, SLC5A12 and actin-beta in T98G cells.

FIGURE 10. Oocytes injected with SLC16A1 cRNA, SLC16A3 cRNA or water were incubated with 5-[³H]oxoproline (60 nM) for 15 min ([H⁺]: 3.2 μM). Data represent means± S.E. for three experiments.

TABLES

TABLE 1.

Missense-type *SLC16A1* variants

Gene	DNA level	Protein level	SNP ID	Reference
<i>SLC16A1</i>	610A > G	Lys204Glu	—	(9)
	1282G > A	Val428Ile	—	(10)
	1414G > A	Gly472Arg	—	(9)
	1470T > A	Asp490Glu	rs1049434	(9-15)

SNP ID: Single nucleotide polymorphism identification (rs) number.

TABLE 2.

Demonstration of proton-coupled electroneutral transport of 5-oxoproline via SLC16A1 in a *Xenopus laevis* oocyte expression system. Uptake of 5-[³H]oxoproline (60 nM) was monitored with 15-min incubation, and SLC16A1-specific transport of 5-oxoproline was calculated by subtracting the uptake in water-injected oocytes from the uptake in cRNA-injected oocytes. For the Na⁺-free condition, Na⁺ was replaced with NMDG. For the Cl⁻-free condition, Cl⁻ was replaced with gluconate. Uptake measurements were made in the presence of KCl (50 mM) and

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CCCP (40 μ M). Uptake measured in the absence of CCCP was taken as 100% (control), and uptake in the presence of CCCP is given as percent of this control value. Uptake measured in the presence of 2 mM KCl was taken as 100% (control), and uptake in the presence of 50 mM KCl is given as percent of this control value. Uptake measured in the presence of Na⁺ was taken as 100% (control), and uptake in the absence of Na⁺ is given as percent of this control value. Uptake measured in the presence of Cl⁻ was taken as 100% (control), and uptake in the absence of Cl⁻ is given as percent of this control value. The experiment was done in triplicate. Data represent means $\pm$ S.E. (^a p <0.05 compared to control, ^b p >0.05 compared to control)

Condition	5-oxoproline uptake
	(% of control)
Na ⁺ -free	91.0 $\pm$ 2.5 ^b
Cl ⁻ -free	95.0 $\pm$ 5.0 ^b
KCl (50 mM)	99.5 $\pm$ 1.4 ^b
CCCP (40 μ M)	24.9 $\pm$ 1.1 ^a

TABLE 3.

Impact of polymorphism (rs1049434) on SLC16A1-mediated lactate uptake. Uptake of lactate (100 μ M) was monitored in oocytes injected with SLC16A1-WT or the indicated mutants. SLC16A1-specific transport of lactate was calculated by subtracting the uptake in water-injected oocytes from the uptake in cRNA-injected oocytes. The experiment was done in triplicate. Data represent means $\pm$ S.E. (^a p <0.05 compared to wild type, ^b p >0.05 compared to wild type)

SLC16A1 mutants	Lactate uptake rate
	(pmol/min/oocyte)
Wild type	14.9 $\pm$ 1.3
Lys204Glu	14.6 $\pm$ 0.4 ^b
Val428Ile	14.7 $\pm$ 0.6 ^b
Gly472Arg	15.5 $\pm$ 1.2 ^b
Asp490Glu	20.1 $\pm$ 0.7 ^a

TABLE 4.

Effects of various conditions on 5-oxoproline uptake by T98G cells. Uptake of 5-oxoproline (60

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nM) was measured in the uptake buffer ([H⁺]: 3.2 μ M except for “[H⁺]: 0.032 μ M”) for 5 min at 37°C under various conditions. Data represent means $\pm$ S.E. (^a p <0.05 compared to control, ^b p >0.05 compared to control)

Conditions	5-oxoproline uptake (% of control)
Na ⁺ -free	103 $\pm$ 2.3 ^b
Valinomycin (50 μ M)	97.5 $\pm$ 5.6 ^b
DEPC (2.5 mM)	9.4 $\pm$ 0.3 ^a
H ⁺ (0.032 μ M)	18.1 $\pm$ 0.4 ^a
CCCP (40 μ M)	14.0 $\pm$ 1.0 ^a

TABLE 5.

Inhibitory effects of various compounds on 5-oxoproline uptake by T98G cells. Uptake of 5-oxoproline (60 nM) was measured for 5 min at 37°C in the absence or presence of various compounds (10 mM). Data represent means $\pm$ S.E. (^a p <0.05 compared to control, ^b p >0.05 compared to control)

Compounds	5-oxoproline uptake (% of control)
Lactate (10 mM)	20.5 $\pm$ 0.2 ^a
<i>p</i> -Aminohippurate (10 mM)	91.3 $\pm$ 1.6 ^b
Proline (10 mM)	96.4 $\pm$ 0.0 ^b
Histidine (10 mM)	103 $\pm$ 3.9 ^b
Glycylsarcosine (10 mM)	93.6 $\pm$ 1.3 ^b

TABLE 6.

Sequence of PCR primers.

Gene	Primer sequences	Expected product size (bp)
SLC16A1	F: 5'-ccattgtggaatgctgtcct-3' R: 5'-cctacttctttcccccaccc-3'	540

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SLC16A3	F: 5'-atcctggggttcattgacat-3'	540
	R: 5'-cttcaggaaatgctccacct-3'	
SLC5A12	F: 5'-agctgagcacctggatcagt-3'	193
	R: 5'-agtgcacccttccaattcac-3'	
Actin-beta	F: 5'-tcctggagaagagctacga-3'	591
	R: 5'-gtcaccttcaccgttcagt-3'	

Figure 1

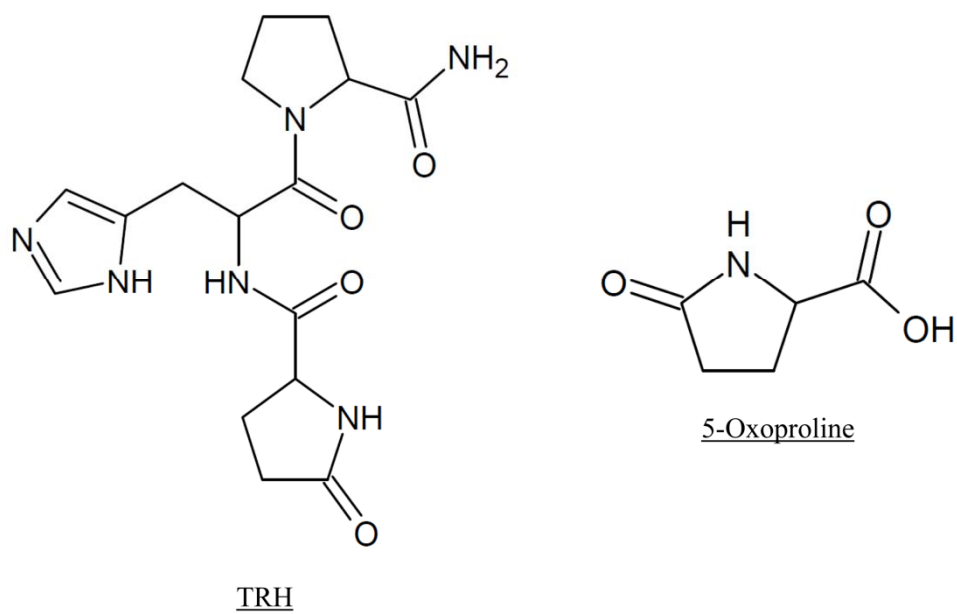


Figure 2

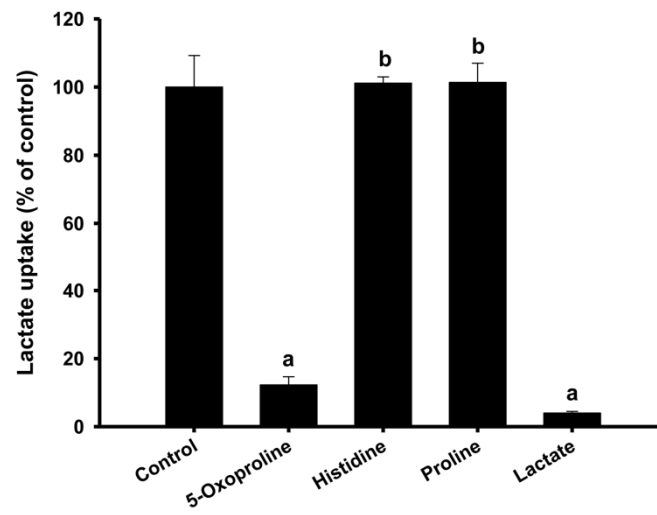


Figure 3

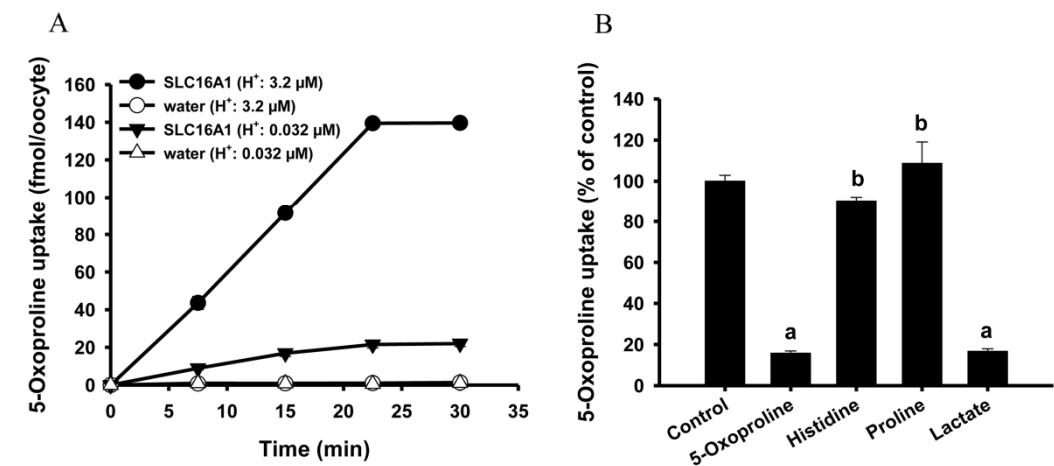


Figure 4

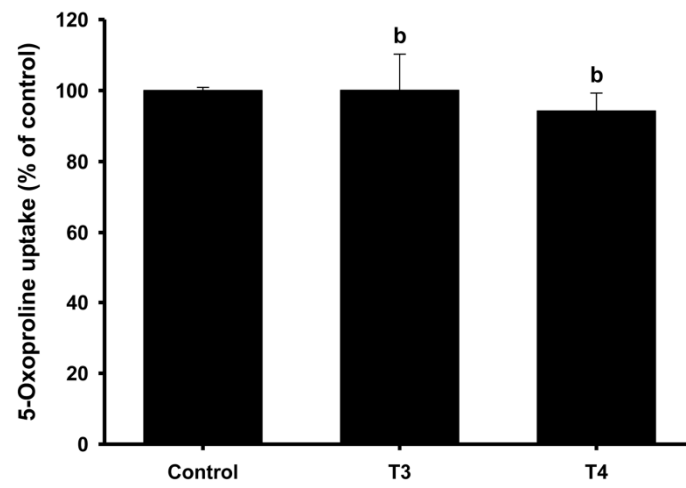


Figure 5

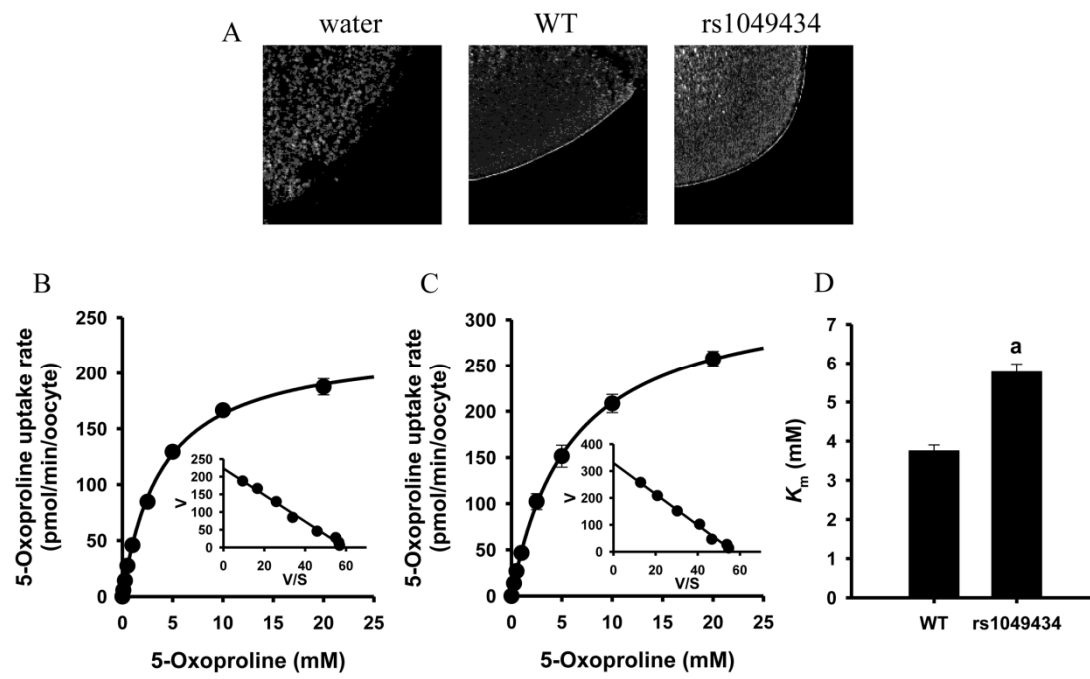


Figure 6

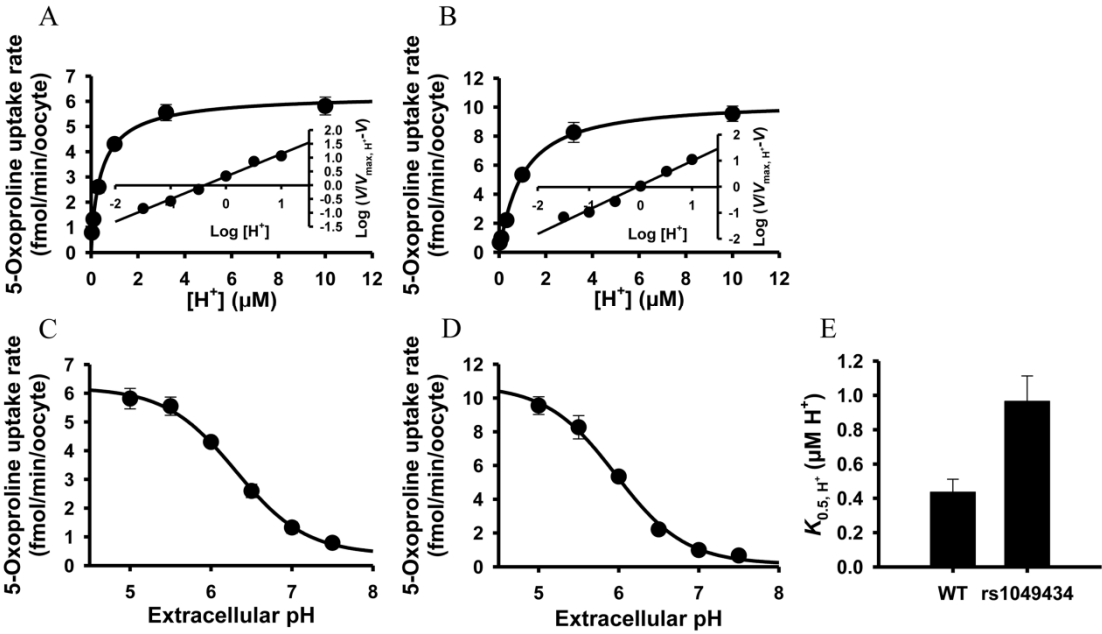


Figure 7

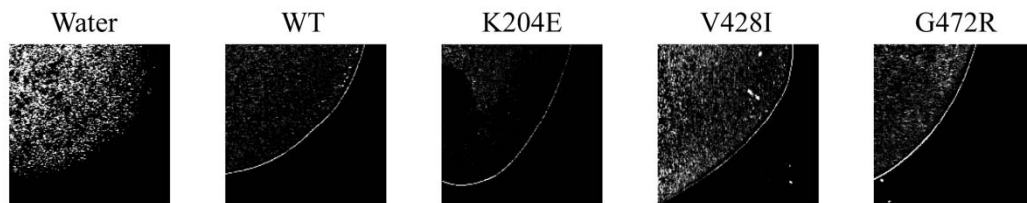


Figure 8

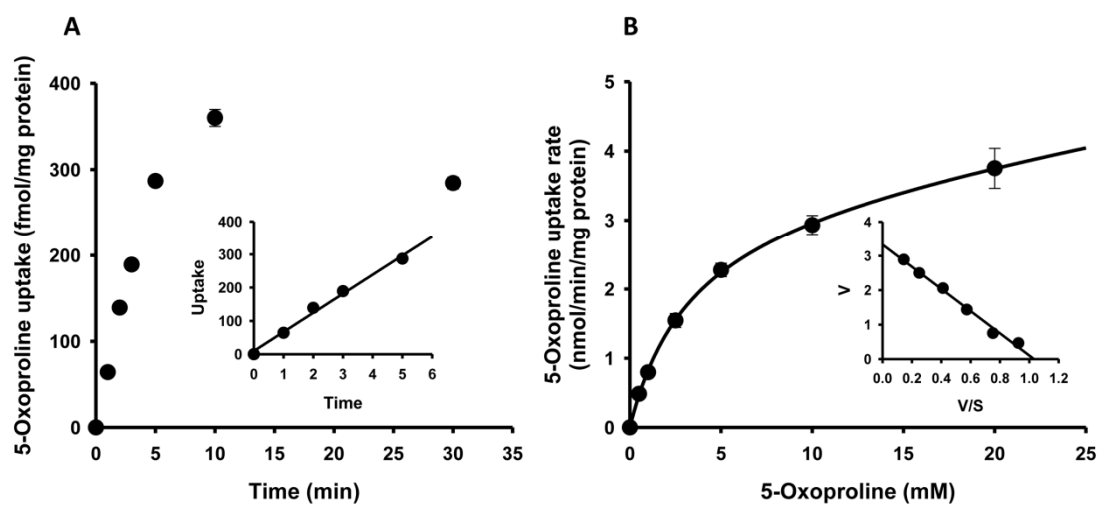


Figure 9

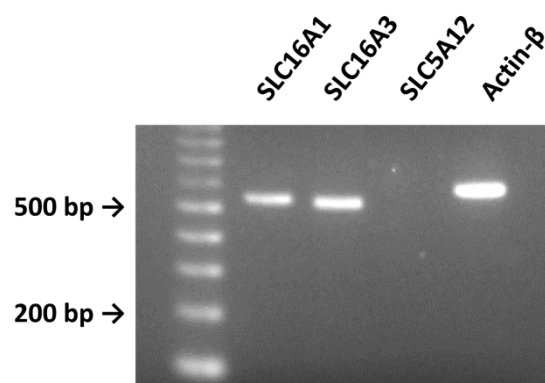


Figure 10

