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Amino Acid Transport System N: Molecular Structure, Distribution and Functional Analysis of Canine SLC38A5 (SNAT5) in Lens Epithelial Cells.

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Abstract: Na-dependent of neutral amino acid transport activity in canine lens epithelial cells (LEC) line was investigated. The transporter activity of glutamine was 11.17 ± 3.17 nmol/mg protein/min, and it was reduced by 75% in the absence of sodium. The full-length cDNA sequence of canine sodium-dependent neutral amino acid transporter 5 (SNAT5) was 2151 bp long and was predicted to encode the 536 amino acid polypeptides. The deduced amino acid sequence of canine SNAT5 showed >80% similarities with those of human and mouse. The RT-PCR analysis indicated that SNAT5 was expressed in liver, kidney and LEC, but not in heart and skin.
Key words: cDNA sequence, distribution, lens epithelial cells, SNAT5.

Introduction

Many amino acid transport systems were distinguished based on differences in their substrate-selectivity, ion-dependence, pH sensitivity, kinetics and regulatory properties, using membrane vesicle preparation or cultured cells[1, 2]. Among them, system N was designated as the transporter of Na^+-dependent neutral amino acids. This designation is now attributed to the transporters termed sodium-dependent neutral amino acid transporters (SNAT), which belong to the SLC38 gene family. The family comprises five members, SLC38A1-5. Among them, SLC38A5 (SNAT5) plays physiological roles including the transfer of glutamine from the astrocyte to the neuron in the CNS, ammonia detoxification and gluconeogenesis in the liver, and the renal response to acidosis.[3]

The lens epithelial cells (LEC) are the progenitors of the lens fibers in vivo and undergo a developmental transition into fiber cells of the lens cortex. This process is characterized by distinct biochemical and morphologic changes such as the synthesis of crystallin proteins, cell elongation, loss of cellular organelles, and the disintegration of the nucleus.[4, 5] LEC possesses a milli-molar order contentration of high glutathione (GSH). As a potent antioxidant, GSH maintains enzymes and protein thiols in their reduced state and scavenges free radicals and other reactive oxygen species. GSH synthesis is dependent on its precursors glutamate, glycine and cysteine. In addition, glutamine, the most abundant free amino acid in plasma, is promptly converted to glutamate by glutaminase. Previously, we developed the lens epithelial cell line originated from mature cataract of dog and reported several characteristics of this cell line.[6]

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Table 1 Sequences of the oligonucleotides used to clone canine SNAT5

Primer		Sequence (5'-3')	Product (bp)
Oligonucleotide			
s-1	sense	AATCAGAGCATTCATGGGACTTG	520
a-1	anti	CTGTAGATGGGAAGGACCTCAGGGT	
GSP1 for 3'RACE	sense	ACACCCACCAGAATCCTGCAGGGCTAG	
GSP2 for 5'RACE	anti	TGCTAGGATAGGGATTGCGTAGGCCGT	
s-2	sense	AAATACCCTGCCCTCTGCCCGTTCTGG	311
a-2	anti	GGACCTCAGGGTGGCACACAAAAGCAA	
GAPDH	sense	ATC ACC ATC TTC CAG GAG CGA GA	192
(AB038240)	anti	GTC TTC TGG GTG GCA GTG ATG G	

(accession number) is indicated.

In this study, we investigated the glutamine, neutral amino acid transport activity of this lens epithelial cell line and determined the cDNA sequence of canine SNAT5, which was found to be expressed in LEC.

Materials and Methods

Animal and cell samples

All experiments were performed according to the guidelines of The Laboratory Animal Care Committee of Azabu University, and were in compliance with the Fundamental Guideline for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions. The canine lens epithelial cell line originated from mature cataract was maintained as described previously[6].

Measurement of arginine transport activity

Radioactive (^{3}H-) glutamine was purchased from American Radiolabeled Chemicals (St Louis, MO, USA). Glutamine uptake was measured as described previously. Simply, the cells were plated in 5×10^5 cells/6-well plate 24 h before the experiment. The cells were washed 3 times with 130 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 10 mM glucose, 15 mM Tris/MOPS, pH 7.4, and 0.1% BSA. Then, medium containing 100 μM glutamine with radioisotope was added and the cells were incubated at 37°C for 10 min. To study Na-dependency, NaCl was replaced by N-methyl-D-glucamine-Cl. Uptake was terminated by washing with ice-cold phosphate-buffered saline. After solubilizing the cells with 1% SDS, the radioactivity was measured with a liquid scintillation counter and protein content was determined by the Micro BCA method. Glutamine uptake was expressed as nmol/mg protein/min.

Determination of cDNA sequence of canine SNAT5

Total RNA was isolated from canine tissues using an RNA extraction solution (Isogen, Nippon Gene, Tokyo, Japan). The primers used in this study are shown in Table 1. The primer set (s1 and a1) for the amplification of a partial canine SNAT5 cDNA were prepared from the conserved sequences between humans and mice (DDBJ accession No. AK291168 and BC024072, respectively). The RT-PCR amplification was performed employing a SuperscriptIII first cDNA system kit (Invitrogen, Carlsbad, CA, USA) with Hot start Ex Taq DNA polymerase (Takara Bio, Kyoto, Japan). The band was excised from the agarose gel and purified using a Wizard SV gel clean-up system (Promega, Tokyo, Japan). The extracted and purified DNA were cloned into a pCR II-TOPO cloning vector (Invitrogen) and sequenced with a BigDye terminator kit ver.3 (Applied Biosystems, Carlsbad, CA, USA). The nucleotide sequence obtained exhibited high similarities (90%) with human SNAT5 cDNA sequences. In order to determine the 3' and 5' regions of cDNA, RACE methods

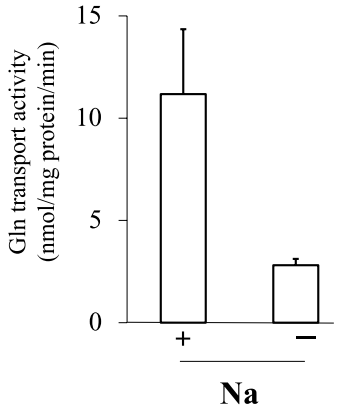

Fig. 1 Glutamine transport activity of LEC in the presence (+) or absence (−) of Na. Values are means and SD of 4 individual experiments.

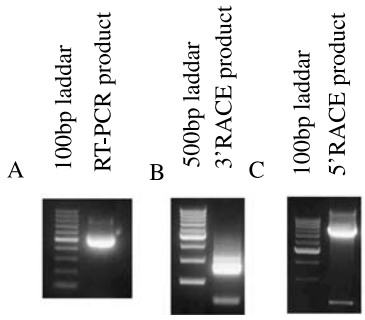

Fig. 2 PCR-based cloning of 5'- and 3'-stretched cDNA clones of canine SNAT5 (A). The electrophoresis of RT-PCR products of canine SNAT5 using primers (s-1 and a-1) (A). 5' and 3' RACE products with the primers are shown in Table 1.

were carried out using a SMARTer RACE cDNA amplification kit (Clontech, Mountain View, CA, U.S.A) and a set of canine SNAT5 gene-specific primers (GSP1 and GSP2).

RT-PCR analysis of SNAT5 mRNA in canine tissues

In order to examine the expression of SNAT5 mRNA in various organs of the dog, we performed RT-PCR using newly designed primers specific to canine SNAT5 (S2 and A2: Table 1). RT-PCR conditions were as follows: 2 min at 94°C, followed by 30 cycles of 10 sec at 94°C, 10 sec at 60°C, and 30 sec at 72°C. Primers and RT-PCR conditions for RT-PCR analysis of canine gapdh GADPH were followed as described elsewhere.[7] PCR conditions were as follows: 30 cycles of three steps; 94C 15 sec, 58C 10 sec and 72C 15 sec.

Results

The glutamine transport activity of canine lens epithelial cell line is showned in Fig. 1. The transport activity was 11.17 ± 3.17 nmol/mg protein min, while the absence of Na reduced to 25% of its transport activity. Figure 2A showed the electrophoresis of RT-PCR products of canine SNAT5 using primers (s-1 and a-1), that were well conserved between human and rodent. The nucleotide sequence comprising 520 bp showed high similarities of 90% to mouse SNAT5 cDNA sequences. The primers used for RACE were prepared from the sequence details listed in Table 1. 3'and 5'RACE products were also shown in Fig. 2 (B, C). The obtained nucleotide sequence corresponding to a full-length canine SNAT5 cDNA was 2151 bp in length and contained an entire open reading frame of 1608 bp, encoding canine SNAT5 of 536 amino acids and having a theoretical mass of 60 kDa (Fig. 2B). Figure 3 shows a comparison of canine SNAT5 with those of reported humans and rodents. The deduced amino acid sequence of SNAT5 showed high similarities of >80% with those of human and mouse. Canine SNAT5 possesses one amino acid (glutamate) insertion between amino acid 14 and 15 of mouse and human SNAT5. Notably the, C-terminus of canine SNAT5 was 33 and 12 amino acids horter than those of human and mouse, respectively. To determine the genomic distribution of each cDNA, the UCSC genome browser site (http://genome.ucsc.edu/) was used to align canine genomic sequences and each cDNA. It was revealed that the cDNA sequence of SNAT5 consisted of 13 exons of 59-276 base pairs in chromosome 27 (Fig. 3B). RT-PCR analysis of SNAT5 expression indicated that SNAT5 is expressed in the in liver, kidney and LEC, but not in heart and skin (Fig. 4).

Discussion

System N is a major Na^+-dependent transport system for zwitterionic amino acids in mammalian cell types, including hepatocytes and astrocytes. In the central nerve system, SNAT1 and SNAT2 are known to be expressed in neurons in the brain.[8, 9] On the other hand, SNAT3 and SNAT5

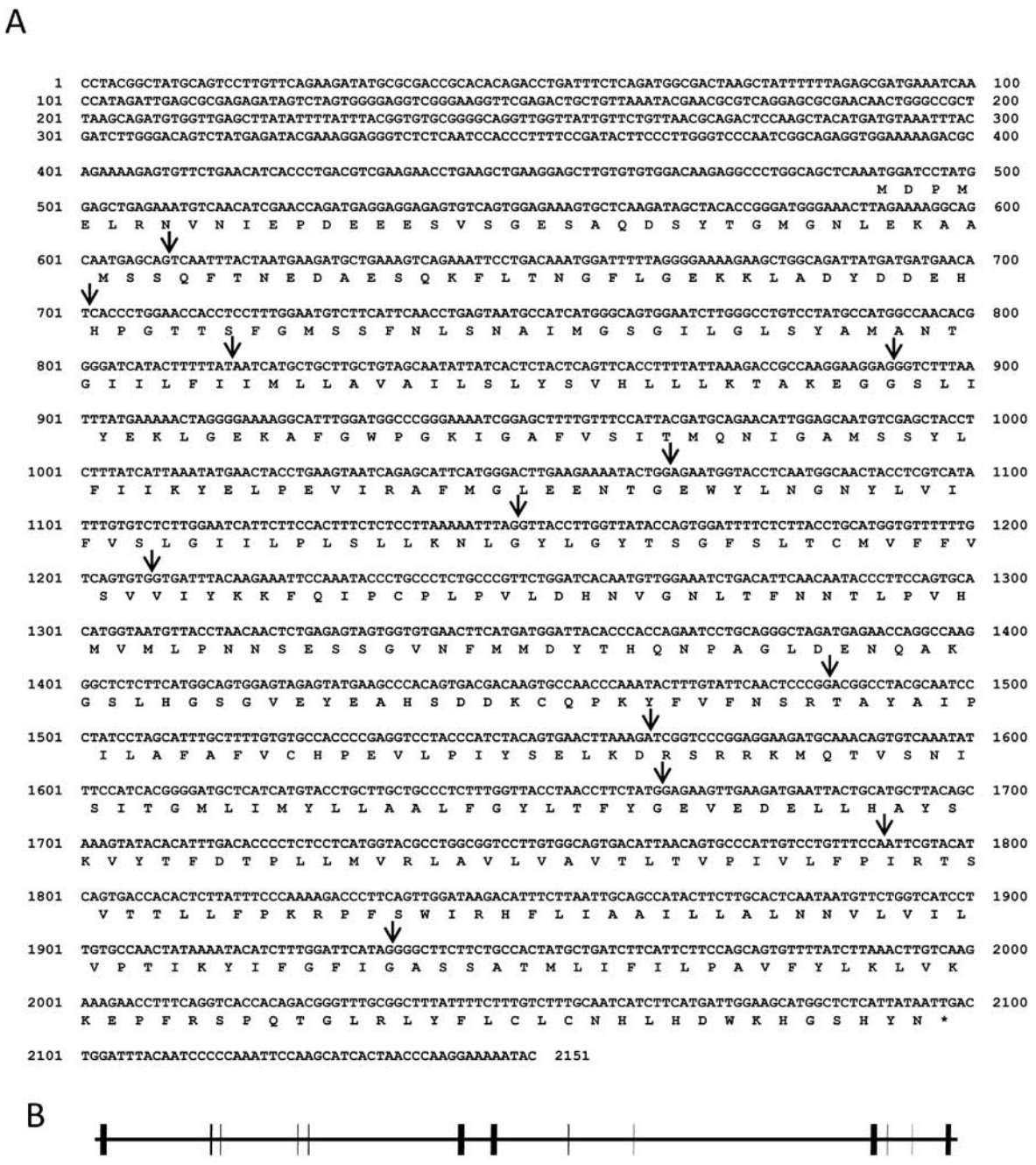

Fig. 3 Nucleotide and deduced amino acid sequences of canine SNAT5 were shown (B). Full-length canine SNAT5 cDNA was 2151 bp in length and contained an entire open reading frame of 1608 bp, encoding canine SNAT5 of 536 amino acids.. The termination codon is shown by an asterisked. Arrows indicates the positions of introns. Alignment of the cDNA sequence with the canine genome chromosome 1 predicts 8 exons of 40-671 base pairs. ■ ; exon, ― ; intron. (B).

have been localized, using immunohistochemistry, exclusively to astrocytes.[10, 11] SNAT1 and 2 as well as SNAT3 and 5 have been suggested to be responsible for glutamine transport in the glutamate/glutamine cycle.[12] This process is crucial for the survival of excitatory neurons as a way of rapidly removing glutamate from the synaptic cleft, to eliminats excitotoxic effect of glutamate.

Cataracts are among the most frequent ophthalmological diseases seen in veterinary clinics. Dogs in particular are more prone to develop lens opacity than other domestic animals. Oxidative stress of lens constituents has been recognized as an important mechanism in the development

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dSNAT5   1:MDPMELRNVNIEPDEEESVSGESAQDSYTGMGNLEKAAMSSQFTNEDAESQKFLTNGFLGEKKLADYDDEHHPGTTSFGMSSFNLSNAIMGSGILGLSYA 100
hSNAT5   1:MDPMELRNVNIEPD-DESSSGESAPDSYIGIGNSEKAAMSSQFANEDTESQKFLTNGFLGKKKLADYADEHHPGTTSFGMSSFNLSNAIMGSGILGLSYA 99
mSNAT5   1:MDPMELNNVSIEPD-GDSCSGDSIQDSYTGMENSDKDAMNSQFANEDAESQKFLTNGFLGKKKLADYADEHHPGMTSFGMSSFNLSNAIMGSGILGLSYA 99
           ******.**.****  .* **.*..***.*..*..*.**.***.***.************.******.******.************************

dSNAT5 101:MANTGIILFIIMLLAVAILSLYSVHLLLKTAKEGGSLIYEKLGEKAFGWPGKIGAFVSITMQNIGAMSSYLFIIKYELPEVIRAFMGLEENTGEWYLNGN 200
hSNAT5 100:MANTGIILFIIMLLAVAILSLYSVHLLLKTAKEGGSLIYEKLGEKAFGWPGKIGAFVSITMQNIGAMSSYLFIIKYELPEVIRAFMGLEENTGEWYLNGN 199
mSNAT5 100:MANTGIILFIIMLLTVAILSLYSVHLLLKTAKEGGSLIYEKLGEKAFGWPGKIGAFISITMQNIGAMSSYLFIIKYELPEVIRAFMGLEENTGEWYLNGN 199
           **************.****************************************.****************************************

dSNAT5 201:YLVIFVSLGIILPLSLLKNLGYLGYTSGFSLTCMVFFVSVVIYKKFQIPCPLPVLDHNVGNLTFNNTLPVHMVMLPNNSESSGVNFMMDYTHQNPAGLDE 300
hSNAT5 200:YLIIFVSVGIILPLSLLKNLGYLGYTSGFSLTCMVFFVSVVIYKKFQIPCPLPVLDHSVGNLSFNNTLPMHVVMLPNNSESSDVNFMMDYTHRNPAGLDE 299
mSNAT5 200:YLVLFVSVGIILPLSLLKNLGYLGYTSGFSLSCMVFFVSVVIYKKFQIPCPLPALDHNNGNLTFNNTLPIHMISLPNDSESSGVNFMMDYAHHNPAGLDE 299
           **..***.**********************.*********************.***..***.****** *...***.****.*******.* *******

dSNAT5 301:NQAKGSLHGSGVEYEAHSDDKCQPKYFVFNSRTAYAIPILAFAFVCHPEVLPIYSELKDRSRRKMQTVSNISITGMLIMYLLAALFGYLTFYGEVEDELL 400
hSNAT5 300:NQAKGSLHDSGVEYEAHSDDKCEPKYFVFNSRTAYAIPILVFAFVCHPEVLPIYSELKDRSRRKMQTVSNISITGMLVMYLLAALFGYLTFYGEVEDELL 399
mSNAT5 300:KQVAGPLHSNGVEYEAQGAEKCQPKYFVFNSRTAYAIPILAFAFVCHPEVLPIYSELKDRSRRKMQTVSNISISGMLVMYLLAALFGYLSFYGDVEDELL 399
           .*..*.** .*******....**.*****************.*********************************.***.***********.***.******

dSNAT5 401:HAYSKVYTFDTPLLMVRLAVLVAVTLTVPIVLFPIRTSVTTLLFPKRPFSWIRHFLIAAILLALNNVLVILVPTIKYIFGFIGASSATMLIFILPAVFYL 500
hSNAT5 400:HAYSKVYTLDIPLLMVRLAVLVAVTLTVPIVLFPIRTSVITLLFPKRPFSWIRHFLIAAVLIALNNVLVILVPTIKYIFGFIGASSATMLIFILPAVFYL 499
mSNAT5 400:HAYSKVYTFDTALLMVRLAVLVAVTLTVPIVLFPIRTSVITLLFPRKPFSWLKHFGIAAIIIALNNILVILVPTIKYIFGFIGASSATMLIFILPAAFYL 499
           ********.*..*****************************.******..****..**.***...****.*****************************.***

dSNAT5 501:KLVKKEPFRSPQTGLRLYFLCLCNHLHDWKHGSHYN---------------------------------                                536
hSNAT5 500:KLVKKETFRSPQKVGALIFLVVGIFFMIGSMALIIIDWIYDPPNSKHHHKEKYFLFLLEMVTSYTPKDI                                  568
mSNAT5 500:KLVKKEPLRSPQKIGALVFLVTGIIFMMGSMALIILDWIYNPPNPNHH---------------------                                547
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Fig. 4 Amino acid sequences of canine SNAT5 were compared with those of human and mouse. Multiple sequence alignments were performed using the Genetyx program (ver. 10). Asterisks and dots indicate identical residues and conservative substitutions, respectively.

of cataracts. Primary defenses that directly protect the lens against oxidative damage include small molecular antioxidants such as GSH. GSH is a tripeptide synthesized by the sequential actions of the enzymes λ-glutamylcysteine synthetase and glutathione synthetase.[13, 14] In the lens, GSH is maintained at an unusually high concentration as result of potent synthesis from precursor amino acids including glutamine. Therefore, active transport of these amino acids into lens cells is essential for protecting the crystalline proteins from oxidative stress which causes lens opacity and eventually development of cataract. Preiously, we reported that all subtypes of glutamate transporters (excitatory amino acid transporter: EAAT 1-5) were expressed in LEC. SNAT5 may be function as glutamine supplier into LEC which have a potent demand for GSH precursor amino acids.

In this study, we confirmed the potent Na-dependent glutamine transport activity in canine LEC, and determined a full-length cDNA sequence of canine SNAT5. RT-PCR analysis indicated that SNAT5 is expressed in liver, kidney and LEC, but not in heart and skin, and this is an expressional pattern was similar to those reported for other mammalians'.[15] Notably, the deduced amino acid sequence of canine SNAT5 showed high similarities with those of

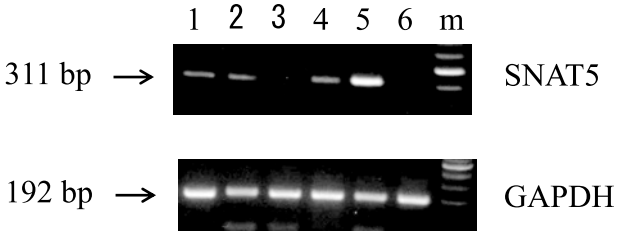

Fig. 5 RT-PCR analysis of canine SNAT5 mRNA in various dog tissues (A). Integrity of RNA was examined by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Lane 1: LEC; lane 2: kidney; lane 3: skin; lane 4: lung; lane 5: liver; lane 6: heart.

human and mouse with the exception of the C-terminus of SNAT5 protein. There may be functional or regulational difference between canine and other mammalians. To our knowledge, this is the first report of SNAT5 expression in LEC. The data clarified in this paper may facilitate the study on neutral amino acid metabolism on the canine ocular surface.

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References

1）Christensen, H.N, Handlogten, M.E., Lam, I., Tager, H.S., Zand, R. A., bicyclic amino acid to improve discriminations among transport systems. *J. Biol. Chem*. **244**: 1510-20 (1969)

2）Christensen, H.N., Role of amino acid transport and countertransport in nutrition and metabolism. *Physiol. Rev*. **70**: 43-77 (1990)

3）Mackenzie B., Erickson J.D., Sodium-coupled neutral amino acid (System N/A) transporters of the SLC38 gene family. *Pflugers Arch*. **447**: 784-95 (2004)

4）Babizhayev, M.A., Costa, E.B., Lipid peroxide and reactive oxygen species generating systems of the crystalline lens. *Biochim. Biochem. Acta* **1225**: 326-37 (1994)

5）Bhat, S.P., The Ocular lens epithelium. *Biosci. Reports*. **21**: 537-63 (2001)

6）Kanemaki, N., Saito, M., Onda, K., Maruo, T., Ogihara, K., Naya, Y., Morishita, T., Ochiai, H., Establishment of a lens epithelial cell line from a canine mature cataract. *Exp. Anim*. **61**: 41-7 (2012)

7）Ochiai, H., Saito, M., Maruo, T., and Kanemaki, N., Molecular cloning of canine excitatory amino acid transporter 5 and its detection in primary lens epithelial cells. *Exp. Anim*. **59**: 449-57 (2010)

8）Sundberg, B.E., Waag, E., Jacobsson, J.A., Stephansson, O., Rumaks, J., Svirskis, S., Alsiö, J., Roman, E., Ebendal, T., Klusa, V., Fredriksson, R., The evolutionary history and tissue mapping of amino acid transporters belonging to solute carrier families SLC32, SLC36, and SLC38. *J. Mol. Neurosci*. **35**: 179-93 (2008)

9）Varoqui, H., Zhu, H., Yao, D., Ming, H., Erickson, J.D., Cloning and functional identification of a neuronal glutamine transporter. *J. Biol. Chem*. **275**: 49-54 (2000)

10）Chaudhry, F.A., Reimer, .RJ., Krizaj, D., Barber, D., Storm-Mathisen, J., Copenhagen, D.R., Edwards, R.H. Molecular analysis of system N suggests novel physiological roles in nitrogen metabolism and synaptic transmission. *Cell* **99**: 69-80 (1999)

11）Cubelos, B., González-González, I. M, Giménez, C., Zafra, F., Amino acid transporter SNAT5 localizes to glial cells in the rat brain. *Glia* **49**: 230-44 (2005)

12）Conti, F., Melone, M., The glutamine commute: lost in the tube? *Neurochem Int*. **48**: 459-64 (2006)

13）Rathbun, W. B., Glutathione Biosyntthesis in the lens and erythrocytes. New York : Elsevier/ North Holland: 169-73 (1980)

14）Valencia, E., Marin, A., Hardy, G., Glutathione-nutritional and pharmacological viewpoint. *Nutrition* **17**: 485-6 (2001)

15）Nakanishi, T., Kekuda, R., Fei, Y. J., Hatanaka, T., Sugawara, M., Martindale, R. G., Leibach, F. H., Prasad, P. D., Ganapathy V. Cloning and functional characterization of a new subtype of the amino acid transport system N. *Am J Physiol Cell Physiol*. **281**: C1757-68 (2001)