

Characterization of an N-system Amino Acid Transporter Expressed in Retina and Its Involvement in Glutamine Transport*

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We report here on the characterization of a mouse N-system amino acid transporter protein, which is involved in the transport of glutamine. This protein of 485 amino acids shares 52% sequence homology with an N-system amino acid transporter, mouse N-system amino acid transporter (mNAT) and its orthologs. Because this protein shares a high degree of sequence homology and functional similarity to mNAT, we named it mNAT2. mNAT2 is predominately expressed in the retina and to a slightly lesser extent in the brain. In the retina, it is located in the axons of ganglion cells in the nerve fiber layer and in the bundles of the optic nerve. Functional analysis of mNAT2 expressed in *Xenopus* oocytes revealed that the strongest transport activities were specific for L-glutamine. In addition, mNAT2 is a Na⁺- and pH-dependent, high affinity transporter and partially tolerates substitution of Na⁺ by Li⁺. Additionally, mNAT2 functions as a carrier-mediated transporter that facilitates efflux. The unique expression pattern and selective glutamine transport properties of mNAT2 suggest that it plays a specific role in the uptake of glutamine involved in the generation of the neurotransmitter glutamate in retina.

Amino acid transporters play essential roles in a variety of cellular processes, including uptake of nutrients, energy, and chemical metabolism, detoxification, and specifically in neurotransmitter cycling (1). Mammalian amino acids transporters identified to date belong to different gene families such as cationic amino acid transporter, γ -aminobutyric acid, and excitatory amino acid transporter (2–4). Many of the gene families identified thus far consist of multiple members that are expressed in a tissue-specific manner. We recently cloned and characterized a mouse N-system amino acid transporter, mNAT,¹ which is dominantly expressed in the liver (5). Two orthologous genes of mNAT have been identified by us and other groups, as human *g17* and rat *SN1* (5–7). The system N amino acid transporter mostly mediates the transport of L-glutamine, L-histidine, and L-asparagine across cell mem-

branes. Glutamine is the most abundant free amino acid in both plasma and cerebrospinal fluid (8). Glutamine serves as a precursor for other amino acids during protein synthesis. Moreover, transport of glutamine is essential for nitrogen metabolism and synaptic transmission (9–12). System N has been shown to be important in transporting glutamine for the control of nitrogen metabolism in the liver (9, 10). Furthermore, in that organ, glutamine is a central intermediate in the detoxification of ammonia and the production of urea (13). Accordingly, we have observed that there is a graded distribution of mNAT expression from the central vein to the portal tract (5).

In the nervous system, glutamine serves as a precursor for the synaptic transmitter, glutamate, both in glutamatergic neurons in the brain (11, 12) and in the retina (14). The N-system transporter, SN1, and the ASC system transporter, ASCT2, are expressed in astroglial cells, and ASCT2 plays a role in the export of glutamine from these cells (6, 15). Thus, it is likely that either one or both of these two transporters mediate the export of glutamine in glial cells. The glutamine released by glial cells is then transported to glutamatergic neuronal cells where glutamine is converted to glutamate (16, 17). The A-system transporter, GlnT/ATA1 has been reported to be present in neurons of the central nervous system (18). Hence, glutamine serves as a precursor for the generation of glutamate in these cells by maintaining the glutamate-glutamine cycle (19, 20).

To identify new member(s) of the N-system transporters, we performed reverse transcription-PCR using primers based on known N-system amino acid transporter sequences to screen the RNAs isolated from mouse tissues. In this study, we report the characterization of a mouse N-system transporter, mNAT2. mNAT2 was found to be predominantly expressed in mouse retina and brain. Its specific expression in the axons of ganglion cells and its selective transport properties suggest that this transporter plays a key role in generation of glutamate and in the maintenance of retinal homeostasis and function.

EXPERIMENTAL PROCEDURES

Materials—mMESSAGE mMACHINE for *in vitro* transcription was obtained from Ambion (Austin, TX). The Marathon cDNA amplification kit was obtained from CLONTECH (Palo Alto, CA). TRI reagent was obtained from Molecular Research Center (Cincinnati, OH). ³H-Labeled L-alanine, L-glutamine, L-glutamate, L-lysine, and L-histidine were purchased from PerkinElmer Life Sciences (Boston, MA). Restriction endonucleases were from New England BioLabs (Revere, MA). Protease inhibitors were obtained from Roche Molecular Biochemicals (Mannheim, Germany). Nitrocellulose membrane was purchased from Schleicher & Schuell (Keene, NH), and Nylon transfer membrane-Hybond H⁺ was from Oncor (Gaithersburg, MD). SDS-polyacrylamide gel electrophoresis standards were from Bio-Rad (Hercules, CA). RNA standards, oligo(dT) primer and Superscript II reverse transcriptase were from Life Technologies, Inc. (Grand Island, NY). Micro BCA Protein Assay Reagent kit was from Pierce (Rockford, IL). Paraformaldehyde (16% stock solution) was from Electron Microscopy Science (Fort

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF184240.

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¹ The abbreviations used are: mNAT, mouse N-system amino acid transporter; MeAIB, N-methylamino-isobutyric acid; ORF, open reading frame; PCR, polymerase chain reaction; GST, glutathione S-transferase; FITC, fluorescein isothiocyanate; kb, kilobase(s).

Washington, PA). Tissue-Tek OCT compound was from Miles Scientific (Naperville, IL). All other chemicals were from either Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Cloning of the *mNAT2* cDNA—The reverse transcription-PCR approach was utilized to identify new member(s) of the mNAT family. RNAs were isolated from mouse brain, liver, kidney, bone, and skeletal muscle using TRI reagent. To obtain the cDNA sequence, polyadenylated RNA was isolated using a mRNA Separator kit (CLONTECH). mRNA was converted to single-stranded cDNA by oligo(dT) and Superscript reverse transcriptase (Life Technologies, Inc.). Several primer pairs were designed based on the homology between the conserved regions of the available N-system transporters, *mNAT* (AF159856), *g17* (U49082), transporter in *Arabidopsis* (U39782) and transposase in *Pseudomonas stutzeri* (AF039534). After PCR amplification over 30 cycles with annealing temperatures of 40 °C, one of the primer pairs (sense primer, 5'-GGTTGCGACAATGTCTGTTAGGAC-3'; antisense primer, 5'-CGTGGGTCGTGAGATGTAGCAC-3') generated a 0.5-kb DNA fragment from mouse brain cDNA. After sequencing (DNA Sequencing Facility, University of Texas Health Science Center, San Antonio), this fragment showed homology to mNAT and g17. A mouse brain cDNA was prepared using the Marathon-cDNA kit (CLONTECH) according to the manufacturer's recommendation. Based on the sequence obtained from this 0.5-kb PCR fragment, two unique primers were designed: 5'-TACACCATGCAGCCGGTTTC-3' to amplify the additional 5' sequence and 5'-GCAAGTTCATCTCAGATCGGGA-3' to amplify further the 3' sequence of this new gene. Subsequently, based on the newly generated additional sequence, ten specific primers were used to generate a complete ORF sequence of *mNAT2*.

Northern Blot Analysis—Northern blots were performed as described (21). Total RNA was extracted from different tissues of adult mice using TRI reagent. Thirty micrograms of RNA was loaded and separated by agarose gel electrophoresis containing formaldehyde and transferred to a Nylon membrane. The membrane was hybridized at 45 °C for 12 h in a hybridization solution containing 50% formamide and ³²P-labeled cDNA probe corresponding to nucleotides 1–287 of mNAT2. The probed membrane was washed in a high stringency condition, 0.1× SSC and 0.1% SDS at 63 °C for 1 h.

Antibody Preparation—Chicken anti-mNAT2 IgG antibody was produced using a glutathione S-transferase (GST)-tagged fusion protein as described previously (21). A DNA fragment encoding amino acids 1–65, which is distinguished from mNAT and its orthologs (5, 6), was produced by PCR using a mNAT2 DNA clone as a template (sense primer, 5'-TCCCCCGGGACCGAATATGATGCATTTC-3'; antisense primer, 5'-GGCCGAATTCGTCGATTCCTTTTCTC-3'). This fragment was inserted into the expression vector, pGEX-2T. The recombinant fusion protein was expressed in *Escherichia coli*, induced by isopropyl-thio-β-D-galactoside, and isolated with GST beads. The purified fusion protein was used to raise polyclonal antisera in chicken (Pocono Rabbit Farm and Laboratory Inc., Canadensis, PA). The antisera generated were affinity-purified by passage through two Sepharose CL-4B columns, GST-conjugated and GST-mNAT2 fusion protein-conjugated, respectively.

Membrane Protein Preparation and Immunoblot Analysis—Crude membrane extracts were prepared from *Xenopus* oocytes and mouse tissues, including retina, liver, brain, and testis as previously described (5, 21, 22). Oocyte homogenates were centrifuged at 10,000 × *g* at 4 °C for 10 min to discard the yolk. The supernatant of oocyte homogenates and mouse tissue homogenates were centrifuged at 100,000 × *g* at 4 °C for 30 min. The membrane pellet was collected, and protein concentration was determined using the Micro BCA Protein Reagent Assay kit (Pierce). Twenty micrograms of protein were loaded and separated on a 10% SDS-polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane. The membrane was probed with a 1:200 dilution of affinity-purified preimmune and anti-mNAT2 antibodies. The primary antibody was detected using 1:1000 dilution of alkaline phosphatase-conjugated rabbit anti-chicken secondary antibody.

Immunofluorescence and Confocal Laser Microscopy—The immunofluorescence detection of mNAT2 was performed as described previously (23). Oocytes and retinal tissue samples were fixed in 2% paraformaldehyde for 2 h, incubated in 30% sucrose in phosphate buffered saline at 4 °C overnight, embedded in OCT, and then frozen in liquid nitrogen. Frozen sections (10-μm thickness) were fixed in acetone at –20 °C for 5 min, incubated with blocking solution (2% normal goat serum, 0.25% Triton X-100, 2% fish skin gelatin, and 1% bovine serum albumin in phosphate buffered saline) for 30 min and then with affinity-purified preimmune or anti-mNAT2 (1:100 dilution in blocking solution) at 4 °C overnight. The primary antibodies were detected by fluorescein (FITC)-conjugated goat anti-chicken Ig (1:500 dilution) for 2 h at

room temperature. Mouse retinal sections were double-labeled with propidium iodide (2 μM) and anti-mNAT2 antibody followed by FITC-conjugated goat anti-chicken secondary antibody. The specimens were analyzed using a confocal laser scanning microscope (model: Fluoview, Olympus). FITC fluorescence was excited at 488 nm by an argon laser and propidium iodide was excited at 543 nm with a HeNe-G laser. The emission filters used were: BA505–525 for FITC fluorescence, BA610 for propidium iodide, and BA660 for phase-contrast.

Expression of *mNAT2* in *Xenopus laevis* Oocytes—To prepare cRNA for oocyte injection, cDNAs were synthesized by PCR and subcloned between the 5'- and 3'-flanking sequences of the *Xenopus* β-globin gene of a *Xenopus* expression vector as described previously (24). The primer pairs used to amplify the entire ORF of mNAT2 were designed with a restriction site *EcoRI* at the 5'-end and *HindIII* at 3'-end; sense primer, 5'-ACGGAATCAAGATGATGCATTTCAAAGTGG-3'; antisense primer, 5'-ATCGAAGCTTCAGTGGCCTTCGTC-3'. PCR products were purified and digested with *EcoRI* and *HindIII* before subcloning into the *Xenopus* expression vector. The constructs were confirmed by sequencing. The plasmids were linearized with *NotI* and *in vitro* transcribed by T7 RNA polymerase using mMACHINE mMESSAGE. Capped cRNA was extracted with phenol/CHCl₃, precipitated with ethanol as described (24), resuspended in diethyl pyrocarbonate-treated H₂O at a concentration of 1.5–2.0 μg/μl and stored at –80 °C prior to use.

Transport Assays—Stage V–VI oocytes from *Xenopus laevis* were dissected and injected with 50 nl of the synthetic cRNA or diethyl pyrocarbonate-H₂O as a control. After 3 days of incubation at 18 °C, functional analyses were performed in groups of five to ten oocytes per assay as described previously (25–27). Oocytes were rinsed briefly in uptake buffer (KCl, 2 mM; MgCl₂, 1 mM; CaCl₂, 1 mM; HEPES, 10 mM; and Tris, 50 mM) in the presence of 100 mM NaCl (Na⁺ buffer), 100 mM choline chloride (choline⁺ buffer), or 100 mM LiCl (Li⁺ buffer). These oocytes were transferred into a 24-well culture dish containing 2 ml of uptake buffer and were incubated for 2–60 min at room temperature. Amino acid transport activities were measured by incubating oocytes in 0.5 ml of uptake buffer in the presence of 50 μM L-amino acids plus corresponding ³H-labeled L-amino acids as tracers for 15 or 30 min. The oocytes were washed four times in cold uptake buffer. These oocytes were lysed in 100 μl of 2% SDS, and the radioactivity accumulated by each oocyte was measured with a scintillation counter (Beckman) in 10 ml of scintillation solution.

The specificity of mNAT2-mediated amino acid uptake was examined using an amino acid competition assay. L-Glutamine uptake (50 μM) was measured in the presence of 5 mM 20 non-radioactive L-amino acids and MeAIB. The Na⁺ dependence and Li⁺ tolerance of L-glutamine transport by mNAT2 were investigated using Na⁺ buffer, choline⁺ buffer, or Li⁺ buffer. The effects of extracellular pH on L-glutamine uptake mediated by mNAT2 were investigated at pH 6.5–8.5 by adjusting the Na⁺ buffer with Tris-base or hydrochloric acid as described (26, 27). L-Glutamine efflux was measured as previously described (28). An average of nine injected oocytes was incubated for 30 min in Na⁺ buffer containing 50 μM L-[³H]glutamine. After washing in Na⁺ buffer containing 50 μM non-radioactive L-glutamine, oocytes were transferred to 300 μl of Na⁺ buffer or Na⁺ buffer containing 1 mM non-radioactive L-glutamine. At each designated time, 200-μl samples were taken to determine the levels of radioactivity and fresh 200 μl of Na⁺ buffer or Na⁺ buffer containing 1 mM non-radioactive L-glutamine were added to assume the volume size of the reaction. The readings from radioactivity measurement were adjusted to compensate for the dilution factors. All experiments were repeated at least three times, and the data collected are presented as S.E.

RESULTS

Molecular Cloning of *mNAT2* and Sequence Analysis—To identify member(s) of N-system transporters, we used PCR homology cloning (see “Experimental Procedures”). We screened cDNA from various mouse tissues using conserved primer pairs. One fragment of 0.5 kb was obtained from brain tissue. This fragment showed 77% nucleotide identity to *mNAT* (nucleotides 166–296), 78% to *g17* (nucleotides 166–258), and 78% to *SN1* (nucleotides 161–289), suggesting that this is a new mouse amino acid transporter. Further cloning using primers derived from this PCR fragment sequence generated a complete ORF of the new gene, which encodes 485-amino acid residues (GenBank[®] accession number AF184240). The alignment of amino acid sequence of mNAT2 with those of N-system

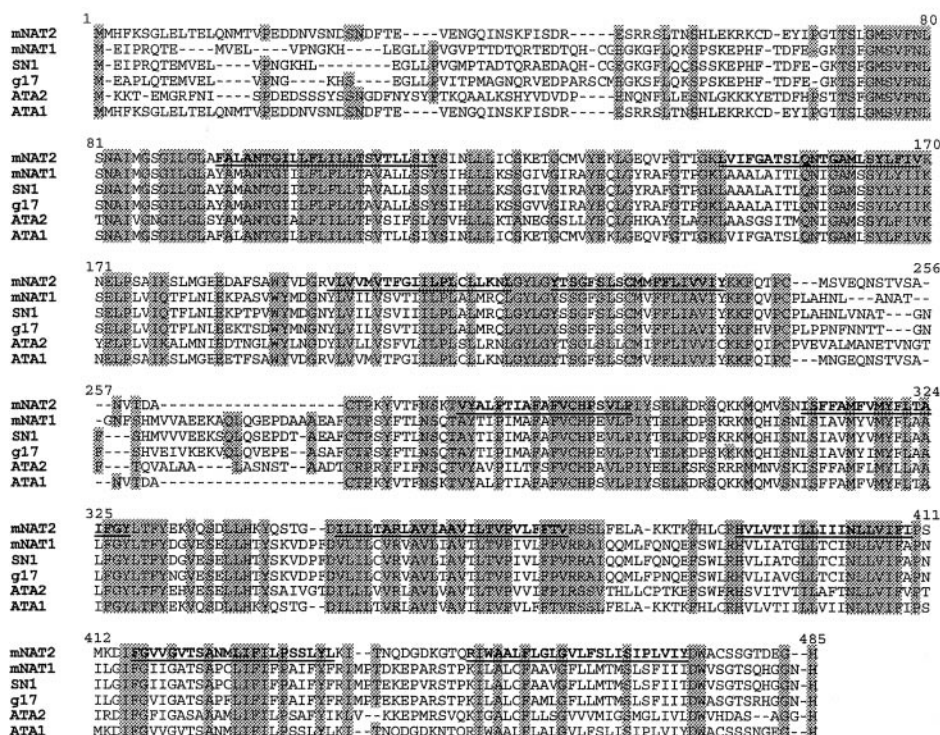


FIG. 1. Amino acid sequences alignment of the members of N- and A-system transporters. The deduced amino acid sequence of mNAT2 is aligned with N-system transporters, mNAT (AF159856), SN1 (AF273025), and g17 (U49082), and A-system transporters, GlnT/ATA1 (AF075704) and ATA2 (AF249673) (GenBank® accession numbers are shown in parentheses). Identical residues are shaded. Putative transmembrane domains of mNAT2 are marked in **boldface** and underlined. Gaps in the amino acid sequence are marked with dashed lines.

transporters, mNAT, SN1, and g17, and A-system transporters, GlnT/ATA1 and ATA2 is shown in Fig. 1. mNAT2 shares 52% homology with mNAT, SN1, and g17. The homologies between mNAT2 and A-system transporters, GlnT/ATA1 and ATA2 are 97 and 50%, respectively. The first seven transmembrane domains are highly conserved between N- and A-system transporters whereas N-terminal regions are highly diverse. Based on hydrophobicity analysis (PSORT version 6, Nakai file server; TMPRED, Swiss, EMBNET; and BCM Search Launcher), mNAT2 like mNAT is a plasma membrane protein (certainty = 87%), with a high probability of having 10 transmembrane domains (Fig. 2A). As the deduced sequence lacks a signal peptide, the long N- and the short C-terminal tails are assumed to be in the cytoplasm (high probability score) (Fig. 2B).

mNAT2 Is Predominately Expressed in Retina and Brain—To elucidate the tissue-specific expression of mNAT2, high stringency Northern blots of equivalent amounts of RNA isolated from various tissues were probed with a labeled mNAT2 DNA fragment. An abundance of ~8.5 kb was detected in mouse retina and brain. Trace levels of mNAT2 expression were detected in spleen, small intestine, and lung whereas there was no detected hybridization in lens, testis, muscle, liver, kidney, and heart (Fig. 3A). Based on the 3'- and 5'-RACE sequencing and Northern blot results, there was a 0.1-kb non-coding sequence at the 5'-end and a 7-kb non-coding sequence at the 3'-end in the extended transcript (8.5 kb).

Western blot analysis with an affinity-purified mNAT2-specific antibody revealed an immunoreactive protein band that was consistent with the predicted molecular mass of M_r 52 kDa_a on crude membrane fractions of brain (Fig. 3B, lane 2) and retina (lane 3). There was no detectable mNAT2 expression in testis (lane 4) and liver (lane 5). The specificity of this protein band was further ascertained by the lack of detected immunoreactivity of the retinal sample by probing with preimmune serum (lane 1). These results confirmed the observation from the Northern blot that mNAT2 was predominantly expressed in the retina and brain.

Differential Expression of mNAT2 in the Retina—Expression

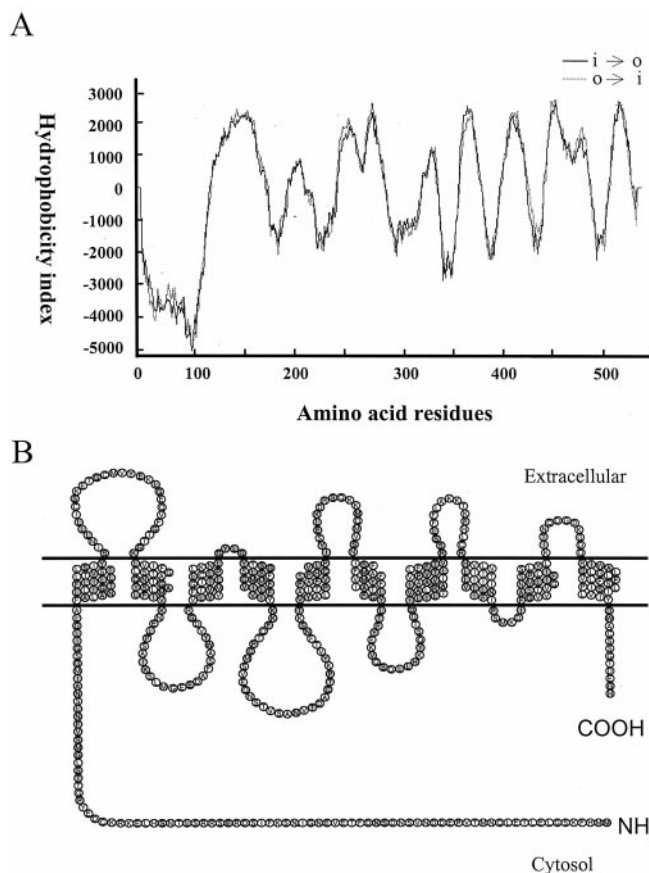


FIG. 2. Hydrophobicity and inferred topology of mNAT2. A, hydrophathy analysis of mNAT2. The two lines in the hydrophobicity plot represent the probability of the orientations: $i \rightarrow o$ represents the orientation from cytoplasmic side to extracellular side; $o \rightarrow i$ represents the opposite orientation. B, amino acid sequence and inferred structure of mNAT2. The most probable transmembrane topology of mNAT2 was deduced based on the high probability score (PSORT version 6). The amino acids conserved with mNAT are shaded.

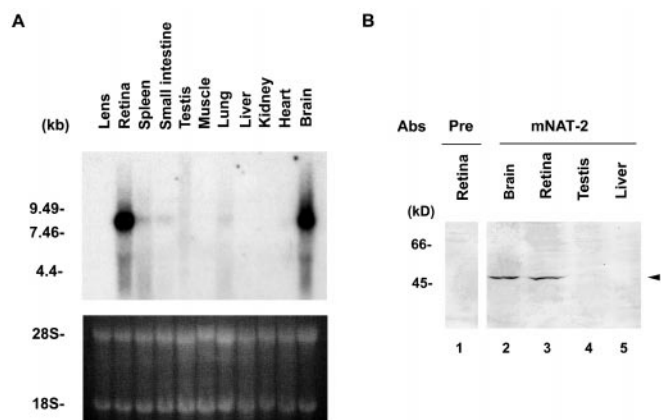


FIG. 3. mNAT2 mRNA and protein levels in mouse tissues. *A*, Northern blot analysis of mNAT2 expression in different mouse tissues. Thirty micrograms of total RNA from different mouse tissues were hybridized with a DNA probe of mNAT2 under high stringency conditions. Loading control of ribosomal RNA (28 S and 18 S) is shown stained with ethidium bromide. The positions of known size standards are shown on the left. *B*, immunoblot of crude membranes isolated from tissues as labeled with 1:200 dilution of affinity purified anti-mNAT2 antibody (lanes 2–5). mNAT2 expression is detected in mouse retina (lanes 3) and brain (lane 2), but not observed in testis (lane 4) and liver (lane 5). Control Western blot with preimmune serum on a membrane fraction of mouse retina (lane 1).

of mNAT2 was detected in the retina with the aid of immunofluorescence confocal microscopy. Based on the published histological illustrations of mouse retina (29), the defined layers of mouse retina in our preparation were visualized in the phase-contrast image (Fig. 4A) and in the corresponding image of cell nuclear staining (Fig. 4B). The immunofluorescence with specific antibody to mNAT2 demonstrates its predominant localization to the axons of ganglion cells in the layer of nerve fibers of the retina (Fig. 4C). Interestingly, the localization of mNAT2 expression is consistent with that of free glutamine, which has been previously shown to occur at the highest levels in ganglion cells and their axons (30, 31). To further confirm the expression of mNAT2 in the nerve fibers of ganglion cells, we examined the localization of mNAT2 in the optic papilla (optic disc) where ganglion cell axons converge to form the optic nerve (Fig. 5). mNAT2 was shown to be localized in the inner layer of the retina and in the optic nerve (Fig. 5, A and B, low magnification). The abundant expression of mNAT2 was clearly demonstrated in the strands of nerve fibers near the optic papilla and in the optic nerve bundles formed by axons of ganglion cells (Fig. 5, C and D, high magnification). No expression of mNAT2 was detected in the connective tissues surrounding the optic nerve (arrow). Together, our results from our functional analysis and expression pattern suggest that mNAT2 expressed at the axon fibers of ganglion cells is likely to participate in the uptake of glutamine in the glutamine-glutamate cycle (19).

mNAT2 Preferably Transports L-Glutamine, L-Histidine, and L-Asparagine—To determine the functional role of mNAT, we expressed mNAT2 in *Xenopus* oocytes, which is one of the model systems most often used to study the properties of transporters (5, 19, 24). Using an affinity-purified mNAT2 antibody, expression of mNAT2 was detected by immunofluorescence and immunoblot analysis as early as 24 h following cRNA injection. Three days after injection, mNAT2 protein was mainly localized in the oocyte plasma membrane, whereas positive signals were undetectable with an affinity-purified preimmune antibody (Fig. 6A). The anti-mNAT2 antibody did not detect positive signals in oocytes injected with water (data not shown). By Western blotting using mNAT2 antibody, a protein band, ≈ 52 kDa was detected in mNAT2 cRNA-injected oocytes (Fig. 6B,

lane 1), which was undetectable in water-injected control oocytes (lane 2). No immunoreactive bands could be detected in mNAT2-expressing oocytes using preimmune antibody (lane 3).

The uptake of ^3H -labeled L-alanine, L-glutamine, L-glutamate, L-histidine, and L-lysine, representing groups of zwitterionic, anionic, and cationic amino acids, was measured in *Xenopus* oocyte system. Compared with control water-injected oocytes, mNAT2-expressing oocytes exhibited uptake of L-glutamine most strongly, followed by L-histidine and L-alanine. A modest increase in L-lysine uptake was observed, whereas there was no increase in L-glutamate uptake (Fig. 7A). These results demonstrate that the most favorable substrate for mNAT2 is L-glutamine followed by L-histidine and L-alanine and suggest that mNAT2 has similar transporting substrate selectivity as the other N-system transporters, mNAT, SN1, and g17 (5–7).

L-Glutamine Transport by mNAT2 Is Not Competed by the A-system-specific Substrate, MeAIB—Because there are substrate overlaps among defined amino acid systems, especially between systems A and N, a competition assay in the presence of all 20 L-amino acids and the A-system-specific substrate, MeAIB (2), was performed (Fig. 7B). As the most optimal substrate for mNAT2, L-glutamine was selected as a model for the competition assay. In this assay, the rate of uptake of L-glutamine was determined in oocytes injected with mNAT2 cRNA in the presence of 5 mM non-radioactive-labeled 20 L-amino acids and MeAIB. L-Glutamine uptake was significantly inhibited by the following amino acids, listed in order of inhibitory potency: L-glutamine \geq L-histidine \geq L-asparagine \geq L-methionine \geq L-alanine \geq L-serine \geq L-cysteine. The A-system-specific substrate MeAIB was unable to significantly block the transport of glutamine, suggesting that mNAT2 is an N-system glutamine transporter and is not an A-system transporter.

mNAT2 Is a Na^+ - and pH-dependent Low-affinity Transporter and Facilitates Efflux of L-Glutamine Transport—To characterize the transport properties of mNAT2, we examined the Na^+ dependence and tolerance for Li^+ substitution of mNAT2. In the presence of Na^+ buffer, L-glutamine uptake into oocytes expressing mNAT2 was significantly greater (close to 90-fold) than the uptake exhibited by the control non-expressing oocytes. Choline ion substitution for Na^+ led to a significant decrease in L-glutamine uptake when compared with uptake in oocytes incubated in Na^+ buffer. Replacement of Na^+ by Li^+ partially restored the stimulatory effect by Na^+ (Fig. 8A). These results demonstrate that L-glutamine uptake mediated by mNAT2 is Na^+ -dependent and partially tolerates replacement of Na^+ by Li^+ , which is consistent with the properties exhibited by N-system transporters as previously described (5, 25, 32).

Extracellular pH in the range between 6.5 and 8.5 affected L-glutamine uptake mediated by mNAT2. The results showed that uptake of L-glutamine exhibited a pH dependence increasing from low to high pH (Fig. 8B). To analyze the effect of pH on the transport rate and substrate affinity of mNAT2, the concentration dependence of L-glutamine uptake mediated by mNAT2 at three pH conditions, pH 7, 7.5, and 8, was investigated (Fig. 8C). At the lower concentration, the rate of uptake was an incremental function of concentration, whereas at higher concentrations, uptake was close to saturation. This observation indicated that L-glutamine uptake in oocytes expressing mNAT2 behaved as a carrier-mediated transport. The kinetic constants were determined: At pH 7, $K_m = 2.40 \pm 0.7$ mM and $V_{\max} = 395 \pm 66$ pmol/min/oocyte; at pH 7.5, $K_m = 0.89 \pm 0.3$ mM and $V_{\max} = 430 \pm 92$ pmol/min/oocyte; at pH 8, $K_m = 0.54 \pm 0.06$ mM and $V_{\max} = 597 \pm 53$ pmol/min/oocyte ($n = 6$). The results demonstrated that pH altered both V_{\max}

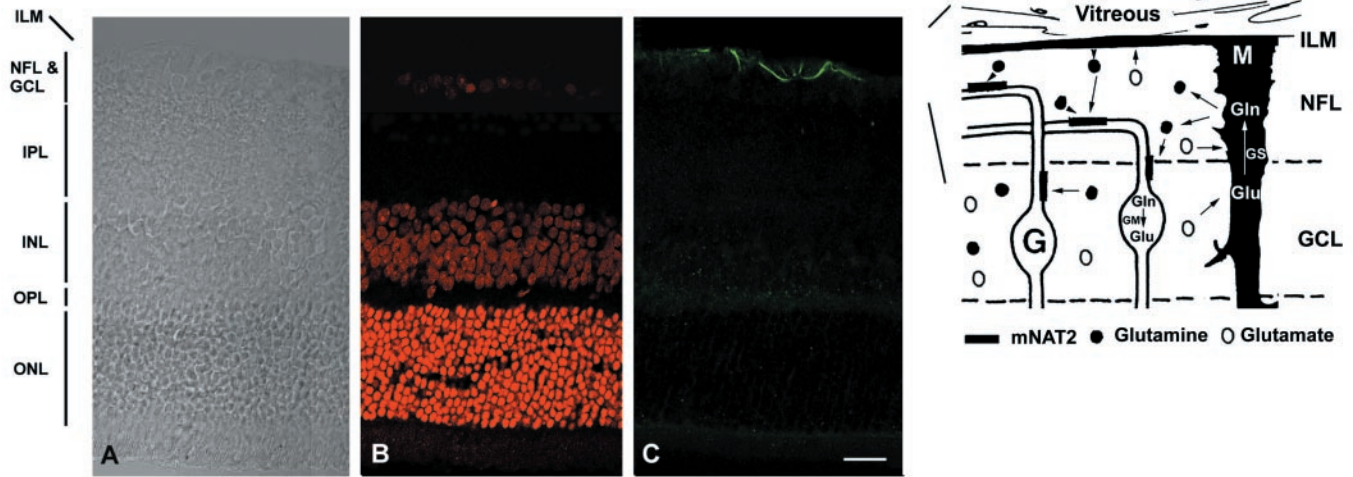


FIG. 4. **Immunofluorescence detection shows that mNAT2 is localized to the axons of ganglion cells in the nerve fiber layer of retina.** A frozen section of adult mouse retina is shown as follows: A, phase contrast; B and C, double labeling with propidium iodide and anti-mNAT2 antibody (1:200 dilution) followed by FITC-conjugated secondary antibody (1:500 dilution), respectively. The following retinal layers are indicated: ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; NFL, nerve fiber layer; and ILM, internal limiting membrane. Bar, 20 μm . The glutamate-glutamine cycling process in the ILM/NFL/GCL layers and the proposed involvement of mNAT2 are illustrated in the inset to the right. Glutamine that is synthesized by glutamine synthetase (GS) is released by Müller (M) cells (16, 17). Notice that free glutamine is most abundant in NFL/GCL layers as shown by other investigators (30, 31). mNAT2 mediates the transport of free glutamine into axons of ganglion (G) cells. In ganglion cells, glutamine is catalyzed by glutaminase (GM) to glutamate resulting in regeneration of this neurotransmitter (16, 17). Glutamate released by ganglion cells is taken up by Müller cells to maintain the glutamine-glutamate cycle.

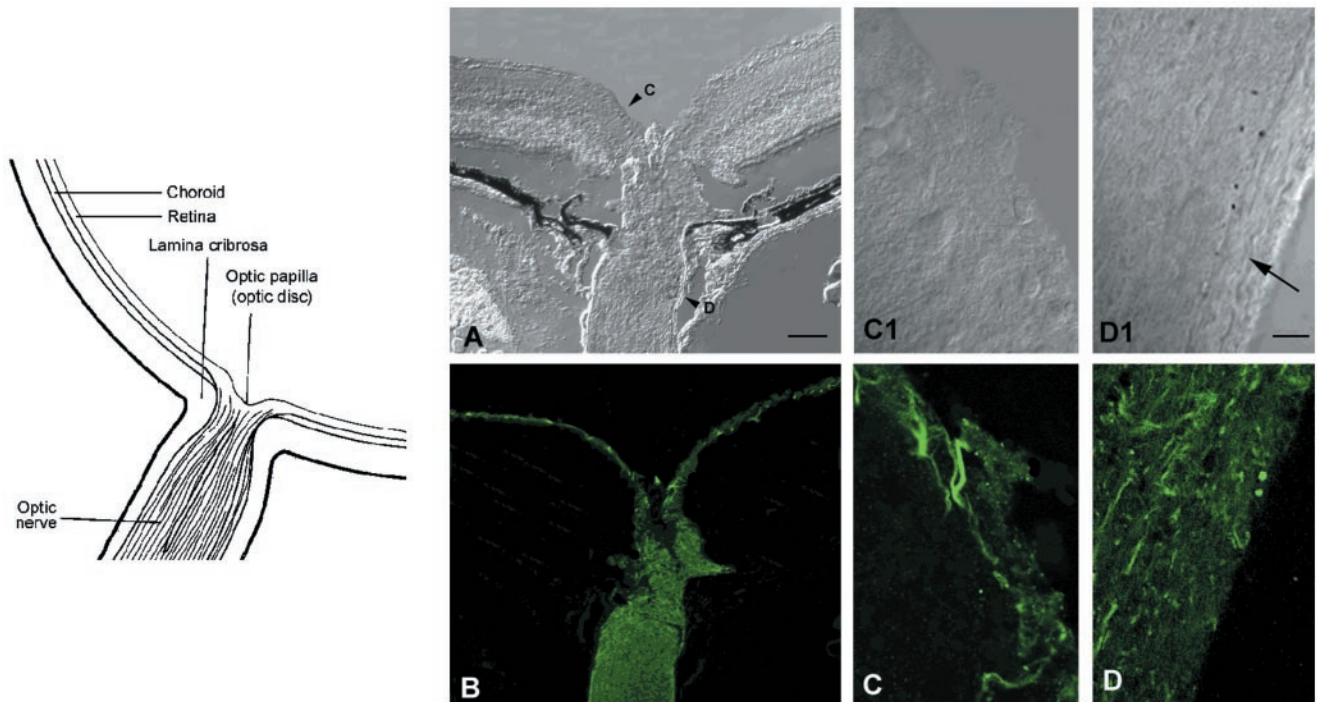


FIG. 5. **Expression of mNAT2 in the mouse optic nerve.** The diagram shown in the inset to the left illustrates the optic papilla region in the retina. A frozen section of adult mouse retina around this region was labeled with anti-mNAT2 antibody (1:200 dilution) followed by FITC-conjugated secondary antibody (1:500 dilution). The images shown are as the following: A (phase-contrast) and B (fluorescence) at low magnification. Bar, 100 μm . The magnified FITC fluorescence images of the portions of the tissue section indicated in A (arrowheads) are shown in C and D. C1 and D1 are the corresponding phase-contrast images of C and D. The connective tissue surrounding optic nerve is indicated in D1 (arrow). Bar, 10 μm .

and K_m of the transport mediated by mNAT2. With the decrease in pH, the substrate affinity (K_m) is decreased significantly, whereas the transport rate (V_{max}) is decreased to a lesser extent. Compared with mNAT (5), mNAT2 has a relatively higher substrate affinity (K_m) and transport capacity (V_{max}).

System N transport mediates efflux, because it can be *trans*-stimulated by its substrates (5, 33). We measured efflux of

L -[^3H]glutamine in mNAT2-expressing oocytes to determine whether the substrate affects efflux of L -glutamine. These oocytes were pre-labeled with L -[^3H]glutamine and incubated in Na^+ buffer in the presence or absence of non-radioactive L -glutamine. As expected, in the presence of external L -glutamine, oocytes expressing mNAT2 showed significantly increased efflux of L -[^3H]glutamine, particularly within the first 2-min time points, as compared with control oocytes incubated

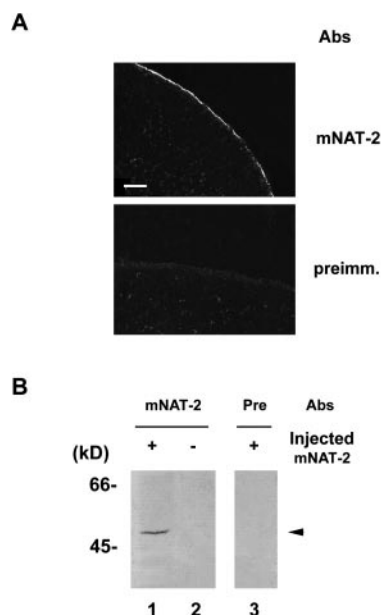


FIG. 6. **Expression of mNAT2 in *Xenopus* oocytes.** *A*, immunofluorescence of mNAT2. Frozen section of *X. laevis* oocytes 3 days after injection with mNAT2 cRNA was immunolabeled with an affinity-purified anti-mNAT2 antibody or preimmune serum and were examined by confocal microscopy at a scanning interval section of 0.5 μm (Bar, 30 μm). *B*, Western blot analysis shows the expression of mNAT2 (arrowhead) in crude membranes from oocytes injected with mNAT2 cRNA (lanes 1 and 2) and water (lane 3) using anti-mNAT2 antibody (lanes 1 and 2) or preimmune antibody (lane 3).

in the absence of external L-glutamine (Fig. 8D). These results suggest that mNAT2, like N-system transporters, can function as a mediator for the efflux of substrates.

DISCUSSION

In this study, we have characterized the second member of the N-system amino acid transport family, mNAT2 expressed in axons of retina ganglion cells. Sequence and functional analyses show that mouse mNAT2 is related to the N-system transporter, mouse mNAT and its orthologs, rat SN1 and human g17 (5–7). The degree of amino acid homology between mNAT2 and mNAT is 52%. The greater degree of divergence between mNAT and mNAT2 is in the N terminus of the transporter. In contrast to mNAT, which has nine transmembrane domains, the predicted topology of that mNAT2 suggests that it has 10 transmembrane segments, placing the N and C termini in the cytoplasm.

The substrate selectivity of mNAT2 conforms to that of the previously characterized N-system transport (32), which prefers substrates with nitrogen on their side chains such as L-glutamine, L-histidine, and L-asparagine. The previously identified first members of the N-system transporter, mNAT and SN1, indeed possess this transport selectivity (5, 6). However, it is not sufficient to classify the of amino acid transporter systems solely on the basis of substrate selectivity. For a given amino acid, multiple transport systems might be involved in its transport across cell membranes. Although L-glutamine is a preferred substrate for the N-system, other systems such as A and ASC can also mediate Na^+ -dependent L-glutamine uptake (34). Therefore, to generate a clear classification, other criteria are required. Both systems N and A are characterized by their pH dependence (5, 6, 32, 35). However, system ASC-mediated transport is pH-insensitive (36). Because we characterize mNAT2 as a pH-dependent transporter, it thus does not belong to the ASC system. Although systems A and N share some substrate overlaps, their preferred substrates are different.

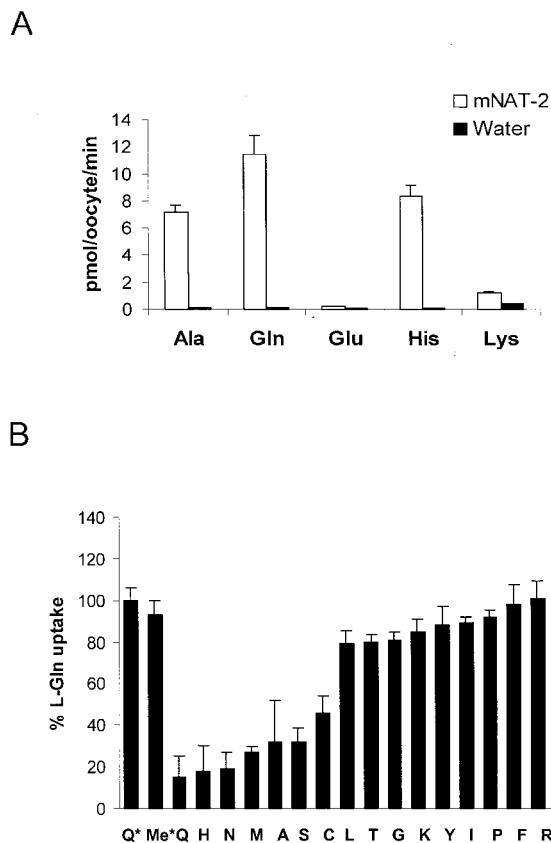
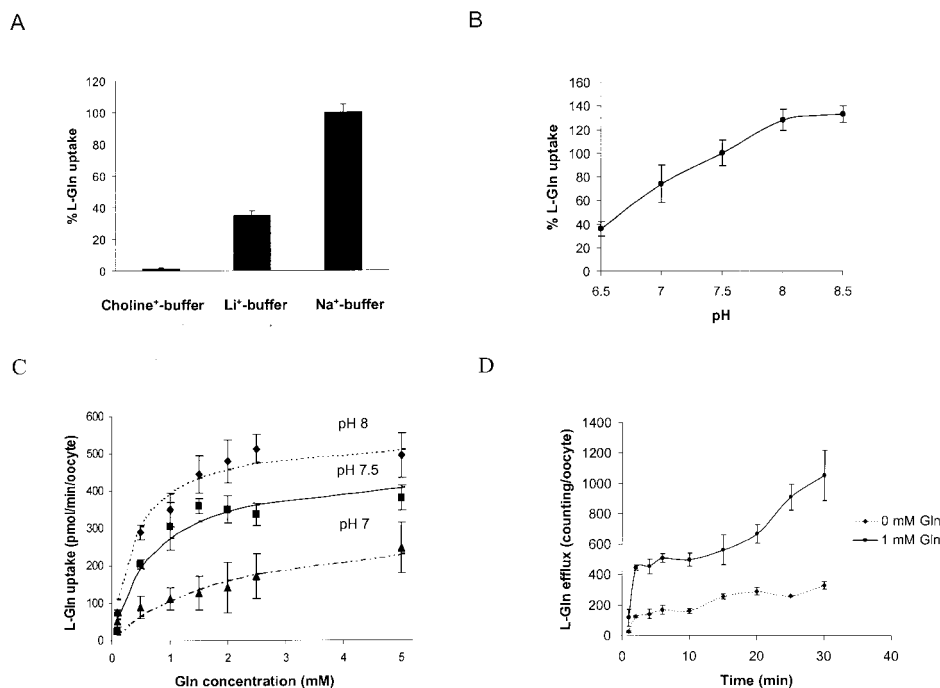


FIG. 7. **Amino acid uptake and competition analyses.** *A*, amino acid uptake rates in mNAT2-expressing oocytes against control, non-expressing oocytes. L-Alanine, L-glutamine, L-glutamate, L-histidine, and L-lysine uptake was measured in uptake buffer containing 50 μM ^3H -labeled amino acid and incubated for 30 min ($n = 7$). *B*, competitive inhibition by 20 L-amino acids and MeAIB on the uptake of 50 μM ^3H glutamine in Na^+ uptake buffer ($n = 6$). The percentage values after subtracting controls injected with water are presented as 50 μM L-glutamine uptake, defined as 100%. *Q, L-glutamine uptake in the absence of non-labeled amino acids; *Me, MeAIB. All data are presented as mean \pm S.E.

System N is characterized by its high selectivity for L-glutamine and L-histidine uptake (5, 6, 32, 35). System A, however preferentially transports short, straight-chain amino acids such as L-alanine, L-glycine, and L-cysteine (37, 38). Another method to distinguish between these two systems is by the amino acid and its related substrate transport competition assay. It has been generally accepted that inhibition by MeAIB and L-histidine can be used to define systems A and N, respectively (2). Therefore, based on the unique features of these two systems, we define the mNAT2 to be a system N instead of A transporter. First, mNAT2 has a strong transport activity for L-glutamine and L-histidine, the preferred substrates for N-system. Second, the activity of L-glutamine transport mediated by mNAT2 can be effectively inhibited by N-system substrates such as L-histidine and L-asparagine but not by MeAIB. Third, uptake of glutamine by mNAT2 can be partially supported by Li^+ . In contrast, system A is not tolerant for Li^+ substitution for Na^+ (5, 6, 27, 32). Finally, mNAT2 mediates efflux of substrates, a distinct feature of system N that differentiates it from system A (33). Taken together, the transport properties suggest mNAT2 belongs to the N-system family of transporters.

mNAT2 also shares sequence homology with the recently identified A-system transporters, rat GlnT/ATA1 (18), rat ATA2 (39), rat SA1 (40), and SAT2 (41). In fact, ATA2, SA1, and SA2 are the same gene. mNAT2 has a very high degree of

FIG. 8. Na⁺, pH, and concentration dependence and efflux analyses of mNAT2. *A*, effect of Na⁺ on the uptake of L-glutamine mediated by mNAT2. Uptake of 50 μM L-[³H]glutamine was measured using choline⁺ buffer or Li⁺ buffer to replace Na⁺ buffer (*n* = 5). *B*, pH dependence of L-glutamine uptake. Uptake of 50 μM L-[³H]glutamine was measured with Na⁺ buffer under the indicated pH (*n* = 5). *C*, concentration dependence of L-glutamine uptake at pH 7, 7.5, and 8. Net uptakes of L-glutamine were measured at different concentrations (0–5 mM) of L-glutamine in Na⁺ buffer at pH 7, 7.5, and 8. *D*, L-glutamine efflux was examined in Na⁺ buffer and in Na⁺ buffer containing 1 mM non-radioactive labeled L-glutamine (*n* = 3). All data are presented as mean ± S.E.



sequence identity to GlnT/ATA1, suggesting that mNAT2 is likely the mouse ortholog of rat GlnT/ATA1. Interestingly, mNAT has a higher sequence identity to N-system transporters than to A-system transporters (52% versus 50%), suggesting that mNAT2 is more closely related to system N transporters. Similar to mNAT2 expression in the retinal ganglion cells, GlnT/ATA1 is expressed only on neurons and is absent from astrocytes (18). In contrast to mNAT2 as an N-system transporter, GlnT/ATA1 is reported to be a member of A-system transporter family. In the reported substrate competition assay of GlnT/ATA1, uptake of L-glutamine is dramatically inhibited by L-histidine, but only weakly inhibited by the A-system-specific substrate, MeAIB (18). This result suggests that, in contrast to the previous interpretation (18), this functional feature of GlnT/ATA1 matches N-system instead of the A-system transport.

We have identified the predominant expression of mNAT2 in the retina and brain. In the retina, mNAT2 is predominantly localized in the nerve fibers of ganglion cells. As illustrated in the insets in Figs. 4 and 5, the nerve fiber layer is composed almost entirely of the axonal projections of the ganglion cells. These axons are aggregated into nerve fiber bundles that pass through the arcades formed by the columns and footplates of Müller cells (42) and converge to form the optic nerve. The internal limiting membrane layer located adjacent to ganglion axon fibers consists mostly of the basal lamina of Müller glial cells. Müller cells are known to uptake glutamate, and interestingly, the glutamate transporter L-glutamate/L-aspartate transporter has been identified in these cells (43). There, glutamate is converted into glutamine by glutamine synthetase and the newly synthesized glutamine is released (16, 17). Two glutamine transporters, SN1 and ASCT2 are expressed in astroglial cells and play a role in the export of glutamine from these cells (6, 15). Thus, it is likely that either or both of these two transporters mediate the export of glutamine in Müller glial cells. The released glutamine by Müller cells is then transported to glutamatergic ganglion cells where glutamine is converted to glutamate via glutaminase (16, 17). Hence, glutamine serves as a precursor for the generation of glutamate in these cells (19, 20). In the previously reported studies using a glutamine immunoreactivity assay, the presence of free glutamine

was most abundantly identified in glutamatergic ganglion cells and their axons in comparison to all other retinal cells (30, 31). Remarkably, the localization pattern of glutamine in the retina matches that of mNAT2 expression. Therefore, based on specific expression pattern and transport function, our results suggest that mNAT2 transports free glutamine into ganglion cells for generation of the glutamate used in neuronal transmission. Further studies will shed light on the function and regulation of mNAT2 in retinal physiology and pathology.

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