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# Expression and Function of System N Glutamine Transporters (SN1/SN2 or SNAT3/SNAT5) in Retinal Ganglion Cells

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## Abstract

**Purpose—**Glutamine transport is essential for the glutamate-glutamine cycle, which occurs between neurons and glia. System N, consisting of SN1 (SNAT3) and SN2 (SNAT5), is the principal mediator of glutamine transport in retinal Müller cells. Mediators of glutamine transport in retinal ganglion cells were investigated.

**Methods—**The relative contributions of various transport systems for glutamine uptake (systems N, A, L, y+L, ASCT, and $ATB^{0,+}$) were examined in RGC-5 cells based on differential features of the individual transport systems. mRNA for the genes encoding members of these transport systems were analyzed by RT-PCR. Based on these data, SN1 and SN2 were analyzed in mouse retina, RGC-5 cells, and primary mouse ganglion cells (GCs) by in situ hybridization (ISH), immunofluorescence (IF), and Western blotting.

**Results—**Three transport systems—N, A, and L—participated in glutamine uptake in RGC-5 cells. System N was the principal contributor; systems A and L contributed considerably less. ISH and IF revealed SN1 and SN2 expression in the ganglion, inner nuclear, and photoreceptor cell layers. SN1 and SN2 colocalized with the ganglion cell marker Thy 1.2 and with the Müller cell marker vimentin, confirming their presence in both retinal cell types. SN1 and SN2 proteins were detected in primary mouse GCs.

**Conclusions—**These findings suggest that in addition to its role in glutamine uptake in retinal glial cells, system N contributes significantly to glutamine uptake in ganglion cells and, hence, contributes to the retinal glutamate-glutamine cycle.

Glutamine plays an essential role in recycling the excitatory neurotransmitter glutamate. In retina, glutamate is the major neurotransmitter for the photoreceptor-bipolar-ganglion cell circuitry. Once released at the synapse, glutamate must be cleared from the extracellular milieu to terminate neurotransmission.[1] Glutamate clearance is mediated largely by excitatory amino acid transporter 1 (EAAT1 [GLAST]), a glutamate transporter found in retinal Müller glial cells.[2] Within Müller cells, glutamate is converted to glutamine by glutamine synthetase, which is then released and subsequently taken up by ganglion cells, where it is hydrolyzed by glutaminase to form glutamate, thus completing the glutamate-glutamine cycle.[3]

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Extensive investigations have been conducted into the mechanisms used by retina to transport glutamate (for a review, see Pow[2]), but relatively few have been conducted into the transport of glutamine. In other tissues, several transporters of glutamine have been identified at the molecular level.[4–6] Of these the A-, N-, and L-systems are most important. Systems A and N are sodium-coupled neutral amino acid transporters (SNAT) of the *SLC38* gene family. ATA1 (SNAT1) and ATA2 (SNAT2) are two isoforms of system A.[7–10] ATA1 and ATA2 function under physiologic conditions as influx transporters, and glutamine is an excellent substrate for ATA1/ATA2.[5] SN1 (SNAT3) and SN2 (SNAT5) are two isoforms of system N.[11–14] They are coupled to $Na^+$ and $H^+$ gradients and mediate a transport process in which $Na^+$ and glutamine move in one direction and $H^+$ moves in the opposite direction. The direction of glutamine flux through SN1/SN2 can be altered, even under normal physiologic conditions, such that they mediate the influx or efflux of glutamine into and out of cells. ATA1/ATA2 can be differentiated from SN1/SN2 based on their ability to transport 2-(methylamino) isobutyric acid (MeAIB), a highly specific substrate for system A but not for system N.[15] System L, a member of the *SLC7* gene family, is a $Na^+$-independent transport system for glutamine and other neutral amino acids. It functions as an amino acid exchanger.[6] Members of this family include LAT1/4F2hc and LAT2/4F2hc.[4,6] These transporters are heterodimeric and consist of a light chain (LAT1 or LAT2) and the glycoprotein heavy chain 4F2hc. Other transport systems for glutamine include $y^+L$, ASCT, and $ATB^{0,+}$. Members of the $y^+L$ system include y+LAT1 (SLC7A7) and y+LAT2 (SLC7A6). They associate with 4F2hc to mediate $Na^+$-independent transport of cationic amino acids and $Na^+$-dependent uptake of neutral amino acids.[16] Transport by family members of this system can be inhibited competitively by arginine and lysine. The ASCT1 (SLC1A4 [SATT]) and ASCT2 (SLC1A5 [$ATB^0$]) transport systems are $Na^+$-dependent and have high affinity for alanine, serine, and cysteine. They exhibit distinct substrate selectivity. In addition to the common substrates of ASCT transporters, ASCT2 also accepts glutamine and asparagine as high-affinity substrates, though ASCT1 does not.[17] ASCT1 and ASCT2 are obligatory exchangers for amino acids and cannot function in a unidirectional manner. The unique amino acid transporter $ATB^{0,+}$ (SLCA14) is energized by $Na^+$ and $Cl^-$ gradients and membrane potential. It has broad substrate specificity and concentrative ability; it recognizes neutral as well as cationic amino acids, and its function is inhibited by arginine and lysine.[18]

In the brain, a number of studies of the molecular basis of glutamine transfer between astrocytes and neurons suggest a dichotomy of function such that astroglial cells use system N to release glutamine, whereas neurons use system A to take up glutamine.[19–21] Interestingly, this dichotomy has been called into question with recent reports suggesting that system A (ATA1/ATA2) may not contribute significantly to the influx of glutamine in cerebral cortical neurons because axons of these neurons were not immunoreactive for the antibodies against these transporter proteins.[22,23]

Regarding analysis of glutamine transport in the retina, Saenz et al.[24] used crude synaptosomal fractions from hamster retina and analyzed glutamine uptake and release. Their data showed that systems L, A, and N contributed to this process. The cellular distribution of these systems within retina, however, was not investigated; thus, it could not be determined which retinal neurons used which of these transporters for glutamine uptake. Boulland et al.[25] localized SN1 immunohistochemically in rat retina to radial fibers, consistent with localization to Müller cells. Recently, we analyzed glutamine transport in retinal Müller glial cells using a rat Müller cell line (rMC-1) and primary cultures of mouse Müller cells.[26] Our functional and molecular data showed that the family members of systems A, N, and L are all expressed in Müller cells and that system N is the principal mediator of glutamine transport in these cells. Because system N can function in the efflux mode, this transport system appears to be the major contributor to the release of glutamine from Müller cells. Our findings are consistent with the reports in brain that system N is the primary glutamine transporter for glial cells.

Interestingly, in 2001, a report was published that claimed that system N was expressed in retinal ganglion cells (RGCs).[27] The investigators had previously cloned the system N family member SN1 in liver. Subsequently, they analyzed retina and reported characterization of the N-system in ganglion cells (which they termed mNAT2). Later analysis of the gene sequence of mNAT2 revealed, however, that it was identical with the gene sequence for ATA1 (a member of the system A, not system N, family). Reviews of the SNAT transport systems now categorize mNAT2 as a system A family member.[5] To our knowledge, no further investigations have been conducted of the mechanisms used by ganglion cells to acquire glutamine. Given the availability of the ganglion cell line RGC-5[28] and the optimization in our laboratory of the isolation of primary ganglion cells cultured from mouse retinas,[29–31] we sought to investigate comprehensively the transporters used by RGCs to take up glutamine. We found that multiple transport systems for glutamine are expressed in these cells but that system N (SN1 and SN2) functions as the major contributor to glutamine uptake and that systems A and L play a less significant role.

## Materials and Methods

### Animals and Cell Culture

Mice (C57Bl/6) purchased from Harlan-Sprague-Dawley (Indianapolis, IN) were maintained in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. In situ hybridization and immunolocalization studies were performed in the tissue of 3-week-old mice. For primary cell culture, ganglion cells were isolated from retinas of C57BL/6 mice approximately 1 day to 3 days old; they were verified for purity and maintained in culture according to our published methods.[29–31] The ganglion cell line RGC-5[28] was the kind gift of Neeraj Agarwal (University of North Texas Health Sciences Center, Fort Worth, TX). RGC-5 cells were maintained at 37°C in a humidified chamber of 95% $O_2$/5% $CO_2$. They were cultured in 75-$cm^2$ flasks in DMEM/F12 supplemented with 10% FBS, 100 U/mL penicillin, and 100 $\mu$g/mL streptomycin. Culture medium was replaced with fresh medium every other day.

### RT-PCR Analysis of Expression of mRNA Transcripts

The presence of specific mRNA transcripts for the amino acid transporters SN1, SN2, ATA1, ATA2, LAT1, LAT2, $y^+$LAT1, $y^+$LAT2, ASCT1, ASCT2, and $ATB^{0,+}$ in RGC-5 cells and primary mouse ganglion cells was evaluated by RT-PCR. Rat and mouse PCR primers were designed based on the published sequences (Table 1). Total RNA was prepared from RGC-5 or primary ganglion cells (TRIzol; Invitrogen-Gibco, Carlsbad, CA). For RT-PCR analysis, 1 $\mu$g total RNA was reverse transcribed using an RNA-PCR kit (Gene-Amp; Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. PCR was performed using 0.4 $\mu$mol each of sense and antisense primers, 2.5 U of buffer (Ampli-Taq DNA polymerase; Applied Biosystems), and the following cycling conditions: 94°C for 3 minutes; 35 cycles of 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute; 1 cycle of 72°C for 10 minutes. The products were size fractionated on an agarose gel and stained by ethidium bromide.

### Uptake of Glutamine in RGC-5 Cells

Uptake of glutamine in RGC-5 cells was measured as described.[26] Culture medium was removed by aspiration, and cells were washed once with uptake buffer (140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, and 25 mM HEPES [pH 8]) and preincubated in 0.25 mL of the same buffer for 10 minutes at 37°C. The medium was replaced with 0.25 mL uptake buffer containing [$^3$H]glutamine (0.5 $\mu$Ci/assay; final concentration of radiolabeled plus unlabeled glutamine during uptake, 5 $\mu$M; GE Healthcare, Amersham, UK), and incubation continued for 15 minutes at 37°C. Uptake was terminated by removal of the

uptake buffer and washing twice with ice-cold uptake buffer. Cells were lysed with 0.5 mL of 1% SDS in 0.2 N NaOH; the lysate was used for measurement of radioactivity by liquid scintillation spectrometry.

### Determination of Relative Contributions of Specific Transport Systems to Glutamine Uptake

The relative contributions of transport systems for glutamine can be determined because of differential substrate selectivity and $Na^+$ dependence of the systems. Systems A and N are $Na^+$ coupled, whereas system L is $Na^+$ independent. Glutamine uptake measured under $Na^+$-free conditions represents uptake through system L and any glutamine entry into cells through diffusion. Glutamine uptake that is $Na^+$ dependent (i.e., uptake in $Na^+$-containing buffer minus uptake in $Na^+$-free buffer) represents the combined uptake through systems A and N. MeAIB is a specific substrate for system A[15]; this model amino acid does not interact with system N. $Na^+$-dependent glutamine uptake that is inhibitable by MeAIB represents the system A–mediated uptake component, whereas the $Na^+$-dependent glutamine uptake that is insensitive to MeAIB represents the system N-mediated uptake component. We used this strategy to determine the relative contributions of systems N, A, and L to glutamine uptake in ganglion cells. Tryptophan is a selective substrate for system L. Glutamine uptake in the presence of 2 mM tryptophan (to inhibit system L contribution) and 5 mM MeAIB (to inhibit system A contribution) in a $Na^+$-containing medium represents system N activity. The glutamine transport systems y+L and $ATB^{0,+}$ can be distinguished from other systems because their function is inhibited by arginine and lysine.

### Kinetic Analyses

Saturation kinetics of glutamine uptake through system N was analyzed by measuring the system N–specific uptake in the presence of increasing concentrations of glutamine. Data were fit to the Michaelis-Menten equation to calculate the kinetic parameters (Michaelis constant, $K_m$ and maximum velocity, $V_{max}$). $Na^+$-activation kinetics of glutamine uptake through system N was analyzed by measuring the system N-specific uptake in the presence of increasing concentrations of $Na^+$. The concentration of $Na^+$ was varied in the uptake buffer by substituting NaCl iso-osmotically with *N*-methyl-D-glucamine (NMDG) chloride. $Na^+$-dependent uptake data were analyzed by the Hill equation to determine the Hill coefficient (*h*, the number of $Na^+$ ions involved in the activation process). Relative affinities of various amino acids for system N were evaluated by competition studies in which system N–specific uptake of [$^3$H] glutamine (5 $\mu$M) was measured in the presence of increasing concentrations of amino acids. The $IC_{50}$ (i.e., concentration of the competitor necessary to cause 50% inhibition of glutamine uptake) was then calculated from the dose-response curves. Because the concentration of glutamine used in these studies was less than 5% of the $K_m$ for the uptake process, the $IC_{50}$ was taken as a close approximation of $K_m$ for the respective amino acids.

### Data Analysis

Uptake experiments were performed in 24-well plates, permitting each condition to be performed in duplicate or triplicate. Each experiment was repeated two to four times. Data analysis (analysis of variance) was performed using the SPSS statistical software package ($P < 0.05$ was considered significant). For data in which variances were equivalent, the least significant difference post hoc test was used; for tests in which they were not equivalent, the Dunnett test was used. Kinetic analysis was performed (Figure P, version 6.0; Biosoft, Cambridge, UK). Data are presented as mean ± SE.

### In Situ Hybridization Analysis of SN1 and SN2

Cryosections of mouse eyes were prepared as described.[30] Sense and antisense probes were prepared using RT-PCR products specific for SN1 and SN2, which were subcloned into the

vector (pGEM-T Easy; Promega, Madison, WI), sequenced, and verified. After linearizing the plasmid with suitable restriction enzymes, the digoxigenin-labeled probes were prepared by in vitro transcription with appropriate RNA polymerases using an RNA-labeling kit (DIG; Roche Applied Science, Indianapolis, IN). Retinal cryosections were subjected to in situ hybridization using these probes according to our method.[30] Cryosections hybridized with the sense riboprobe served as the negative control.

### Preparation of SN1 and SN2 Antibodies

Polyclonal antibodies against SN1 and SN2 were generated in rabbit using the services of a commercial firm (Proteintech Group, Inc., Chicago, IL). The antigenic peptides were ELKKDPSKRKMQHIS (amino acid residues 309–322 in mouse SN1) and LLFPSKAFSWPRHVA (amino acid residues 363–377 in mouse SN2). Peptide sequences were selected such that there was no significant homology between the two peptides or with other members of the *SLC38* gene family. To determine the specificity of the antibodies, cloned mouse SN1 and mouse SN2 were expressed heterologously in HRPE cells (a human retinal pigment epithelial cell line), and immunofluorescence studies were performed with these cells in parallel with vector-transfected cells. The anti–SN1 and anti–SN2 antibodies yielded positive immunofluorescence signals only in cells transfected with SN1 cDNA and SN2 cDNA, respectively, whereas cells transfected with vector alone showed no positive immunofluorescence (data not shown).

### Immunodetection of SN1 and SN2 in Intact Retinal Tissue and in Cultured Cells

Eyes were harvested from 3-week-old mice and immediately flash frozen (without fixation) in OCT embedding compound, and cryosections were prepared as described.[30] Retinal cryosections were fixed at 4°C for 10 minutes in 4% paraformaldehyde/PBS, washed three times in PBS for 5 minutes, and blocked (PowerBlock; Biogenex, San Ramon, CA) for 1 hour. Sections were incubated in a humidified chamber at 4°C overnight with a rabbit polyclonal antibody against SN1 or SN2 (dilutions 1:100 for both antibodies). Rat anti-mouse monoclonal antibody against Thy 1.2 (1:250; BD Biosciences, San Jose, CA), a marker for RGCs,[32] and mouse anti-human monoclonal antibody against vimentin (1:250; Chemicon, Temecula, CA), a marker for retinal Müller cells,[33] were used in dual-labeling experiments to determine whether SN1 or SN2 colocalized with these cell markers. As negative controls, some sections were incubated with primary antibodies that had been neutralized with antigenic peptides against which the antibodies were originally generated. As a positive control, liver, which is known to express SN1 and SN2,[5,13,14] was isolated from mice and prepared for cryosectioning and immunofluorescence detection. For detection of immunopositive signals, retinal and liver sections were incubated with Alexa Fluor 555–conjugated donkey anti–rabbit IgG or Alexa Fluor 488–conjugated donkey anti–mouse IgG (Invitrogen, Carlsbad, CA) secondary antibodies (1:1500). Slides were washed in PBS, incubated with DAPI, coverslipped, and viewed by epifluorescence using a microscope (Axioplan-2; Carl Zeiss, Oberkochen, Germany) equipped with a specialized program (Axiovision; Carl Zeiss) and an HRM camera. To detect SN1 and SN2 in cultured RGC-5 and primary mouse ganglion cells, the cells were cultured on coverslips, air dried for 5 minutes, fixed in 4% paraformaldehyde in PBS for 5 minutes, washed three times for 5 minutes with PBS, and incubated for 5 minutes in 0.1% triton X-100. Cells were blocked with 4% donkey serum plus block (PowerBlock; BioGenex) and incubated with the antibodies against SN1 and SN2 followed by secondary antibodies, as described for the tissue sections. Cells were analyzed by epifluorescence. As negative controls to determine nonspecific binding, the SN1 and SN2 primary antibodies were incubated with the appropriate antigenic peptide and were used in immunohistochemistry experiments.

### Western Blot Analysis of SN1 and SN2 in Ganglion Cells

Western blot analysis was performed to detect SN1 and SN2 in RGC-5 cells and primary cultures of mouse ganglion cells. Proteins were isolated and subjected to SDS-PAGE according to our method.[29–31] Prestained standards (SeeBlue Plus2; Invitrogen) were loaded onto adjacent lanes of the gel. Protein concentrations were estimated according to the Lowry method.[34] Nitrocellulose membranes (Hybond-ECL; Amersham Biosciences, Piscataway, NJ), to which the separated proteins had been transferred, were incubated with SN1 and SN2 antibodies (1:250). They were then probed with a secondary horseradish peroxidase–conjugated goat anti–rabbit IgG antibody (1:3000; Santa Cruz Biotechnology, Santa Cruz, CA), and proteins were visualized using a detection system (SuperSignal West Pico Chemiluminescent Substrate; Pierce Biotechnology, Rockford, IL). SN1 and SN2 have both been detected in liver[6,13,14] and in retinal Müller cells.[26] Thus, as a positive control, protein was harvested from liver cells and Müller cells and was used in companion immunoblotting experiments. To confirm the specificity of the antibody, the primary SN1 and SN2 antibodies were preadsorbed with the antigenic peptide and then used for Western blotting.

## RESULTS

### Gene Expression Analysis

Specific mRNA transcripts for the amino acid transporters SN1, SN2, ATA1, ATA2, LAT1, LAT2, $y^+$LAT1, $y^+$LAT2, ASCT1, ASCT2, and $ATB^{0,+}$ were analyzed in RGC-5 cells by RT-PCR using the rat primers listed in Table 1. Similar studies were performed in primary ganglion cells harvested from mouse retina. The mouse primers for these transporters are provided in Table 1. Of the amino acid transporters analyzed, all except $ATB^{0,+}$ were expressed in RGC-5 and primary ganglion cells (Fig. 1).

### Identity of Transport Systems Contributing to Glutamine Uptake in RGC-5 Cells: Functional Studies

Uptake of glutamine was studied in RGC-5 cells in the presence and absence of $Na^+$ to determine the contributions of $Na^+$-dependent compared with $Na^+$-independent transport processes. Glutamine uptake in these cells was predominantly $Na^+$ dependent (Fig. 2A). The contribution by a $Na^+$-independent process was less than 20% of the total uptake. This minor component consisted of a noncarrier-mediated diffusional process and system L. Uptake through system L was sensitive to inhibition by excess tryptophan. The $Na^+$-independent uptake of glutamine was inhibited almost 80% by 2 mM tryptophan, suggesting that glutamine uptake in RGC-5 cells in the absence of $Na^+$ occurred partly through diffusion (approximately 20%) and mostly through system L (approximately 80%). The contribution of system A to glutamine uptake was then evaluated by the ability of MeAIB to inhibit the uptake process. As expected, glutamine uptake in the absence of $Na^+$ was not inhibited by MeAIB (Fig. 2B) because MeAIB is a specific substrate only for the $Na^+$-dependent system A. In the presence of $Na^+$, glutamine uptake was inhibited by MeAIB to some extent; the maximal inhibition at excess MeAIB was approximately 20%. Multiple measurements with several independent cell cultures led to the conclusion that system L contributed approximately 10% and system A contributed approximately 20% to the total uptake of glutamine in RGC-5 cells when measured in the presence of $Na^+$. The $Na^+$-dependent and MeAIB-insensitive transport process was the principal contributor to glutamine uptake in these cells, constituting approximately 70% of total uptake. $Na^+$ dependence and insensitivity to MeAIB are the defining characteristics of system N. These data thus provide the first functional evidence for the expression of system N in this neuronal cell line.

We then investigated the substrate specificity of system N in these cells. Uptake of glutamine, which occurred specifically through system N, was measured by monitoring uptake in a $Na^+$-

containing medium in the presence of 5 mM MeAIB (to inhibit uptake through system A) and 2 mM tryptophan (to inhibit uptake through system L). We tested in competition experiments the ability of various amino acids (5 mM) to inhibit glutamine uptake under these conditions (Fig. 3A). Anionic amino acids (glutamate and aspartate) and cationic amino acids (lysine and arginine) did not have any significant effect on glutamine uptake. Neutral amino acids, with the exception of phenylalanine, caused significant inhibition. The most potent inhibitory amino acids were cysteine, alanine, asparagine, serine, methionine, leucine, and unlabeled glutamine. This substrate specificity closely matches that of system N. Dose-response studies provided data on the relative affinities of these neutral amino acids to system N in RGC-5 cells (Fig. 3B). $IC_{50}$ values for the inhibition process were in the following order: leucine (3.97 ± 0.63 mM) > methionine (0.94 ± 0.18 mM) > serine (0.88 ± 0.16 mM) > asparagine (0.75 ± 0.08 mM) > alanine (0.56 ± 0.11 mM) > cysteine (0.31 ± 0.06 mM). Data showing that glutamine uptake was not inhibited by arginine or lysine eliminates systems y+L and $ATB^{0,+}$ as mediators of glutamine transport in ganglion cells.

The kinetic features of glutamine uptake through system N were then investigated. Uptake of glutamine in RGC-5 cells was saturable, and the data fit reasonably well to a transport model consisting of a single saturable process (Fig. 4A). The $K_m$ was 0.41 ± 0.09 mM, and the maximal velocity was 6.2 ± 0.9 nmol/mg protein/15 minutes. The relationship between glutamine uptake rate and $Na^+$ concentration was hyperbolic, with a Hill coefficient of 1.1 ± 0.1 (Fig. 4B), indicating the involvement of 1 $Na^+$ in the activation process. We also studied the effect of extracellular pH on the uptake process (data not shown). The uptake rate increased steadily when the extracellular pH was changed from 6 to 8.5. The increase was 60% when uptake was compared between pH 6 and pH 8.5. The transport process mediated by system N is known to involve the transfer of $Na^+$ and glutamine in one direction and the transfer of $H^+$ in the opposite direction. Therefore, when glutamine influx is measured in the presence of extracellular $Na^+$, uptake is expected to be stimulated in the presence of an alkaline extracellular medium, which creates an outwardly directed $H^+$ gradient across the plasma membrane.

Taken collectively, the data from the studies described provide strong evidence that system N is the major contributor to glutamine uptake in RGC-5 cells. Functional evidence in support of this includes $Na^+$ dependence, insensitivity to MeAIB inhibition, and stimulation by extracellular alkaline pH. RT-PCR with RNA isolated from RGC-5 cells indicated that both SN1 and SN2 isoforms are expressed in this cell line (Fig. 1). These isoforms exhibit almost similar substrate specificity and kinetic features, and the uptake processes mediated by the two isoforms cannot be differentiated at the functional level. Thus, additional experiments were undertaken to analyze whether SN1, SN2, or both components of system N were expressed in intact retina and in primary ganglion cells.

### Expression of SN1 and SN2 in Mouse Retina: In Situ Hybridization

To determine whether the genes encoding SN1 and SN2 were present in ganglion cells in native retinal tissue, in situ hybridization was performed in mouse retinal cryosections using digoxigenin-labeled antisense riboprobes. The expression patterns of SN1 and SN2 mRNA are similar to each other (Fig. 5). Both genes are expressed in cells of the ganglion cell layer, in soma of some cells of the inner nuclear layer, and in the inner segments of photoreceptor cells. Neither SN1 nor SN2 is expressed in RPE (the brownish signal, characteristic of the C57Bl/6 mouse, reflects the pigment of this cellular layer). Incubation of sections with sense probes showed no expression.

### Expression of SN1 and SN2 in Mouse Retina: Immunofluorescence Studies

As a positive control, immunodetection for SN1 and SN2 was performed using cryosections of mouse liver, a tissue known to express both proteins.[6,13,14] Figure 6A shows a hematoxylin-

eosin–stained liver for reference and a higher magnification inset. Both SN1 and SN2 were detected in this tissue (Figs. 6B, 6C). The antibodies detected the proteins intracellularly, with some labeling observed at the plasma membrane. Using the antibodies generated against SN1 and SN2, immunofluorescence analysis was then performed in cryosections of mouse retinas. SN1 and SN2 were detected in several retinal layers, consistent with the widespread gene expression observed in the in situ hybridization data (Fig. 5). The antibody against SN1 labeled cells of the ganglion cell layer and the soma of some cells of the inner nuclear layer (Fig. 6D). Consistent with the reports of Boulland et al.,[25] SN1 was not detected in the RPE. As shown in Figure 6I, the SN2 antibody labeled the ganglion cell layer and the nerve fiber layer. SN2 was also detected in the cells of the inner nuclear layer and in the inner plexiform layer. Incubation of cryosections with antibodies preincubated with antigenic peptides yielded no immunopositive reaction (Figs. 6H, 6L). We showed earlier by functional analyses that Müller cells use system N to mediate glutamine uptake.[26] Hence, we were interested in assessing the extent to which SN1 and SN2 colocalized with the glial cell marker vimentin. The secondary antibody, directed against vimentin, fluoresces green, whereas the secondary antibody against the SN1 and SN2 fluoresces red. Vimentin labels the radial glial fibers and the glial end feet, as shown in Figures 6E and 6J. The merged image of labeling of vimentin-positive/SN1-positive cells is shown in Figures 6F and 6G. SN1 does not appear to colocalize significantly with Müller cell radial fibers. The merged image of labeling of vimentin-positive/SN2-positive cells is shown in Figure 6K. SN2 colocalized with the Müller cell radially oriented fibers, though not to the Müller cell end feet. Arrowheads mark the radial fiber distribution of the two proteins (Fig. 6K). These data provide additional support for the presence of system N in the retinal glial cells and support our earlier functional study findings of glutamine transport in Müller cells.[26]

Subsequent immunohistochemical experiments analyzed SN1/SN2 in ganglion cells, the focus of this study, and their colocalization with the ganglion cell marker Thy 1.2. As shown in Figures 7A and 7D, Thy 1.2 has a labeling pattern (green fluorescence) consistent with the distribution of ganglion cells and their processes (labeling some cell bodies within the ganglion cell layer, axons of the nerve fiber layer, and dendritic processes of the inner plexiform layer but minimal labeling in the remainder of the retina[32]). The labeling pattern of SN1 (red fluorescence) shows positive immunoreactivity surrounding the cell bodies of the ganglion cells (Fig. 7B). Colocalization occurs with Thy1.2, especially of the ganglion cell axonal fibers in the nerve fiber layer as shown in the merged image (Fig. 7C), which is best viewed at a higher magnification (Fig. 7D). The areas of yellow-orange fluorescence denote the colocalized SN1 and Thy1.2 (Figs. 7C, 7D). Labeling of Thy 1.2 and SN2 is shown separately in Figures 7E and 7F, respectively. In the merged images, the yellow-orange fluorescence reflects the overlap of the green fluorescence (Thy 1.2) and the red fluorescence (SN2; Fig. 7G), which can be seen best at higher magnification (Fig. 7H). These data provide strong in vivo evidence for the presence of system N in ganglion cell neurons.

### Immunodetection of SN1 and SN2 in Cultured Ganglion Cells

Because our functional studies were performed in the ganglion cell line RGC-5, we performed immunofluorescence studies to confirm the presence of SN1 and SN2 proteins in these cells (Fig. 8A). In addition, we used primary ganglion cells isolated from mouse retina to assess these proteins. These studies detected robust expression of SN1 and SN2 in RGC-5 cells and in primary cultures of mouse retinal ganglion cells (Fig. 8A). The purity of primary ganglion cells has been confirmed, establishing their identity as ganglion cells.[30] RGC-5 cells do not develop extensive axonal processes in culture, but primary ganglion cells do. We found both isoforms of system N to be expressed in the primary ganglion cells, not only in the cell body but also in axonal processes. RGC-5 labeling for the proteins appeared to be punctate at the membrane, with some intracellular detection especially for SN2. Western blotting was

performed with protein isolated from RGC-5 cells and primary ganglion cells (Fig. 8B). In addition, system N is known to be present in Müller cells[26] and liver.[13,14] Thus, we isolated protein from primary Müller cells and liver to serve as positive controls. Using the SN1 antibody, a band of the appropriate molecular weight (approximately 55 kDa) was detected in the RGC-5 cell line, primary ganglion cells, Müller cells, and liver cells. The broader band labeling observed for liver is consistent with observations reported by Solbu et al.[35] A protein band of approximately 50 kDa was detected in RGC-5, primary ganglion, primary Müller, and liver cells when membranes were probed with the antibody generated against SN2. These data confirm the presence of system N in ganglion cells. In each case, 30 $\mu$g protein was loaded per lane, and the gels were reprobed for $\beta$-actin as an internal loading control. RGC-5 cells appeared to have more SN1 than primary ganglion cells or Müller cells; however, the amount of SN2 was similar between the cell types. To establish the fidelity of the antibodies, neutralized antibodies were used. For this, the SN1 and SN2 antibodies were preincubated with excess amounts of respective antigenic peptides that were used to generate the antibodies, and these neutralized antibodies were then used to probe membranes prepared from proteins isolated from RGC-5 cells. With both SN1 and SN2, the respective expected protein band was eliminated when the neutralized antibodies were used (Fig. 8C).

## DISCUSSION

Glutamine is required by excitatory retinal neurons, including ganglion cells, for conversion to glutamate, the major neurotransmitter in this tissue. Characterization of transport systems responsible for glutamine entry into retinal cells complements our understanding of transport mechanisms for glutamate and is relevant to the retinal glutamate-glutamine cycle. The present study represents the first comprehensive analysis of glutamine uptake by retinal ganglion cells and was facilitated by the availability of the RGC-5 cell line and primary ganglion cells.

Data obtained from RT-PCR analysis showed expression of a number of candidate glutamine transporters in RGC-5 cells, including systems N, A, L, y+L, and ASCT. $ATB^{0,+}$ is not expressed by the cells and thus is not expected to contribute to glutamine uptake in this cell type. Interestingly, though ASCT was expressed in RGC-5 cells, it was unlikely to play a significant role in glutamine uptake in ganglion cells because it is an obligatory exchanger that works in a bimodal fashion. In other words, it has the ability to mediate glutamine influx in exchange for an intracellular amino acid, but it also mediates the efflux of glutamine (again, in exchange for an amino acid). Thus, this ASCT is not ideal for the mediation of extracellular glutamine into neuronal cells; rather, its predicted role is to maintain amino acid equilibrium in the cells.

The studies then focused on determining contributions to glutamine transport by the transport systems expressed in RGC-5 cells, namely systems A, N, L, and y+L. Because glutamine uptake was not inhibited competitively by arginine or lysine, system y+L was eliminated from consideration as a transporter for glutamine in ganglion cells. Functional studies showed that most of the glutamine taken up by ganglion cells is $Na^+$ dependent with only a small contribution (approximately 10%) from the $Na^+$-independent system L. Interestingly, only a small portion of glutamine uptake was inhibited by MeAIB, suggesting that system A did not play a major role in glutamine uptake. The finding that system A was expressed and contributed to glutamine uptake in ganglion cells, even though only approximately 20%, is consistent with the observations of Gu et al.[27] that mNat (ATA1) is present in these cells. Their studies analyzed glutamine uptake in the xenopus oocyte expression system, not in ganglion cells; hence, they did not speculate on the relative contributions of various glutamine transporter systems to glutamine uptake in ganglion cells.

Based on the molecular and functional data obtained in the present study, it appears that system N is the primary transporter of glutamine uptake in the RGC-5 cells, contributing approximately 70% to this process. Given that system N is more commonly considered a glutamine transporter for glial cells, we analyzed the presence of system N in intact retina and in primary ganglion cells. System N expression was robust in intact retina, with genes for SN1 and SN2 displaying a similar pattern of RNA expression. Immunohistochemical analysis colocalized SN1 and SN2 with Thy1.2, a ganglion cell-specific marker supporting their location in ganglion cells. Interestingly, in our hands, SN2 colocalized more abundantly in radial Müller fibers (as indicated by intense vimentin staining) than did SN1. Generally, however, the pattern of antibody staining for SN1 was similar to that of SN2, with expression in the ganglion cell layer, inner plexiform layer, and cell soma of the inner nuclear layer. The similar distribution of the two proteins may reflect that these two transporters complement each other to facilitate glutamine uptake with redundancy in transporter expression to ensure adequate glutamine uptake. In the primary ganglion cells, SN1 and SN2 were abundantly detected immunocytochemically and by immunoblotting.

Our finding that the key contributor to glutamine uptake in ganglion cells is through system N rather than system A is of interest given the prevailing theory, put forth from studies performed in brain, suggesting that neurons use system A to take up glutamine whereas glial cells use system N to release glutamine. This theory links system A and system N to the functional coupling between neurons and glia in terms of the glutamate-glutamine cycle. Our earlier studies in Müller cells were consistent with this theory and showed that system N is the major glutamine transporter system in these cells.[26] The present studies show unequivocally that system N is expressed robustly and is the major glutamine transporter in retinal ganglion cells. Although system A contributes to glutamine uptake in RGCs, it does so to a smaller extent.

It is intriguing to speculate on the roles of the two transporter systems, A and N, in the retina. System A always functions in the influx mode because of its energization by the transmembrane electrochemical $Na^+$ gradient. In contrast, system N can function in influx mode or in efflux mode because it is an electroneutral transport system, with involvement of $H^+$ as a transportable substrate. Depending on the concentration gradients for glutamine, $Na^+$, and $H^+$ across the plasma membrane, the transport system is capable of mediating glutamine influx or glutamine efflux. The ability of system N to function in the efflux mode is the cornerstone for the model describing its participation in the glutamate-glutamine cycle.[5,19,36] Astrocytes release glutamine as part of this cycle, and the transport features of system N fit well with its postulated role in the efflux of glutamine from these cells. This role is supported by the findings that SN1 is expressed in astrocytes in the brain[5,20] and in the retina.[25] The labeling pattern for SN1 observed in the retinal sections in the present study may reflect expression in astrocytes, though the present study did not examine this question. In the glutamate-glutamine cycle between neurons and astrocytes, there is no role of glutamine release from the neuronal cells. Thus, in ganglion cells, system N likely mediates the influx rather than the efflux of glutamine. Presumably, intracellular concentrations of glutamine play a critical role in determining the transport mode of system N in Müller cells compared with ganglion cells. Müller cells express glutamine synthetase robustly; therefore, these cells synthesize glutamine, resulting in high intracellular concentrations of this amino acid. This generates an outwardly directed glutamine gradient across the plasma membrane, favoring the efflux mode for the transport function of system N in these cells. In contrast, ganglion cells express glutaminase, which converts glutamine to glutamate, reducing intracellular concentrations of glutamine and leading to an inwardly directed glutamine gradient across the plasma membrane. This would favor the influx mode for the transport function of system N. Interestingly; recent studies[22,36] in brain have also questioned whether system A is the sole mediator of glutamine influx in cortical neurons. Thus, the data reported herein may be relevant to the glutamate-glutamine cycle in the brain.

In summary, systems L and A together contribute approximately 30% of the glutamine uptake in ganglion cells, whereas system N contributes approximately 70%. SN1 and SN2, isoforms of system N, are expressed robustly in retinal ganglion cells. The present data and the results of our previously published study[26] suggest that system N may play a dual role in the glutamate-glutamine cycles that operate between Müller cells and ganglion cells in the retina such that in Müller cells, system N functions as an efflux transporter, releasing glutamine into the extracellular space. The same transporter functions as the major influx transporter in ganglion cells mediating the uptake of glutamine.

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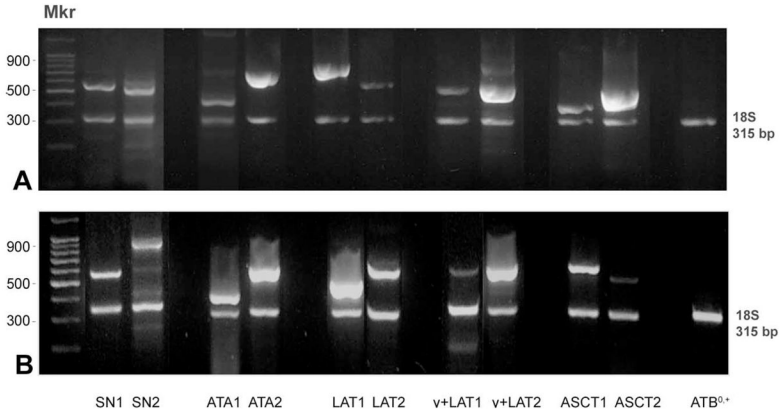


**Figure 1.**

RT-PCR analysis of RGC-5 mRNA transcripts. RNA was isolated from rat RGC-5 cells (**A**) and mouse primary ganglion cells (**B**) and was subjected to RT-PCR using the primer pairs specific for rat or mouse SN1, SN2, ATA1, ATA2, LAT1, LAT2, $y^+$LAT1, $y^+$LAT2, ASCT1, ASCT2, and $ATB^{0,+}$, shown in Table 1. RT-PCR products were subjected to gel electrophoresis and subsequently stained with ethidium bromide. DNA standards (100 bp to ~12.2 kbp) were run in parallel. 18S RNA (315 bp) was the internal control.

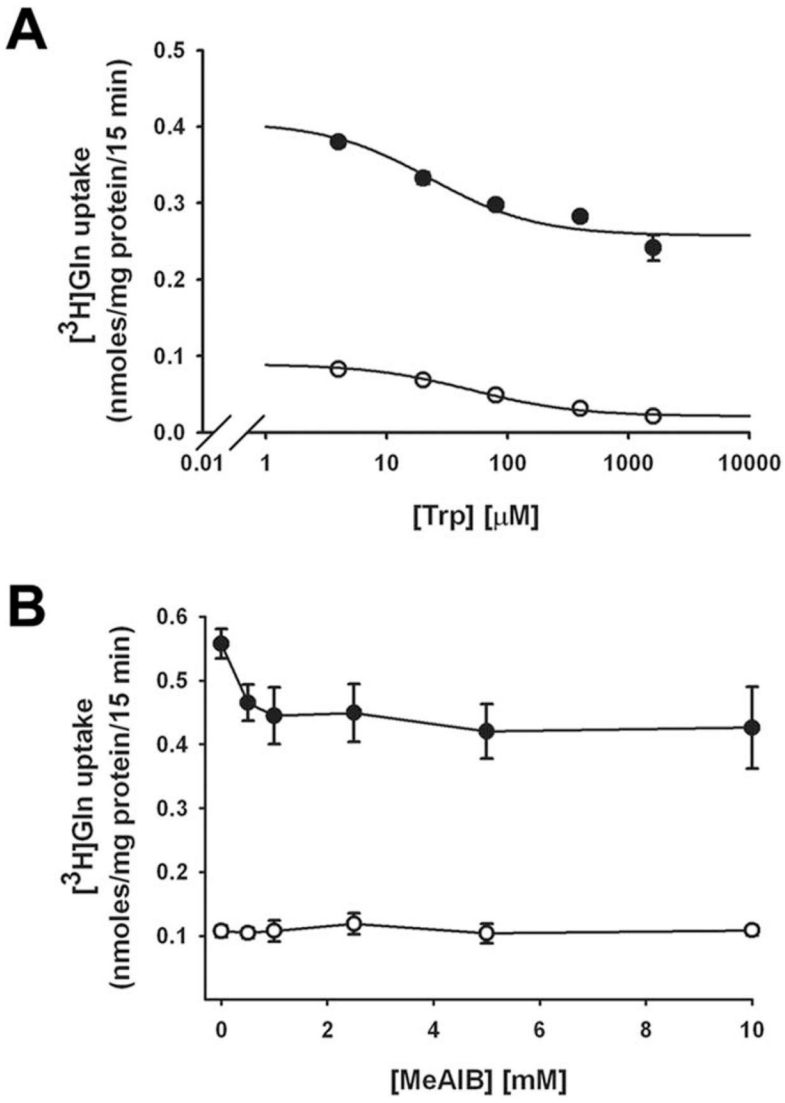


**Figure 2.**

Relative contribution of system L and system A to glutamine uptake in RGC-5 cells. Uptake of glutamine (5 $\mu$M) was measured in confluent cells for 15 minutes in the presence of NaCl (●) or in the presence of *N*-methyl-D-glucamine chloride (○). (**A**) Uptake was measured in the presence of increasing concentrations of tryptophan, a system L–selective substrate. (**B**) Uptake was measured in the presence of increasing concentrations of MeAIB, a system A–selective substrate. Values represent mean ± SEM for six determinations from two independent experiments.

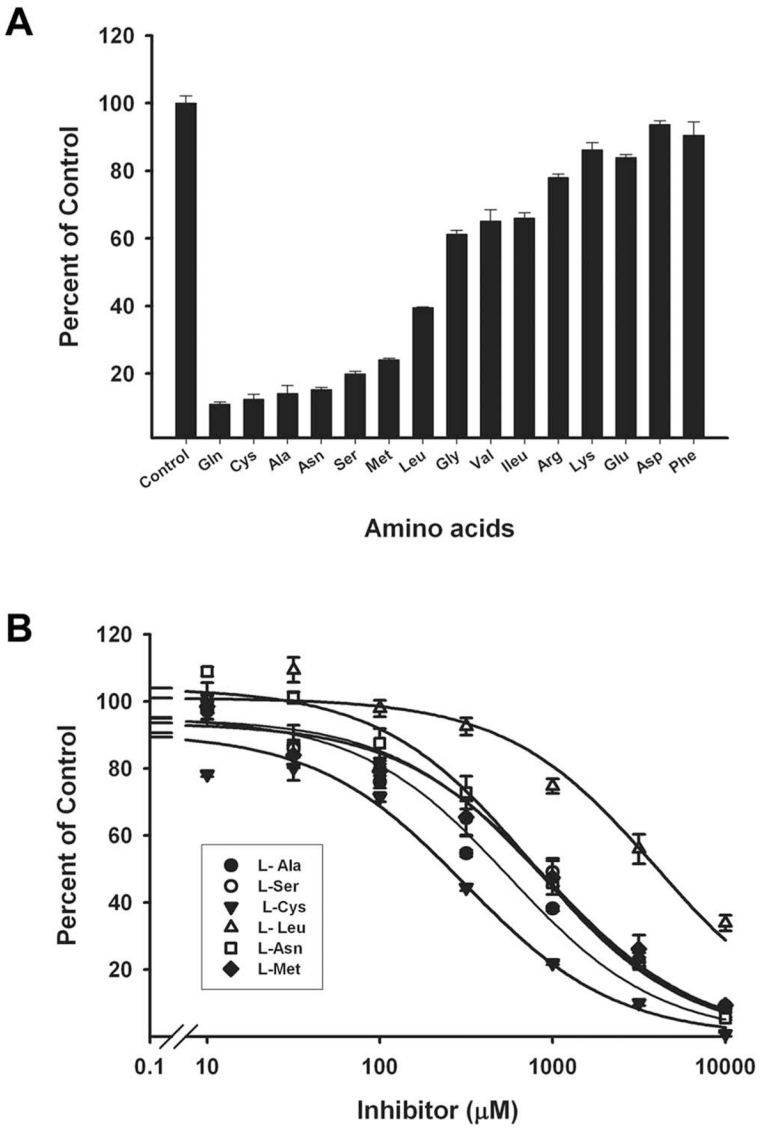


**Figure 3.**

Substrate specificity of the uptake process that was primarily responsible for glutamine uptake in RGC-5 cells. Uptake of [$^3$H]glutamine (concentration of radiolabeled plus unlabeled glutamine, 5 $\mu$M) was measured in confluent cells for 15 minutes in the presence of NaCl, 2 mM tryptophan (to inhibit uptake through system L), and 5 mM MeAIB (to inhibit uptake through system A). (**A**) Uptake was measured in the presence of various unlabeled amino acids (5 mM). (**B**) Uptake was measured in the presence of increasing concentrations of six different amino acids that were most potent in inhibiting glutamine uptake. In both cases, uptake values measured in the absence of competing amino acid substrates were taken as 100%. Values represent mean ± SEM of six determinations from two independent experiments.

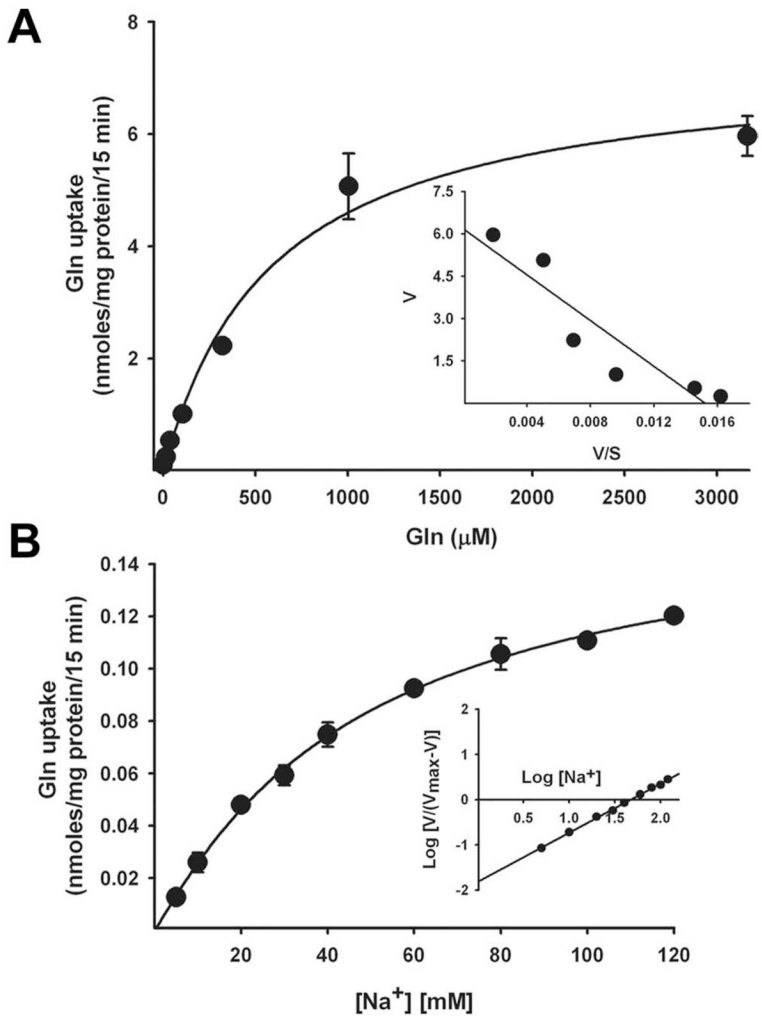


**Figure 4.**

Saturation kinetics and $Na^+$-activation kinetics of glutamine uptake through system N. Uptake of glutamine was measured for 15 minutes in confluent cultures of RGC-5 cells in the presence of NaCl, 2 mM tryptophan, and 5 mM MeAIB. (**A**) Uptake was measured in the presence of increasing concentrations of glutamine. *Inset*: Eadie-Hofstee plot. (**B**) Uptake was measured in the presence of increasing concentrations of $Na^+$. *Inset*: Hill plot. Values represent mean ± SEM of six determinations from two independent experiments.

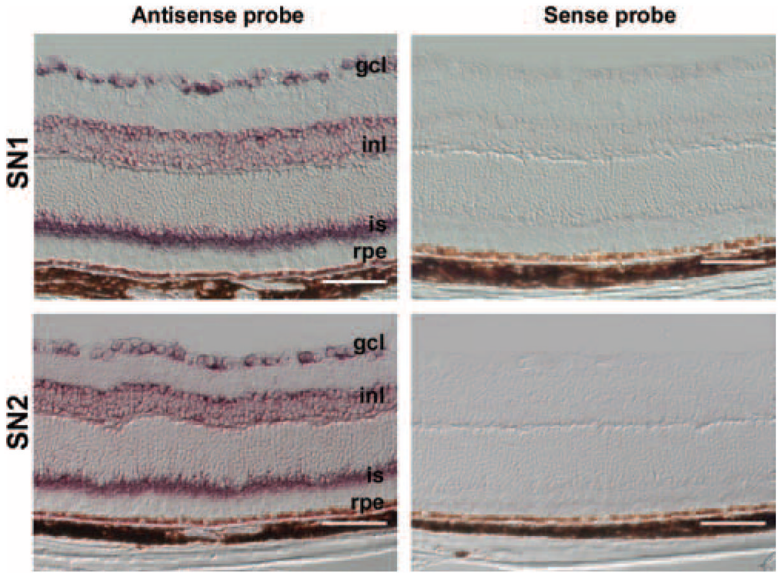


**Figure 5.**

SN1 and SN2 expression in intact mouse retina. Mouse retinal cryosections were subjected to in situ hybridization using anti-sense digoxigenin-labeled SN1 and SN2 riboprobes. The intense *brownish-purple stain* indicates a positive reaction. No specific signal was detected when retinal cryosections were probed with sense digoxigenin-labeled SN1 and SN2 riboprobes (negative control). The *dark brown signal* in RPE reflects pigmentation. gcl, ganglion cell layer; inl, inner nuclear layer; is, inner segments of photoreceptor cells; rpe, retinal pigment epithelium. Scale bar, 50 $\mu$m.

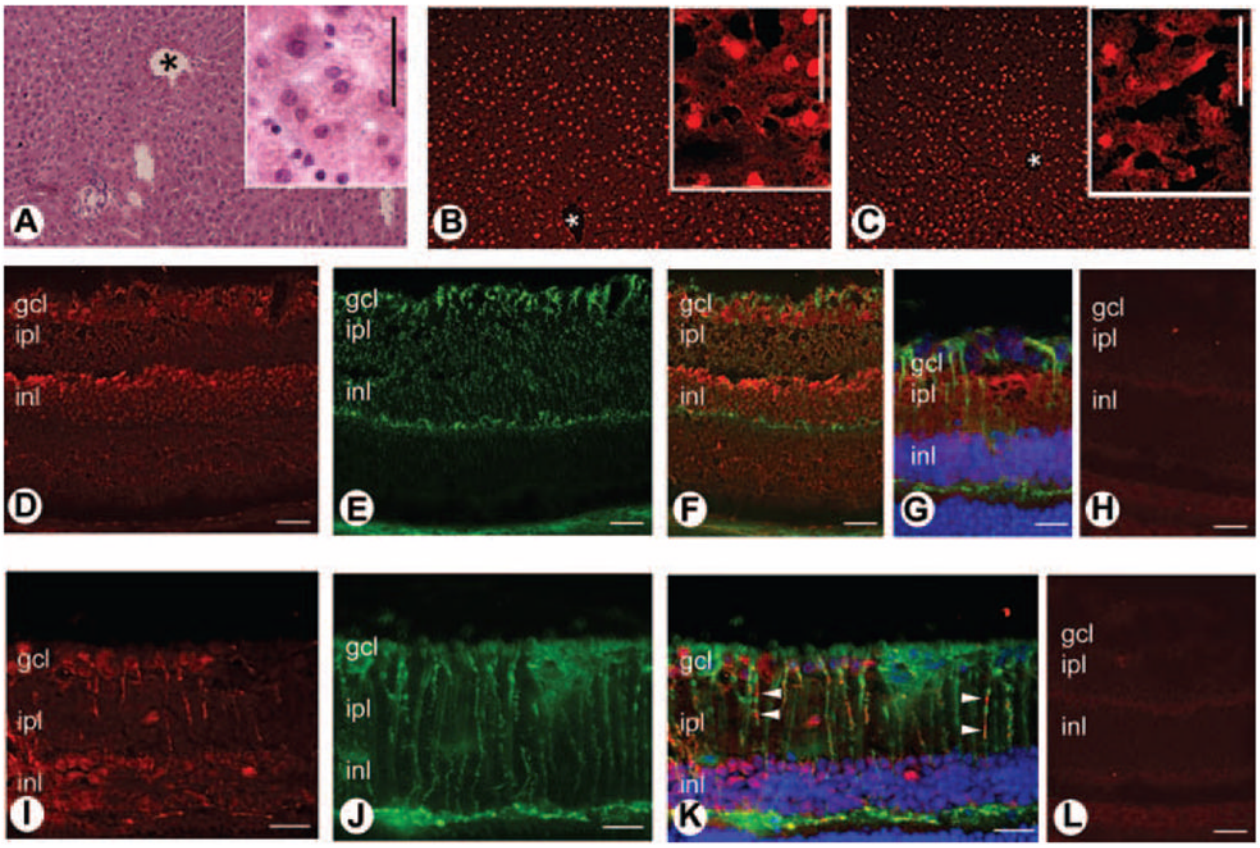


**Figure 6.**

Immunodetection of SN1 and SN2 in Müller cells. (**A**) Hematoxylin and eosin–stained sections of mouse liver showing the central vein (*asterisk*) and the laminae of hepatic cells. Immunohistochemical assays detected SN1 (**B**) and SN2 (**C**) in these cells (*red fluorescence*). Low-power images (**B**, **C**) show widespread detection of SN1 and SN2. *Insets*: higher magnification images. Scale bar, 20 $\mu$m. (**D**–**H**) Retinal cryo-sections subjected to immunohistochemistry to detect SN1 (**D**, *red fluorescence*) or vimentin (**E**, *green fluorescence*). (**F**) Merged images of the two fluorescent labels. (**G**) Higher magnification of merged image with DAPI staining of nuclei (*blue*). (**H**) Immunostaining using the primary antibody preadsorbed with the blocking peptide for SN1 (control). (**I**–**L**) Retinal cryosections subjected to immunohistochemistry to detect SN2 (**D**, *red fluorescence*) or vimentin (**J**, *green fluorescence*). (**K**) Merged images of the two fluorescent labels and DAPI (*blue*) to detect nuclei. *Arrowheads*: radial fibers positive for SN2 and vimentin. (**L**) Immunostaining using the primary antibody preadsorbed with the blocking peptide for SN2 (control). gcl, ganglion cell layer; ipl, inner plexiform layer; inl, inner nuclear layer. Scale bars: (**D**–**F**, **H**–**L**) 50 $\mu$m; (**G**) 15 $\mu$m.

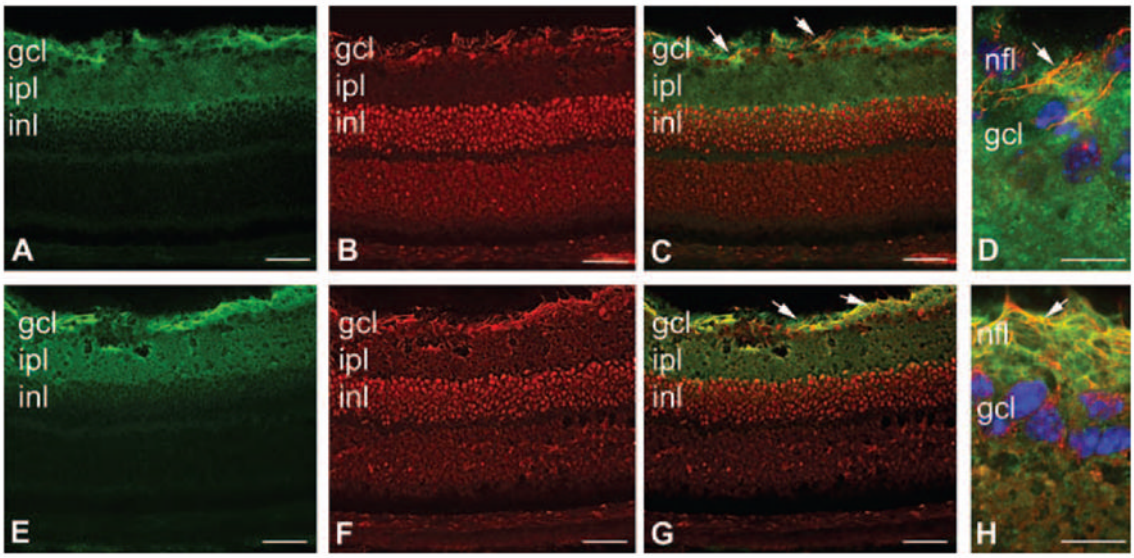


**Figure 7.**

Immunodetection of SN1 and SN2 in ganglion cells. Retinal cryosections subjected to immunohistochemistry to detect the ganglion cell marker Thy1.2 (**A**, *green fluorescence*) or SN1 (**B**, *red fluorescence*). (**C**) Merged image shows colocalization of SN1 with Thy1.2 (*orange-yellow fluorescence*). (**D**) Higher magnification of merged image with DAPI staining of nuclei (*blue*). *Arrows*: examples of colocalization. (**E**–**H**) Retinal cryosections subjected to immunohistochemistry to detect SN2 (**F**, *red fluorescence*) and to determine colocalization with Thy1.2 (**E**, *green fluorescence*). Merged images of the two fluorescent labels (**G**) reflect colocalization. *Arrowheads*: nerve fiber layers positive for SN2 and Thy 1.2 (*orange-yellow*), seen better at higher magnification (**H**). gcl, ganglion cell layer; ipl, inner plexiform layer; inl, inner nuclear layer; nfl, nerve fiber layer. Scale bars: (**A**–**C**, **E**–**G**) 50 $\mu$m; (**D**, **H**) 15 $\mu$m.

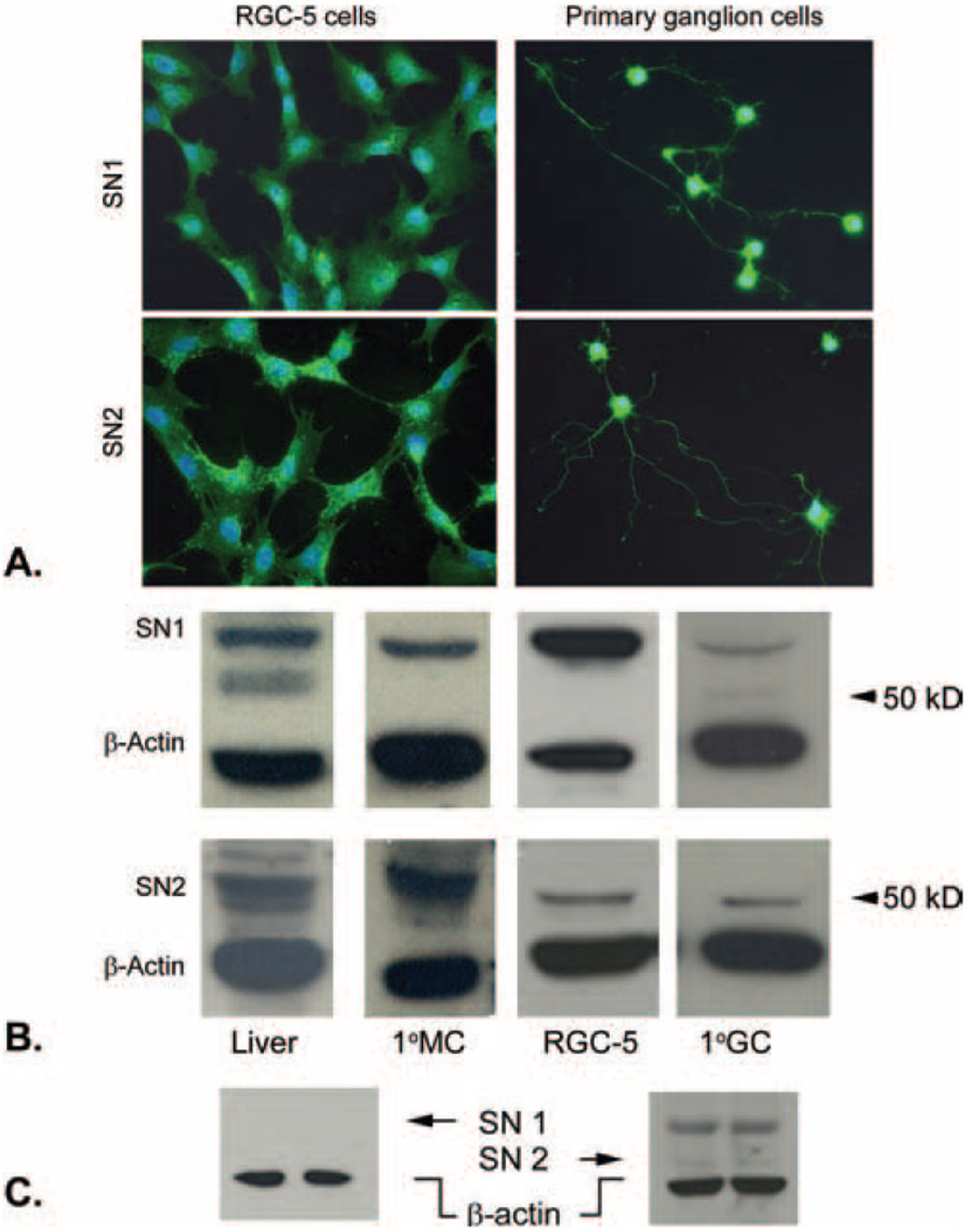


**Figure 8.**

Immunodetection of SN1 and SN2 in RGC-5 and primary mouse ganglion cells. (**A**) Immunofluorescence detection of SN1 and SN2 was performed in RGC-5 cells and primary ganglion cells harvested from neonatal mice. Antibodies were detected using the Alexa Fluor 488–conjugated secondary antibody. Intense green fluorescence reflects the positive reaction in the cell bodies and their processes. (**B**) Western blot detection of SN1 and SN2 in proteins isolated from liver, primary mouse Müller cells, RGC-5, and primary mouse ganglion cells. Proteins were isolated, subjected to SDS-PAGE, transferred to membranes, and incubated with antibodies against SN1 ($M_r$ = ~55 kDa) or SN2 ($M_r$ = ~50 kDa). Band sizes detected for SN1 and SN2 are consistent with the published sizes of these proteins. $\beta$-Actin ($M_r$ = ~45 kDa)

served as the loading control. (**C**) Immunodetection of SN1 (*left*) and SN2 (*right*) in RGC-5 cells, the primary antibodies against SN1 and SN2, were incubated with the respective antigenic peptide before immunoblotting. Note that though the positive control (*β*-actin) is clearly visible, the expected bands for SN1 and SN2 are not. Blocking by the antigenic peptide indicates the specificity of the antibody.

**Table 1**
Sequences of RT-PCR Primers

| Gene Name | NCBI Accession No. | Primer Sequence | Predicted Band Size (bp) |
|---|---|---|---|
| *SN1* | | | |
| Mouse | AF159856 | Forward: 5′-TCGGCTACCTGGGTTACTC-3′<br>Reverse: 5′-GGGAACAGAACAATCGGAACTG-3′ | 538 |
| Rat | AF273025 | Forward: 5′-GCGGTTGCCCTGTTGTCTAGC-3′<br>Reverse: 5′-GGGATGGTGTATGCCGTCTGT-3′ | 563 |
| *SN2* | | | |
| Mouse | NM_172479 | Forward: 5′-CTGCCCACCACCCGTAA-3′<br>Reverse: 5′-CCAAAGGTCGCTGTGAGTCCAT-3′ | 850 |
| Rat | AF276870 | Forward: 5′-CTGCGCCAGTGTTGTAGGAA-3′<br>Reverse: 5′-CAGCTGCTATTAAACGCCTGC-3′ | 429 |
| *ATA1* | | | |
| Mouse | BC030378 | Forward: 5′-GGTATCTTGGCTACACGAGTGG-3′<br>Reverse: 5′-CGCCTGTGCTCTGGTACTTGTG-3′ | 396 |
| Rat | NM_138832 | Forward: 5′-ATGTCACCTTCAACTCAA-3′<br>Reverse: 5′-AATCACAAGGAGTATGATG-3′ | 407 |
| *ATA2* | | | |
| Mouse | NM_175121 | Forward: 5′-GCTGCTCTTTATCCTCCCGTCT-3′<br>Reverse: 5′-ACATCGAATGGTTCCGTTAGGC-3′ | 533 |
| Rat | AF249673 | Forward: 5′-TGAATGGCACCTTTACCC-3′<br>Reverse: 5′-CTTCCAATCATCACGACTACGC-3′ | 691 |
| *LAT1* | | | |
| Mouse | AB023409 | Forward: 5′-AGTGGGCTGCCTGTCTACTTC-3′<br>Reverse: 5′-TGACCCAAATGCACGCTACAAA-3′ | 466 |
| Rat | AB015432 | Forward: 5′-CCTGCCTCTGCGTGCTACT-3′<br>Reverse: 5′-TTCACCTTGATGGGACGCT-3′ | 784 |
| *LAT2* | | | |
| Mouse | NM_016972 | Forward: 5′-CATCGGCCTCGTTGCTCT-3′<br>Reverse: 5′-GGCCCAGAACAGCAGGTAGAT-3′ | 616 |
| Rat | AB024400 | Forward: 5′-CTGCTCACATGGGTCAACT-3′<br>Reverse: 5′-CGTGGATCATGGCTAACAC-3′ | 565 |
| *y+LAT1* | | | |
| Mouse | NM_011405 | Forward: 5′-AATCCTGGCCAGTGACGCT-3′<br>Reverse: 5′-GCGGAGGAACAGTGGTCGTT-3′ | 553 |
| Rat | AB020520 | Forward: 5′-GCACCAAGTATGAAGTGGC-3′<br>Reverse: 5′-CCACTTGACATAGGCACAG-3′ | 536 |
| *y+LAT2* | | | |
| Mouse | NM_178798 | Forward: 5′-GCTTCATTGCCTTTATCCGTCT-3′<br>Reverse: 5′-AAATGTCACAGCCACAGCGTCA-3′ | 563 |
| Rat | NM_001107424 | Forward: 5′-AGCCAGGGAGCTTGGGAG-3′<br>Reverse: 5′-CCACTTGACATAGGCACAG-3′ | 556 |
| *ASCT1* | | | |
| Mouse | NM_018861 | Forward: 5′-TCCCTCGGGCGTCTG-3′<br>Reverse: 5′-GACATGGCCCAGCATAGATG-3′ | 594 |
| Rat | AB103401 | Forward: 5′-GTTTGCGACGGCTTTTGCGACCTG-3′<br>Reverse: 5′-GCATCCCCTTCCACGTTCACCACA-3′ | 399 |
| *ASCT2* | | | |
| Mouse | NM_009201 | Forward: 5′-GGTGGTCTTCGCTATCGTCT-3′<br>Reverse: 5′-CCGTTTAGTTGTGCGATGAA-3′ | 516 |
| Rat | AJ132846 | Forward: 5′-GCGCCTGGGCCCTGCTCTTTTT-3′<br>Reverse: 5′-ACAATCTTGCCGGCCACCAGGAAC-3′ | 478 |
| $ATB^{0,+}$ | | | |
| Mouse | AF320226 | Forward: 5′-CTGGCTTGGCTCATAGTTGG-3′<br>Reverse: 5′-AGGAACCCTCATGCGTTTCA-3′ | 615 |
| Rat | NM_001037544 | Forward: 5′-CTGCCATGGGCTAATTGTTC-3′<br>Reverse: 5′-CCGGATATATGAGCCATGTG-3′ | 674 |