Bidirectional substrate fluxes through the System N (SNAT5) glutamine transporter may determine net glutamine flux in rat liver

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System N (SNAT3 and SNAT5) amino acid transporters are key mediators of glutamine transport across the plasma membrane of mammalian cell types, including hepatocytes and astrocytes. We demonstrate that SNAT5 shows simultaneous bidirectional glutamine fluxes when overexpressed in *Xenopus* oocytes. Influx and efflux are both apparently Na^+ dependent but, since they are not directly coupled, the carrier is capable of mediating net amino acid movement across the cell membrane. The apparent K_m values for glutamine influx and efflux are similar (~1 mM) and the transporter behaviour is consistent with a kinetic model in which re-orientation of the carrier from outside- to inside-facing conformations (either empty or substrate loaded) is the limiting step in the transport cycle. In perfused rat liver, the observed relationship between influent (portal) glutamine concentration and net hepatic glutamine flux may be described by a simple kinetic model, assuming the balance between influx and efflux through System N determines net flux, where under physiological conditions efflux is generally saturated owing to high intracellular glutamine concentration. SNAT5 shows a more periportal mRNA distribution than SNAT3 in rat liver, indicating that SNAT5 may have particular importance for modulation of net hepatic glutamine flux.

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System N is a major Na^+-dependent transport system for zwitterionic amino acids in mammalian cell types, including hepatocytes and astrocytes. System N has a relatively limited substrate specificity with preference for transport of glutamine, histidine and asparagine, in which respect it is suggested to have particular importance for regulating physiologically important glutamine exchanges between different cell types in liver and brain (Taylor *et al.* 1999; Bode, 2001; Chaudhry, Reimer & Edwards, 2002*a* for review). The sodium-coupled neutral amino acid transporters (SNAT; Mackenzie & Erickson, 2004) of the SLC38 gene family resemble the classically described System N and System A transport activities in terms of their functional properties and patterns of regulation. Two isoforms of System N transporter (SNAT3 and SNAT5; previously termed SN1 and 2, respectively) have been identified to date, both of which are expressed predominantly in liver and brain (Fei *et al.* 2000; Nakanishi *et al.* 2001*a*,*b*; Mackenzie & Erickson, 2004). A transport stoichiometry of 1 Na^+:1 amino acid in exchange for 1 H^+ is predicted for SNAT3 on the basis of most investigations (Mackenzie & Erickson, 2004 for review); this is despite large currents associated with both SNAT3 and SNAT5 expression in oocytes which appear to arise from uncoupled ion conductances (at least in the case of SNAT3; Chaudhry *et al.* 2001). In contrast, System A transporters mediate the uptake of a wide range of neutral amino acids, coupled to the uptake of Na^+ with a stoichiometry of 1:1. Three distinct isoforms of the System A transporter have been cloned to date (SNAT1, SNAT2 and SNAT4; also known as SAT1–3 or ATA1–3, respectively, amongst previous nomenclatures; Reimer *et al.* 2000; Sugawara *et al.* 2000*a*,*b*; Varoqui *et al.* 2000; Yao *et al.* 2000). All SNAT family transporters studied to date exhibit marked pH sensitivity, with influx depressed as external pH is lowered within the physiological range (pH 7.0–7.8). Such dependence on external pH may have important physiological implications for tissue amino acid and protein metabolism, as well as for regulation of glutamatergic and GABAergic neurotransmission (Chaudhry *et al.* 1999, 2001, 2002*b*).

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Bidirectional transport of glutamine through hepatic amino acid transport mechanisms has been reported previously (Pardridge, 1977; Fafournoux *et al.* 1983; Häussinger *et al.* 1985). Nevertheless, it is only recently that the possibility of a central role for System N in the overall process has been recognized, following the demonstration that SNAT3 is inherently reversible for both H^+ and glutamine fluxes under physiologically relevant conditions (Chaudhry *et al.* 1999; Gu *et al.* 2000; Bröer *et al.* 2002) and the realization that other hepatic glutamine transport processes (System ASC and L type) use obligatory exchange mechanisms (Bode, 2001; Bröer & Brookes, 2001 for review). Nevertheless, major unanswered questions about the functional mechanism of System N remain, notably what is the K_m for intracellular substrates and to what extent is net amino acid flux through the transporter linked to gradients and concentrations of cosubstrates? In addition, given that relative expression of SNAT5 is at least equal to that of SNAT3 in liver (Nakanishi *et al.* 2001*a*,*b*) and that System N is regarded as quantitatively the most important carrier of glutamine at the liver sinusoidal membrane (Kilberg *et al.* 1980; Low *et al.* 1992), do the kinetic properties of System N transporter isoforms and their intrahepatic distribution help explain the changing pattern of glutamine distribution and exchange between plasma and liver cells when glutamine loads alter due to different nutritional and metabolic circumstances (Fafournoux *et al.* 1983; Häussinger *et al.* 1985)? Physiological study of System N from a kinetic rather than thermodynamic perspective also attains greater importance in view of recent evidence that slippage of the SNAT3 transport mechanism (that is, *trans*-membrane reorientation of the empty or partly loaded carrier) represents a significant proportion of transport cycles (Bröer *et al.* 2002), thus precluding simple thermodynamic analysis. We show in this study that the K_m for glutamine transport by SNAT5 appears to be similar at both membrane faces, facilitating reversibility of the carrier under physiological circumstances; this allows us to produce a more detailed explanation of the mechanisms underlying hepatic glutamine balance *in vivo*.

Methods

Animals and materials

Female toads (*Xenopus laevis*) were obtained from the South African *Xenopus* facility (Noordhoek, South Africa). Female Wistar rats were purchased from Bantin & Kingman (Hull, UK). Collagenase A was purchased from Roche Diagnostics (Mannheim, Germany). L-[^{3}H]-serine, L-[^{3}H]-glutamine and [^{14}C]-sucrose were obtained from NEN (PerkinElmer Life Sciences, Cambridge, UK). All other chemicals were obtained from Sigma (Poole, UK).

Preparation of cRNA

Plasmid DNA was linearized with HindIII (for pSPORT1 – rat SNAT5, Nakanishi *et al.* 2001*a*) or HincII (for pTLN2 – rat SNAT2, Yao *et al.* 2000). cRNA was then synthesized *in vitro* using the T7 or SP6 mMessageMachine kit (Ambion, Austin, TX, USA), as appropriate. The cRNA was purified by phenol–chloroform–isoamyl alcohol extraction, followed by precipitation with sodium acetate and ethanol.

Isolation of oocytes and cRNA expression

Xenopus laevis oocytes were isolated by collagenase treatment (Peter *et al.* 1996) of ovarian tissue dissected from toads killed by anaesthetic (2-aminoethylbenzoate) overdose with destruction of the brain according to UK Home Office guidelines. Stage V–VI (prophase-arrested) oocytes were selected and maintained at 18°C in Modified Barth's Medium (MBM) as previously described (Peter *et al.* 1996). Oocytes were injected with 50 ng cRNA using a positive displacement microinjector (World Precision Instruments, Sarasota, FL, USA). Oocytes were incubated at 18°C for 2–3 days to allow full expression of the cRNA before investigation of overexpressed transport activity.

Measurement of amino acid fluxes in *Xenopus* oocytes

Influx of radiolabelled amino acid tracer in *Xenopus* oocytes was measured as previously described (Peter *et al.* 1996) at 22°C in transport buffer (mM: 100 NaCl, 2 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 3 Hepes, 3 Tris, 3 Mes, pH adjusted to predetermined values from 6.5 to 8.0 by altering the amounts of Hepes, Tris and Mes). NaCl was replaced by LiCl or tetramethylammonium chloride (TMACl) in certain experiments. Individual measurements on 8–11 oocytes were made for each uptake experiment.

Efflux of radiolabelled amino acid tracer in *Xenopus* oocytes was measured as follows (Peter *et al.* 1999). Oocytes were injected with 50 nl buffer containing L-[^{3}H]histidine or L-[^{3}H]glutamine and incubated for 30 min in MBM to allow healing of the plasma membrane. They were then transferred to individual wells of a 24-well plate and tracer loss to transport buffer was monitored for timed periods of up to 90 min. The rate constant for tracer efflux was estimated as the slope of a plot of $\ln(C_t/C_0)$ against time (Peter *et al.* 1999), where C_0 is the level of intracellular [^{3}H]-activity at the start of the experiment (calculated by summation of the radioactivity left in the oocyte at the end of the experiment and sequential losses of radioactivity by efflux to wells) and C_t is intracellular [^{3}H]-activity at the end of each experimental time period (calculated by difference). Individual measurements on six to

eight oocytes were made for each efflux experiment. Preliminary experiments showed that the efflux rate constant for L-[^{3}H]glutamine was unaffected by coinjection of the glutaminase inhibitor 6-diazo-5-oxo-norleucine (at a final concentration of 1 mM in oocyte assuming a cytosol volume of 0.5 μl; Taylor *et al.* 1996), indicating that glutamine catabolism in *X. laevis* oocytes was sufficiently low to allow use of L-[^{3}H]glutamine as a tracer for studying the amino acid efflux properties of rSNAT5 using our protocols

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Liver tissue was excised from a rat killed by cervical dislocation according to UK Home Office guidelines and total liver RNA was isolated using the TRIZOL reagent (Invitrogen/Life Technologies, Paisley, UK). Oligonucleotide primer pairs were synthesized by MWG-Biotech (Milton Keynes, UK) as follows (SNAT3: forward, GTG GTA CAT GGA TGG CAA CTA and reverse, CGT CGT ACC GGG AAC AGA AC; SNAT5: forward, GTT ATT GGC ACC TTC CTG and reverse, CAT CCT GCG CTG AGT AG). RT-PCR was performed using the Access RT-PCR system (Promega, Southampton, UK) according to the manufacturer's protocol using the following PCR amplification conditions: 48°C for 45 min (1 cycle); 94°C for 2 min (1 cycle); 94°C for 30 s, 58°C for 30 s, 68°C for 1 min (40 cycles); 68°C for 7 min (1 cycle). RT-PCR was performed in duplicate tubes, in the presence or absence of AMV reverse transcriptase and an aliquot of RT-PCR product was subject to analytical restriction digestion. Products were run on 2.5% (w/v) agarose gels and visualized under UV light using ethidium bromide.

In situ hybridization

Liver tissue excised from a rat killed by cervical dislocation according to UK Home Office guidelines was fixed at 4°C overnight in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) before being methanol dehydrated and embedded in wax. *In situ* hybridization was performed using standard techniques. Tissue sections (10 μm thick) were baked at 65°C for 30 min, dewaxed in xylene, then rehydrated through graded methanol washes before being postfixed in 4% PFA. Sections were prehybridized in a humid chamber containing 50% formamide in saline buffer at 55° for 1 h. Digoxigenin (DIG)-labelled RNA probes (antisense and sense) were prepared by *in vitro* transcription of linearized DNA plasmids (pSPORT1 *N* rat SNAT5, Nakanishi *et al.* 2001*a*; and pBSIIKS+ *N* rat SNAT3, Chaudhry *et al.* 1999) using the DIG RNA-labelling kit (Roche) with appropriate RNA polymerases. DIG-labelled riboprobes were preheated to 95°C before being applied to the sections and incubated overnight at 65°C. Riboprobes were detected using alkaline phosphatase labelled anti-DIG antibody (Roche) and visualized by nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indotyl phosphate (NBT/BCIP) colour reaction using standard protocols.

Rat liver perfusion

Overnight-fasted adult rats were anaesthetized using 60 mg kg^{-1} pentobarbitone sodium, prepared for *in situ* liver perfusion as previously described (Taylor & Rennie, 1987), then killed by anaesthetic overdose followed by exsanguination according to UK Home Office guidelines. Livers were perfused through the hepatic portal vein with Krebs–Henseleit buffer (pH 7.4 and 37°C unless specified) containing 1% bovine serum albumin (Miles Fraction V) and 15% rejuvenated human erythrocytes (prepared using material obtained from Blood Transfusion Unit, Ninewells Hospital, Dundee, UK). The perfusate was gassed with 95% O_2–5% CO_2 and pumped without recirculation at 1.1 ± 0.15 ml (g liver)$^{-1}$ min^{-1}.

Measurement of amino acid flux in perfused liver

Unidirectional amino acid tracer influx was measured using a rapid, paired-tracer dilution technique (Taylor & Rennie, 1987; Yudilevich & Mann, 1982). [^{3}H]-Glutamine and [^{14}C]-sucrose (extracellular marker) were mixed in 0.1 ml perfusate (0.2 MBq total radioactivity; ^{3}H:^{14}C activity ratio of 5:1) and rapidly injected into the portal inflow. Hepatic venous effluent was collected as 0.15 ml aliquots into 0.15 ml 10% perchloric acid over a 2 min period and processed for dual-channel liquid scintillation counting. [^{3}H]-Tracer uptake by erythrocytes had a negligible effect on tissue tracer uptake over the brief experimental sampling period and venous recovery of the extracellular marker ([^{14}C]-sucrose) exceeded 95%. Isotope dilution profiles of the tracers yielded the time course of amino acid fractional extraction (FE), where FE = 1 − (% ^{3}H dose recovered/% ^{14}C dose recovered). The permeability × surface area (PS) product of the liver sinusoidal barrier was calculated from the maximal unidirectional FE (UFE) as perfusate flow × −ln(1 − UFE). Unidirectional flux of amino acid was estimated as PS product × influent perfusate [AA] and net hepatic AA flux as perfusate flow × (influent [AA] − effluent [AA]), where [AA] represents amino acid concentration. The kinetic characteristics of AA transport were investigated by measuring tracer UFE over the final 2 min of successive 10 min exposure periods to perfusates containing 0.01–50 mM unlabelled glutamine, presented in random order. Transport K_m and V_{max} values were obtained from data by fitting to a rectangular hyperbola using iterative procedures (GraphPad Prism3 software). The effects of putative amino acid transport inhibitors

were assessed in a semiquantitative manner by comparison of tracer FE profiles in the presence or absence of 50 mM unlabelled inhibitor in the 0.1 ml injectate.

HPLC analysis of sample amino acid concentrations

Free amino acid concentrations were determined by reverse-phase high-performance liquid chromatography (HPLC) using a Waters PicoTag system. Oocytes or liver tissue and perfusate samples were processed for amino acid analysis as follows. Five oocytes per experiment were incubated for 2–6 h in MBM (pH 7.5) containing amino acid(s); after the desired incubation period, oocytes were rapidly washed in distilled water, transferred to an Eppendorf tube and residual water removed; oocytes were then homogenized in 20 μl ice-cold water. Perfused liver tissue was snap-frozen using tongs prechilled in liquid nitrogen, powdered using a chilled pestle and mortar and a weighed aliquot of powder homogenized in four volumes of water. For oocyte/liver homogenates and fresh untreated samples of influent and effluent liver perfusate, an equal volume of 20% perchloric acid was added and the sample was vortexed and centrifuged (15 000*g*, 4°C, 10 min) to pellet the precipitated protein. The deproteinized supernatants were transferred to clean Eppendorf tubes, neutralized using 4 M KOH, clarified by centrifugation (15 000*g*, 4°C, 30 min) and derivatized with phenylisothiocyanate (PITC) to produce phenylthiocarbamyl amino acids. Samples were injected onto the PicoTag HPLC column and resolved according to the manufacturer's standard protocols, with postcolumn UV (254 nm) detection of phenylthiocarbamyl amino acids.

Presentation of results

Results are expressed as mean values ± S.E.M. for n measurements or experiments. To test for statistical significance, the difference between mean values was assessed using Student's unpaired t test. Where multiple comparisons were required, analysis of variance (ANOVA) was performed and differences were determined using least significant difference. Significance was assigned at $P < 0.05$.

Results

Bidirectional transport properties of rSNAT5 expressed in *Xenopus* oocytes in relation to intracellular and extracellular substrate concentrations

Amino acid influx. Expression of rat SNAT5 in *Xenopus* oocytes resulted in a substantial increase in uptake of glutamine, serine and histidine (Fig. 1 and Table 1). Amino acid transport activity of SNAT5 exhibited marked pH sensitivity, with influx of substrate increasing with pH in the range 7.0–8.0 (Fig. 1*A*). This is not a generalized response of the experimental system, since there was no effect on glutamine transport by overexpressed System L (4F2hc-IU12 coexpression, Christie *et al.* 2002) between pH 7.0 and 8.0 (data not shown). All subsequent measurements of SNAT5 transport activity were measured at the optimum pH value of 8.0, unless otherwise stated. Serine was shown to be an excellent substrate of rat SNAT5, confirming previous studies of SNAT5 transporters (Nakanishi *et al.* 2001*a*,*b*); in fact serine appears to be preferred to glutamine as a substrate for rat SNAT5 as well as for the structurally related rat SNAT2, having a lower K_m and higher V_{max} for transport in both cases (see Table 1). Amino acid transport activity of SNAT5 (System N) is distinguished from that of SNAT2 (System A) in being Li^+ tolerant (Fig. 2*A*), insensitive to inhibition by MeAIB and proline, but markedly sensitive to inhibition by histidine and glutamate-γ-monohydroxamate (Fig. 2*B* and *C*). The estimated $K_{0.5}$ for glutamate-γ-monohydroxamate inhibition of SNAT5 (0.4 mM) is similar to that reported previously for System N in liver cells (Low *et al.* 1993) and purified liver sinusoidal membrane vesicles (Low *et al.* 1991) and confirms the utility of this compound as an inhibitor of System N transporter. In addition, rSNAT5 was much less sensitive to the membrane depolarizing effects of high external K^+ than rSNAT2. Net 0.5 mM L-[^{3}H]serine influx through SNAT5 or SNAT2 transporters overexpressed in *Xenopus* oocytes (after subtraction of equivalent values for water-injected oocytes; 30 min uptake at pH 8) measured at a fixed NaCl concentration of 50 mM (and using TMACl as appropriate to retain nominal 100 mM salt concentration) was significantly decreased by raising the external KCl concentration from 2 to 50 mM in the case of SNAT2 (58 ± 15% decrease; mean ± S.E.M. for 11 oocytes from one batch, $P < 0.05$)

Table 1. K_m and V_{max} values for influx of substrates by rat SNAT5 and SNAT2 transporters overexpressed in *Xenopus* oocytes, measured in NaCl buffer

Transporter	Substrate	pH	K_m (mM)	V_{max} (pmol per oocyte (30 min)$^{-1}$)
SNAT5	Glutamine	8.0	0.99 ± 0.28	500 ± 63
SNAT5	Serine	8.0	0.73 ± 0.14	701 ± 34
SNAT5	Histidine	7.5	0.21 ± 0.11	252 ± 26
SNAT2	Glutamine	8.0	1.80 ± 0.49	901 ± 88
SNAT2	Serine	8.0	0.70 ± 0.08	1384 ± 44

Individual K_m and V_{max} values were calculated using influx data obtained from a single batch of oocytes.

but not SNAT5 (10 ± 8% decrease; data not shown). This result is consistent with the view that the amino acid transport mechanism of System N transporters is electroneutral whereas that of System A transporters is rheogenic (Chaudhry *et al.* 2001; Bröer *et al.* 2002).

Amino acid efflux. The cumulative losses of L-[^{3}H]histidine and L-[^{3}H]glutamine tracers from *Xenopus* oocytes could each be described in terms of a single rate constant for efflux over periods of up to 90 min under our experimental conditions (see Fig. 3*A*). The rate constant for L-[^{3}H]glutamine efflux from SNAT5-expressing oocytes was substantially higher than that of controls (Fig. 3*A*); similarly, the rate constant for L-[^{3}H]histidine efflux was four times higher in SNAT5-expressing oocytes

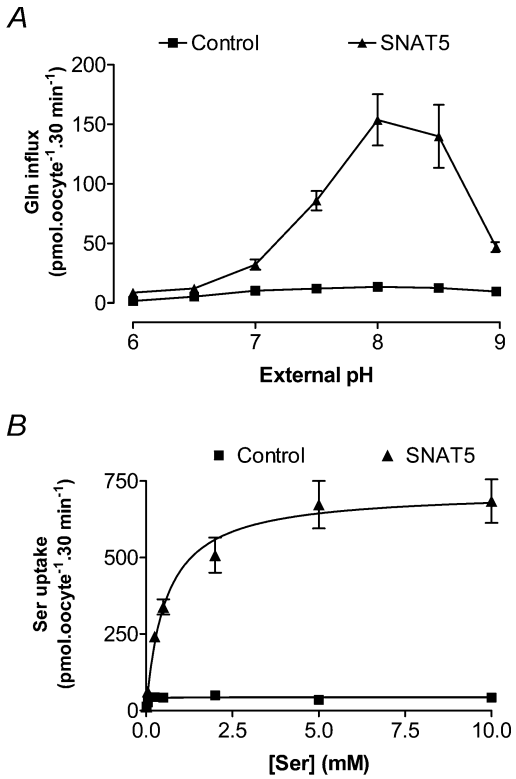

Figure 1. Amino acid influx in oocytes expressing SNAT5
A, the effect of altered external pH on 0.5 mM [^{3}H]-glutamine influx by SNAT5- and water-injected oocytes in NaCl transport buffer. Values are means ± S.E.M. for 10–12 oocytes from a single batch (similar results were obtained in 3 other batches). *B*, serine influx into SNAT5-expressing oocytes as a function of external amino acid concentration. Oocytes expressing SNAT5 or uninjected controls were incubated in NaCl transport buffer (pH 8.0) containing 0.01–10 mM L-[^{3}H]serine for 30 min. Each point represents mean influx ± S.E.M. for 8–11 oocytes from 1 batch. To calculate K_m and V_{max} for SNAT5-mediated amino acid flux, values for uninjected oocytes were subtracted from the corresponding values for SNAT5-expressing oocytes and the data analysed by fitting of a hyperbola using nonlinear regression procedures (GraphPad Prism3); the best-fit hyperbola is indicated on the figure (see also Table 1).

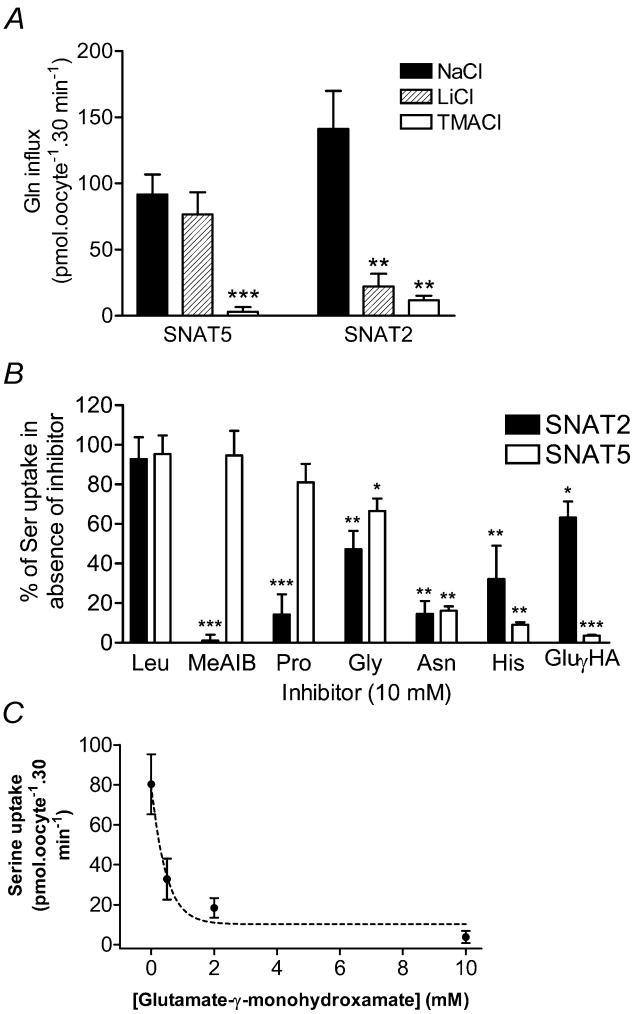

Figure 2. Cation and amino acid substrate selectivity of SNAT5 in comparison with SNAT2
A, influx of 0.5 mM L-[^{3}H]glutamine in control, SNAT5- and SNAT2-expressing oocytes was measured over 30 min in transport buffer (pH 8.0) with each of 3 different major cation chlorides (at 100 mM). Results are presented as means ± S.E.M. for 8–11 oocytes from 1 batch. $^{**}P < 0.01$, $^{***}P < 0.001$ from respective NaCl (control) value after subtraction of corresponding value measured in uninjected oocytes. *B*, effect of unlabelled amino acids and analogues on influx of 0.5 mM L-[^{3}H]serine by SNAT5 and SNAT2 transporters overexpressed in *Xenopus* oocytes (NaCl transport buffer, pH 8.0). Equivalent values for water-injected oocytes were subtracted from raw influx data before calculation of percentage uptake relative to that measured in the absence of putative inhibitor. Results are presented as means ± S.E.M. for 8–11 oocytes from 1 batch. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ from respective value in absence of inhibitor. *C*, concentration dependence of the inhibitory effect of glutamate-γ-monohydroxamate (GluγHA) on influx of 0.5 mM L-[^{3}H]serine by SNAT5 transporter overexpressed in *Xenopus* oocytes (NaCl transport buffer, pH 8.0). Values for water-injected oocytes have been subtracted and results are presented as means ± S.E.M. for 8–11 oocytes from 1 batch. The estimated $K_{0.5}$ for inhibition is 0.4 mM.

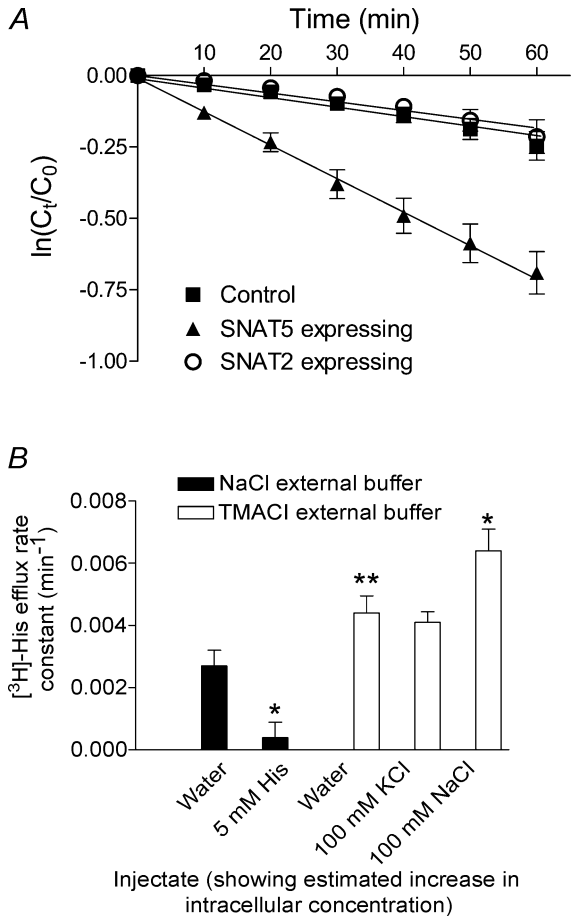

Figure 3. Efflux of substrate amino acids from oocytes expressing SNAT5 but not SNAT2

A, representative result in which oocytes expressing SNAT5 (▲) or SNAT2 (○) and water-injected controls (■) were injected with 50 nl L-[^{3}H]glutamine and incubated for 30 min in MBM to allow healing of the plasma membrane. They were then transferred to individual wells of a 24 well plate and tracer loss was monitored in NaCl transport buffer (pH 8.0) for 60 min. Results are plotted as $\ln(C_t/C_0)$ against time ± S.E.M. for 8 oocytes, where C_0 is the level of radioactivity (c.p.m.) at the start of the experiment and C_t is the level of radioactivity at the end of each experimental time period. These values were calculated by the summation of radioactivity left in the oocyte at the end of the experiment and sequential losses of radioactivity to wells by efflux. *B*, effect of changes in intracellular concentrations of SNAT5 substrates (His, Na^+) on efflux of L-[^{3}H]histidine from SNAT5-expressing oocytes. Oocytes were injected with 50 nl solution containing L-[^{3}H]histidine tracer and either unlabelled L-histidine (to give nominal final concentrations of 5 mM unlabelled L-histidine in oocyte), NaCl or KCl (each increasing intra-oocyte salt concentration by 100 mM). They were then incubated for 30 min in MBM to allow healing of the plasma membrane, transferred to individual wells of a 24 well plate and tracer loss was monitored in NaCl (■) or TMACl transport buffer (□) at pH 8.0. Values for water-injected oocytes have been subtracted and results are expressed as mean efflux rate constant per minute ± S.E.M. for 6–8 oocytes. Similar results were seen in other equivalent experiments using different batches of oocytes. *$P < 0.05$, difference from water injectate value with same buffer; **$P < 0.05$, difference from water injectate value with NaCl external buffer. Basal intracellular histidine concentration was approximately 0.05 nmol per oocyte (equivalent to 0.1 mM).

than controls (0.0023 *versus* 0.00057 min^{-1}; data not shown). These results directly demonstrate: (a) that efflux of substrate amino acids through SNAT5 does occur; and (b) that the mechanism involved does not require the presence of external amino acid. In sharp contrast, there was no difference between efflux of L-[^{3}H]glutamine from SNAT2-expressing oocytes compared to control oocytes under our experimental conditions (Fig. 3*A*), although functional levels of SNAT5 and SNAT2 expression were similar as judged by amino acid influx assays. Efflux of amino acids through SNAT5-expressing oocytes appeared to be saturable, since co-injection of unlabelled histidine with histidine tracer reduced the rate constant for tracer efflux (Fig. 3*B*), presumably due to competition between the two for a common efflux mechanism.

Removal of extracellular Na^+ (i.e. incubation of the cells in TMACl as opposed to NaCl buffer) significantly increased the efflux rate constant for histidine (mean increase 63%; see Fig. 3*B*); this might be viewed conventionally in terms of a stimulation of coupled Na^+–histidine efflux down an outwardly directed Na^+ concentration gradient but, alternatively, it could reflect a *trans*-inhibitory effect of Na^+ itself in the absence of external amino acid cosubstrate (as considered further in the Discussion). An increase in intracellular Na^+ concentration achieved by co-injecting NaCl (sufficient to increase intracellular NaCl by approximately 100 mM) and L-[^{3}H]histidine into oocytes incubated in TMACl buffer was associated with a further (45%) increase in efflux of histidine compared to KCl- or water-injected SNAT5-expressing oocytes (Fig. 3*B*). L-[^{3}H]Histidine efflux through SNAT5 was not significantly affected by change in extracellular pH over the range pH 6.0–8.0 in either NaCl or TMACl buffer, when measured in the absence of extracellular amino acid (Fig. 4*A* and *B*). Such changes in extracellular pH are unlikely to result in a significant change in intraoocyte pH (Rahman *et al.* 1999), therefore the overall charge on the intracellular pool of free histidine will be largely unaffected during these experiments. Addition of 2 mM unlabelled glutamine to external NaCl buffer produced a *trans*-stimulatory effect on L-[^{3}H]histidine efflux at pH 8, the pH optimum for glutamine influx by SNAT5, but not at lower pH values (Fig. 4*A*). In sharp contrast, 2 mM external glutamine added to Na^+-free (TMACl) buffer produced a significant *trans*-inhibition of histidine efflux at pH 8 (Fig. 4*B*), although not at pH 6. There was no difference between the efflux of L-[^{3}H]glutamine from SNAT2-expressing oocytes compared to that in control oocytes when the Na^+ concentration gradient was reversed by injection of NaCl solution (as described above), either in the presence or absence of external 2 mM glutamine (data not shown).

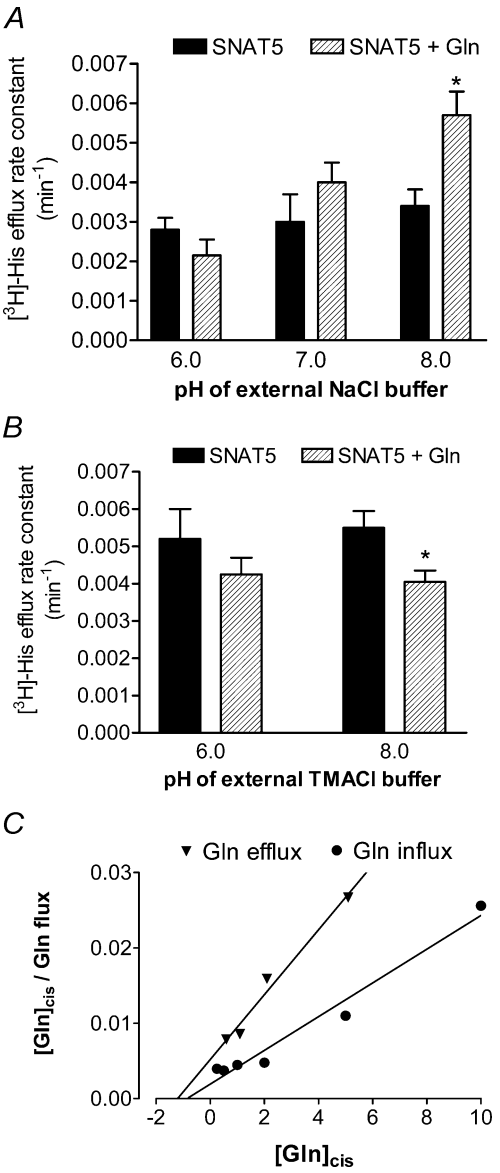

Figure 4. Kinetics characteristics and pH sensitivity of SNAT5
A and *B*, effect of changes in extracellular pH and glutamine concentration on efflux of L-[^{3}H]histidine from SNAT5-expressing oocytes. Oocytes were injected with 50 nl solution containing L-[^{3}H]histidine tracer, then incubated for 30 min in MBM to allow healing of the plasma membrane. Oocytes were transferred to individual wells of a 24 well plate and tracer loss was monitored in either NaCl (*A*) or TMACl transport buffer (*B*) under the conditions stated (2 mM external glutamine as indicated). Values for water-injected oocytes have been subtracted and results are expressed as mean efflux rate constant per minute ± S.E.M. for experiments in 3 batches of oocytes. $^*P < 0.05$, difference between values ±2 mM Gln at pH 8. *C*, Hanes plot of glutamine fluxes measured for SNAT5 overexpressed in oocytes, where $[Gln]_{cis}$ represents glutamine concentration at the *cis*-face of the plasma membrane (intracellular [Gln] for efflux, extracellular [Gln] for influx). L-[^{3}H]Glutamine fluxes were measured in NaCl transport buffer (pH 8.0) using methods described in the text and Table 2 legend. Values shown are means for 6–8 oocytes at each point (after subtraction of flux in water-injected oocytes) for a single batch of oocytes. Lines were fitted using GraphPad Prism3 software, describing SNAT5 kinetic characteristics as follows: glutamine influx $K_m = 0.86$ mM and $V_{max} = 447$ pmol per oocyte (30 min)$^{-1}$; and glutamine efflux $K_m = 1.2$ mM and $V_{max} = 232$ pmol per oocyte (30 min)$^{-1}$.

The specific properties of glutamine efflux from SNAT5-expressing oocytes were further investigated by co-injecting L-[^{3}H]glutamine with quantities of unlabelled glutamine calculated to increase the intracellular concentration by 0.5, 1, 2 and 5 mM. The background intracellular concentration of glutamine is approximately 0.05 nmol per oocyte (0.1 mM; as determined by HPLC analysis), representing only a minor contribution to the total intracellular glutamine concentration in these experiments. The rate constant for L-[^{3}H]glutamine efflux by SNAT5 was progressively reduced by increase in intracellular glutamine concentration up to the putative 5 mM value (Table 2), consistent with the operation of a saturable efflux mechanism. Calculation of the magnitude of glutamine efflux through SNAT5 in oocytes containing various concentrations of unlabelled glutamine, as the product of rate constant for efflux and cell glutamine concentration (pmol per oocyte) assuming oocytes contain a single glutamine pool (Table 2), allowed the K_m and V_{max} for glutamine efflux to be estimated as 1.2 mM and 232 pmol per oocyte (30 min)$^{-1}$, respectively (Fig. 4*C*). The K_m values for glutamine efflux and influx by SNAT5 therefore appear to be of similar order, although the estimated V_{max} value for glutamine efflux by SNAT5 is lower than that for influx under experimental conditions (Fig. 4*C*).

Bidirectional transport of the SNAT5 substrate glutamine in perfused rat liver in relation to rSNAT5 transport properties

Expression of System N (SNAT3 and SNAT5) transporters in rat liver. We have confirmed that rat liver expresses mRNA for both SNAT3 and SNAT5 using RT-PCR (Fig. 5). The PCR primers used were designed within the open reading frame of transporter DNA sequence to generate PCR products spanning several exons. Identity of both SNAT3 and SNAT5 PCR products was confirmed by restriction digest analysis.

Perfused liver studies. Individual paired-tracer dilution profiles for [^{3}H]-glutamine and [^{14}C]-sucrose in livers perfused at constant flow (Fig. 6*A*) were used to produce a time course of fractional glutamine tracer extraction (Fig. 6*B*). Substantial extraction of glutamine tracer from portal perfusate (~0.7 tracer UFE) was observed, comparable to that measured *in vivo* (Pardridge, 1977) at similar rates of portal fluid flow.

Table 2. Glutamine efflux from oocytes via overexpressed SNAT5 at different intracellular glutamine concentrations

Intracellular glutamine concentration* (pmol per oocyte)	Rate constant for glutamine efflux through SNAT5 (min^{-1})	Glutamine efflux through SNAT5 (pmol per oocyte (30 min)$^{-1}$)
50† (0.1 mM)	0.0088 ± 0.0016	13.2
300 (0.6 mM)	0.0085 ± 0.0012	76.5
550 (1.1 mM)	0.0078 ± 0.0027	128.7
1050 (2.1 mM)	0.0042 ± 0.0010	132.3
2550 (5.1 mM)	0.0025 ± 0.0008	191.3

*Oocytes were coinjected with L-[^{3}H]glutamine solution containing unlabelled L-glutamine to give final [glutamine] as indicated. Values in parentheses represent estimated intracellular [Gln] in mM, calculated assuming a water space of 0.5 μl per oocyte (Taylor *et al.* 1996). L-[^{3}H]Glutamine efflux was measured in NaCl transport buffer (pH 8.0) and results are expressed as mean efflux rate constant for glutamine per minute ± S.E.M. for 6–8 oocytes, after subtraction of values measured in oocytes not expressing rSNAT5. Similar results were observed in a second batch of oocytes. Unidirectional glutamine efflux through SNAT5 was calculated from rate constant × intracellular [Gln]. †Basal intracellular glutamine concentration measured in water-injected oocytes.

The permeability × surface area (PS) product of the liver sinusoidal barrier for glutamine tracer (Fig. 6*C*) was progressively reduced as the concentration of unlabelled glutamine in the perfusate was increased, as expected for a saturable transport mechanism. Calculated glutamine influx at a physiologically relevant perfusate concentration of 0.5 mM was primarily Na^+ dependent, substantially Li^+ tolerant and reduced at acidic pH (Fig. 7*A*); uptake under Na^+-free conditions also included a saturable component (data not shown). [^{3}H]-Glutamine tracer FE was substantially reduced by co-injection of 50 mM unlabelled His, Gln, Asn and Ser (in descending order of potency), but D-Gln was without effect (Fig. 6*B* and Table 3). This protocol produced an estimated peak concentration of 3.3 mM unlabelled amino acid at the liver sinusoidal membrane (see legend to Table 3) and the 46% inhibition of [^{3}H]-glutamine tracer FE seen under these conditions is similar to that observed for comparable glutamine concentrations (2 or 5 mM) under steady-state conditions (Fig. 6*C*). The above results are consistent with previous observations indicating that Systems N and L are the major contributors to glutamine influx in liver cells (Kilberg *et al.* 1980; Low *et al.* 1992). We therefore analysed the data of Fig. 6*C* in terms of a two-site binding model using Prism 3.0 software (Graphpad), which generated the following kinetic characteristics: for System N, $K_m = 1.7$ mM, $V_{max} = 1.7$ μmol (g liver)$^{-1}$ min^{-1}. For System L, $K_m = 5.6$ mM and $V_{max} = 0.8$ μmol (g liver)$^{-1}$ min^{-1}.

System L (LAT1 and LAT2) operates typically as an obligate amino acid exchanger (Meier *et al.* 2002) which would be inactive in the absence of external amino acid substrate and would also generate predominantly glutamine–glutamine homo-exchanges under our experimental conditions. The System L2 (LAT3) transporter also expressed in liver mediates facilitated diffusion, but glutamine is not a preferred substrate of this transporter (Babu *et al.* 2003). On this basis, it seemed reasonable to assume that unidirectional glutamine efflux through System L in our perfused liver preparation would at most only match influx by the same route and hence any net glutamine efflux should be through a different mechanism, such as the reversible System N transporters. Net glutamine efflux in the absence of extracellular glutamine was −0.45 μmol (g liver)$^{-1}$ min^{-1} (see Fig. 7*B*). Net glutamine flux in perfused livers switched from efflux to influx at perfusate [Gln] of approximately 0.55 mM. Hepatic [glutamine] at a physiological perfusate glutamine concentration of 0.5 mM (3.95 μmol (g liver)$^{-1}$; see Fig. 7*B*) was similar to that measured *in vivo* (Häussinger *et al.* 1985), but changed markedly after 10 min perfusion with either 0 or 5 mM glutamine (2.95 μmol (g liver)$^{-1}$ decrease and 6.4 μmol (g liver)$^{-1}$ increase, respectively).

Distribution of SNAT3 and SNAT5 within the liver acinus. *In situ* hybridization of DIG-labelled riboprobes for SNAT3 and SNAT5 mRNAs in rat liver sections revealed differences in the intrahepatic distribution of expression of the two transporters. The overall level of probe hybridization (as judged by NBT/BCIP staining of whole sections) was similar for both transporter mRNAs but, at the level of a liver acinus, SNAT5 mRNA showed a relatively diffuse distribution within periportal regions (Fig. 8, left-hand panels), wheras SNAT3 mRNA showed a pattern of distribution concentrated within the perivenous region bordering central veins (Fig. 8, right-hand panels).

Discussion

The expression of rat SNAT5 (SN2) cRNA in *Xenopus* oocytes leads to an accelerated, Na^+-dependent accumulation of glutamine, histidine and serine. The expression of SNAT5, but not SNAT2 (SAT2), also leads to an increase in efflux of ^{3}H-labelled glutamine and histidine from oocytes, demonstrating that System N (SNAT5) but not System A (SNAT2) is readily capable of mediating bidirectional fluxes of amino acid substrates. The ability of SNAT5 to mediate both accumulation of glutamine from an external supply and efflux of glutamine into amino acid-free medium demonstrates the important capability of System N to facilitate *net* movement of amino acid across the plasma membrane both in and out of cells. The estimated K_m for efflux of glutamine from SNAT5-expressing oocytes is very similar to the K_m for

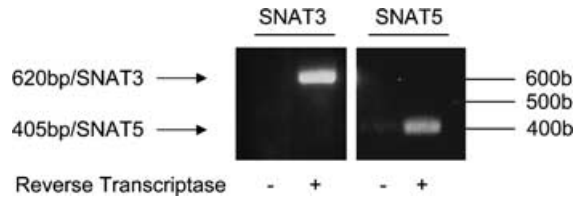

Figure 5. RT-PCR products detected in rat liver using probes specific for SNAT3 and SNAT5
Both RT-PCR products were of the predicted size and their identity was confirmed using specific restriction enzyme digests. No PCR products were detected in the absence of a reverse transcriptase step.

influx (approximately 1 mM; Fig. 4*C*). The K_m for histidine efflux by SNAT5 expressed in oocytes (0.33 mM; estimated using the same technique as for glutamine, data not shown) is also similar to the measured K_m for histidine influx (0.21 mM; see Table 1). The SNAT5 transporter thus differs from other amino acid transporters such as the System L exchangers LAT1- and LAT2-4F2hc, which have markedly different affinities for substrate influx and efflux. These latter transporters recognize the same amino acids on either side of the membrane, but the affinity on the intracellular side appears to be markedly lower than on the extracellular face of the membrane (Meier *et al.* 2002). The influx K_m values for amino acid substrates of SNAT5 measured in the present study are broadly consistent with those reported previously for both human and rat orthologues (Nakanishi *et al.* 2001*a*,*b*) although our values, measured using radiotracer techniques, are slightly lower than those obtained from measurement of substrate-induced currents (Nakanishi

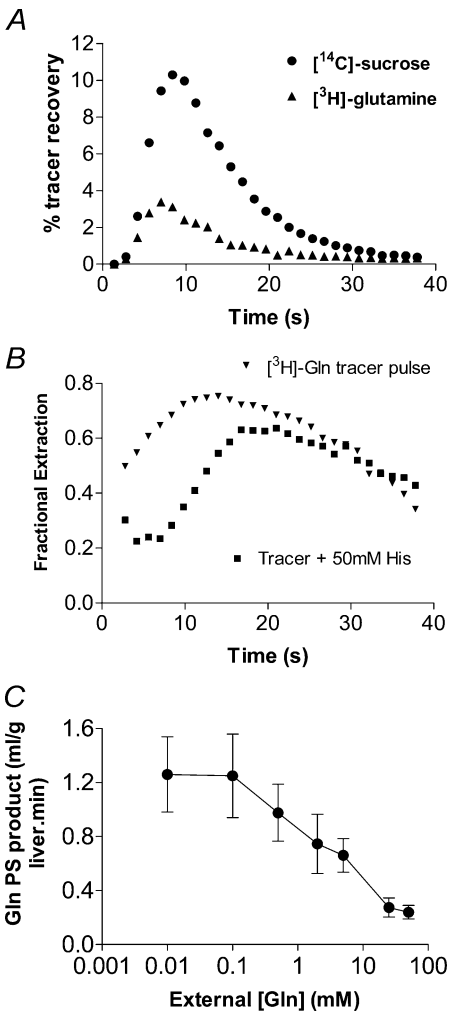

Figure 6. Glutamine tracer extraction by perfused rat liver
A, representative paired-tracer isotope-dilution profiles for [^{3}H]glutamine and [^{14}C]sucrose in perfused rat liver (perfusate flow of 0.9 ml (g liver)$^{-1}$ min^{-1}). *B*, time course of fractional extraction of [^{3}H]glutamine tracer in perfused rat liver in presence and absence of 50 mM L-histidine in the injectate. *C*, permeability × surface area (PS) product of L-glutamine in perfused rat liver as a function of perfusate glutamine concentration (means ± S.E.M., $n = 4$–6 perfusions).

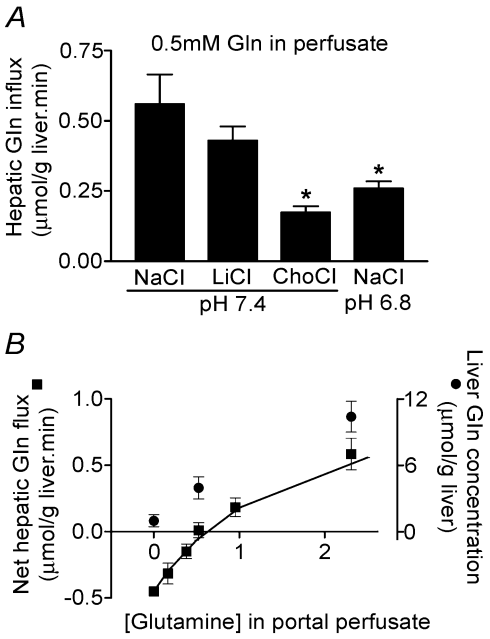

Figure 7. Glutamine fluxes in perfused rat liver
A, effect of changing external cation or perfusate pH on 0.5 mM glutamine influx in perfused rat liver (means ± S.E.M., $n = 4$–6 perfusions). $^*P < 0.05$ from value in NaCl at pH 7.4. *B*, net glutamine flux (■, means ± S.E.M., $n = 4$–8 perfusions) and liver glutamine concentration (●, means ± S.E.M., $n = 5$ livers) in perfused rat liver as a function of influent (portal) glutamine concentration after a 10 min perfusion period. The continuous line represents the predicted flux based on calculated System N (SNAT5 plus SNAT3) kinetic properties in perfused rat liver (excluding any contribution of other routes to net glutamine flux; see text for additional details); that is, $K_m = 1.7$ mM, $V_{max} = 1.7$ μmol (g liver)$^{-1}$ min^{-1} for influx and V_{max} of -0.45 μmol (g liver)$^{-1}$ min^{-1} for efflux. This simple model assumes that intracellular glutamine appearance is sufficiently high to effectively saturate the glutamine efflux mechanism, which is likely to hold under physiological circumstances when intracellular glutamine pools are not depleted (here net release matches net synthesis). Hepatic portal glutamine concentration (generally 0.35–0.65 mM) is lower than that in the general circulation due to net glutamine extraction by the intestine. The model also accurately predicts the increase in intrahepatic glutamine concentration after a 10 min perfusion with 5 mM glutamine.

Table 3. Inhibition of [^{3}H]-glutamine tracer uptake by co-injected amino acids in perfused rat liver

Injectate composition*	Maximum percentage inhibition of tracer extraction
[^{3}H]Gln tracer	0 (control)
[^{3}H]Gln +50 mM L-Gln	46 ± 5 (5)
[^{3}H]Gln +50 mM D-Gln	2 ± 7 (4)
[^{3}H]Gln +50 mM L-His	65 ± 4 (4)
[^{3}H]Gln +50 mM L-Asn	43 ± 6 (3)
[^{3}H]Gln +50 mM L-Ser	31 ± 4 (3)

Values shown are means ± S.E.M. (number of livers). *Estimated peak concentration of amino acid at the liver sinusoidal membrane with 50 mM pulse injection of unlabelled amino acid into influent perfusate (based on tracer recovery data such as shown in Fig. 6) was 3.3 mM.

et al. 2001*a*). Histidine has the highest affinity of all natural amino acid substrates for SNAT5 when measured by radiotracer flux (present study and Nakanishi *et al.* 2001*b*), although it is surprisingly poor at inducing SNAT5-dependent currents (Nakanishi *et al.* 2001*a*), and serine appears to be equally as good a substrate as glutamine. This is somewhat different to the situation for SNAT3 (SN1), in which histidine and glutamine each have the same K_m value of approximately 1.5 mM (Fei *et al.* 2000; Gu *et al.* 2000) and serine is not a preferred substrate (Mackenzie & Erickson, 2004). In other respects (for example, extent of pH, Na^+ and Li^+ dependence), the two System N transporter isoforms SNAT3 and SNAT5 appear to exhibit similar kinetic properties.

We have shown directly that SNAT5 has similar apparent affinity for amino acids at both faces of the cell membrane. If this is also the case for Na^+ binding ($K_{0.5}$ for external Na^+ stimulation of SNAT5 transport activity is 11 mM; Nakanishi *et al.* 2001*a*), then observed differences in V_{max} for amino acid influx and efflux through SNAT5 may largely reflect the presence of subsaturating intracellular Na^+ concentration (approximately 10 mM in oocytes; Costa *et al.* 1989) under normal conditions. This would be consistent with our observation that elevation of intracellular [Na^+] stimulates glutamine efflux. The availability of internal Na^+ may therefore be an important factor regulating flux through SNAT5 as already suggested for SNAT3 (Bröer *et al.* 2002). Equally, an increase in intracellular concentration of glutamine also increases glutamine efflux through SNAT5 until the efflux mechanism becomes fully saturated.

The transport activities of both SNAT3 and SNAT5 have been shown directly to include coupled counter-transport of H^+ (Chaudhry *et al.* 1999, 2001; Nakanishi *et al.* 2001*a*), which may render their mechanisms electroneutral and also facilitate physiologically relevant bidirectional flux of amino acids. This proposed mechanism is consistent with our observation that influx of serine through SNAT5 (but not SNAT2, which does not transport H^+ and is thus rheogenic) is largely unaffected by changes in membrane potential. Similar observations have been reported for rat SNAT3 (Chaudhry *et al.* 1999, 2001), although a slight decrease in activity in highly depolarized cells is noted for both SNAT5 (Nakanishi *et al.* 2001*a*) and SNAT3 (Bröer *et al.* 2002). We now also show that changes in extracellular pH in the range 6–8 have no significant effect on the unidirectional efflux of [^{3}H]-labelled histidine in the absence of extracellular amino acid substrate. In contrast, a decrease in intracellular pH stimulates amino acid influx through SNAT5 (Nakanishi *et al.* 2001*a*), revealing an apparent asymmetry in the SNAT5 transport mechanism with respect to *trans*-effects of pH, which is consistent with the view that the transmembrane pH gradient *per se* is not a primary driving force for the unidirectional efflux of amino acids through SNAT5 (as already suggested in the case of SNAT3; Bröer *et al.* 2002). It should be noted that there is unlikely to be any significant change in intracellular pH under the conditions used in our experiments (Rahman *et al.* 1999).

The observation that amino acid efflux through SNAT5 occurs in the absence of both external Na^+ and glutamine indicates that the empty (or H^+-bound) carrier, after offloading substrates to the external medium, reorientates from the outward-facing to inward-facing conformation fairly readily, thus helping to account for the relatively weak coupling between influx and efflux modes of SNAT5 transport activity. Carrier reorientation may be an important rate-determining step in the overall System N transport cycle and it is important to note that the presence outside the cell of either glutamine or Na^+ in the absence of the other produces effects consistent with *trans*-inhibition of amino acid efflux through SNAT5. Furthermore, the addition of glutamine to NaCl buffer under conditions (pH 8) eliciting maximum glutamine influx only *trans*-accelerates glutamine efflux through SNAT5 to levels observed in the absence of both external substrates (that is, in TMACl buffer). Nevertheless, these results demonstrate directly that amino acid influx and efflux through SNAT5 may occur simultaneously. Mouse SNAT3 (mNAT) also shows *trans*-stimulation of amino acid efflux in NaCl buffer (Gu *et al.* 2000). Our observations are consistent with a model in which fully loaded and empty SNAT5 carriers reorientate conformation at similar rates at pH 8, but where the binding of only one external substrate to the carrier retards reorientation until either the second substrate attaches or the first substrate dissociates from its binding site without being translocated. This type of mechanism would minimize reorientation of the partly loaded carrier. This model is binding-order independent, although an ordered binding model of SNAT3 function has been proposed (Bröer *et al.* 2002) in which amino acid binds before Na^+,

alongside a slippage mode in which the carrier translocates without binding protons (which would generate a non-stoichiometric inward current). There is clearly no translocation of amino-acid loaded SNAT5 carrier in the absence of Na^+ (as there is negligible amino acid tracer influx under Na^+-free conditions). Both Na^+ and amino acid are required for H^+ flux through System N (SNAT3) as judged by intracellular pH change (Chaudhry *et al.* 1999) and it is possible that H^+ only binds after Na^+ and amino acid have bound or translocated. The SNAT3 transport mechanism may include an exchange of Na^+ since glutamine uptake is reported to be accompanied by cotransport of two or three Na^+ ions with a simultaneous release of preloaded $^{22}Na^+$ (Bröer *et al.* 2002). At least with respect to reorientation of SNAT5 to the inwardly facing conformation, movement of the empty (or H^+-bound) carrier appears to be independent of external pH, whereas the fully loaded carrier shows significant dependence on external pH (see Fig. 4*B*). This is consistent with the observation that amino acid influx through SNAT5 is markedly inhibited by lowering external pH (Fig. 1; also Nakanishi *et al.* 2001*a*,*b*), a feature conserved among all members of the SNAT family studied to date (Fei *et al.* 2000; Sugawara *et al.* 2000*a*; Varoqui *et al.* 2000) which may involve an inhibitory allosteric effect of H^+ on the influx mechanism (Bröer *et al.* 2002). The overall result of extracellular acidification will therefore be a change in the net flux of amino acid substrate through System N (SNAT5 and SNAT3) due to the predominant inhibitory effect on the influx mechanism; indeed, it has been shown directly that acidification of the external medium makes the SNAT3 transporter isoform 'run in reverse' and release preloaded glutamine (Bröer *et al.* 2002).

The glutamine transport capacity of intact, perfused rat liver is sufficient to extract over 70% of total delivery from a physiological portal load of 0.35–0.7 mM glutamine and has kinetic properties consistent with System N being the major overall contributor (that is, Na^+-, Li^+- and pH-dependent glutamine influx having histidine and asparagine as competitors). Hepatocytes constitute the predominant exchangeable amino acid pool in the liver (Häussinger, 1990; Bode, 2001 for reviews) and, given that System N is the only amino acid transport mechanism capable of mediating substantial net glutamine fluxes under our experimental conditions, we (amongst others, see Bode, 2001) ascribe net hepatic glutamine fluxes (to a first approximation) in terms of movements through System N of hepatocytes. Both System N transporter isoforms (SNAT5 and SNAT3) are expressed in mammalian liver (e.g. see Fig. 5) at similar relative mRNA abundance (Nakanishi *et al.* 2001*a*,*b*). Our *in situ* hybridization studies have revealed that SNAT5 mRNA is expressed largely in periportal (glutamine-consuming) hepatocytes. In contrast, SNAT3 mRNA expression increases along the rat liver acinus from periportal to perivenous (glutamine-producing) hepatocyte regions, consistent with the expression pattern for SNAT3 protein observed in mouse liver (Gu *et al.* 2000). Assuming that mRNA localization reflects protein expression, our observations establish the likelihood of an important role for SNAT5 in hepatic glutamine exchanges consistent with our functional measurements of System N transport in perfused liver; for example, the apparent preference of histidine as a substrate/inhibitor and the significant interaction of serine with glutamine influx. Histidine has also previously been reported to be a

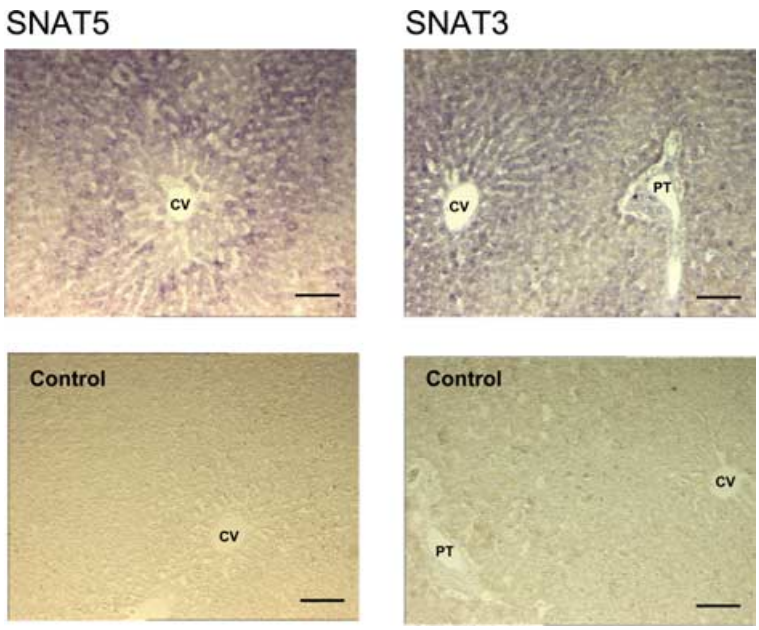

Figure 8. *In situ* hybridization of DIG-labelled riboprobes to SNAT5 and SNAT3 mRNAs in rat liver
Hybridization of antisense riboprobes (upper panels) on wax tissue sections was detected colourimetrically using alkaline phosphate-labelled anti-DIG antibody. Specificity of detection was confirmed by use of the respective sense riboprobe as a control (lower panels). SNAT5 mRNA distribution is largely diffuse within periportal regions, whereas SNAT3 mRNA distribution is graded towards the perivenous region bordering each central vein. Scale bars represent 50 μm. CV, central vein; PT, portal triad.

potent inhibitor of both glutamine uptake and release in perfused rat liver (Häussinger *et al.* 1985), having half-maximal effects at concentrations (0.15–0.4 mM histidine) of very similar order to the histidine K_m value reported here for SNAT5. This raises the possibility that histidine (or indeed serine) availability, as well as SNAT5 activity, may have a regulatory role in hepatic glutamine and nitrogen turnover. In addition, the localization of histidase in periportal hepatocytes (Sano *et al.* 1997) alongside the histidine-preferring SNAT5 transporter may have particular significance for modulation of histidine metabolism.

Our results are consistent with the idea that net hepatic glutamine balance, which is of particular importance for modulation of whole-body glutamine turnover and nitrogen metabolism in health and disease, may largely be dictated by the activity of transport System N (Häussinger, 1998; Bode, 2001 for review). The observations that influx and efflux through System N transporters may be largely decoupled from one another and occur simultaneously mean that fluxes of glutamine in periportal and perivenous liver cells may be independent but remain summative for overall hepatic glutamine balance. Changes in glutamine delivery to liver cells (either exogenous or endogenous supplies) are associated with significant and rapid alterations in intracellular glutamine concentration (Häussinger *et al.* 1985; Lenzen *et al.* 1987; see also Fig. 7*B*), consistent with a regulatory role for glutamine transport at the plasma membrane in hepatic glutamine metabolism. Our observation that the K_m for amino acid binding to SNAT5 is similar at both membrane faces may help explain the reversibility of the carrier under physiological circumstances. Intrahepatic glutamine concentration *in vivo* normally remains above 3.5 μmol (g liver)$^{-1}$ or approximately 7 mmol l^{-1} intracellular water (Häussinger *et al.* 1985); that is, sufficiently high to keep glutamine efflux through System N near saturation. Thus, under normal circumstances the major variable contributing to changes in net hepatic glutamine flux will be glutamine influx (a process proportional to portal [Gln] in such situations), making hepatic glutamine balance highly sensitive to circulating [Gln] so long as uptake and synthetic processes are capable of maintaining sufficiently high intracellular [Gln]. In particular, the glutamine pool of periportal cells is critically dependent on supply from the portal plasma, which is delivered at least partly through SNAT5. In the absence of external glutamine supply, periportal cells rapidly become glutamine depleted (Häussinger *et al.* 1985), presumably accounting for the reduction in liver glutamine concentration we observe after perfusion with glutamine-free medium (Fig. 7*B*). The measured changes in intracellular [Gln] in response to addition or removal of external glutamine are modelled remarkably well in terms of a simple reversible System N carrier, in which influx and efflux modes are saturable but not coupled kinetically, in both perfused liver (Fig. 7*B*) and SNAT5-expressing oocytes (Fig. 9). Net hepatic glutamine flux in the liver is effectively determined by [glutamine] in portal plasma according to this kinetic model. Net glutamine flux in perfused livers switched from efflux to influx at perfusate [Gln] of approximately 0.55 mM, a similar value to that at which the flux mediated by SNAT3 reverses (Chaudhry *et al.* 1999).

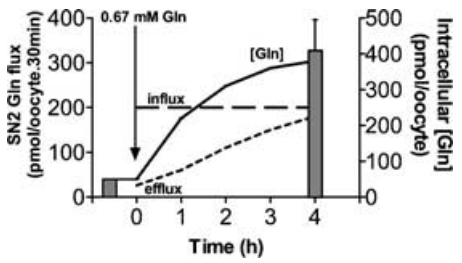

Figure 9. Predicted and measured changes in intracellular glutamine concentration in *Xenopus* oocytes overexpressing SNAT5 (SN2) over a 4 h period following exposure to 0.67 mM L-glutamine in MBM

Predicted changes are based on a simple kinetic model in which glutamine influx and efflux through SNAT5 overexpressed in *Xenopus* oocytes are decoupled and intracellular [glutamine] changes with time as a function of the net glutamine flux. Glutamine accumulates in the oocyte as a function of constant glutamine influx from an external supply (0.67 mM) and a progressively stimulated glutamine efflux. Over a 4 h period, the increase in intracellular [Gln] predicted by this model (based on net intracellular accumulation) is close to that measured under experimental conditions.

The sources of glutamine for net efflux in the absence of extracellular glutamine (amounting to -0.45 μmol (g liver)$^{-1}$ min^{-1}) are the intracellular glutamine stores, protein breakdown and (in the small perivenous hepatocyte population) *de novo* glutamine synthesis. Glutamine production by liver from protein breakdown (0.1 μmol (g liver)$^{-1}$ min^{-1}; recalculated from data obtained from isolated hepatocytes originally expressed per dry weight cells; Low *et al.* 1993) is not sufficient to account for this rate of release from the large periportal hepatocyte population, hence a substantial depletion of the intracellular glutamine pool occurs. In contrast, the sum of endogenous glutamine synthesis (0.15–0.4 μmol (g liver)$^{-1}$ min^{-1}; Häussinger *et al.* 1983, 1985) and glutamine produced from protein breakdown in perivenous hepatocytes would be sufficient to maintain the observed rate of glutamine release. Glutamine entering periportal cells is either catabolized or accumulated and indeed net accumulation from 5 mM glutamine (6.4 μmol (g liver)$^{-1}$ over 10 min) is matched by net glutamine uptake over the same period (0.59 μmol (g liver)$^{-1}$ min^{-1}; see Fig. 7*B*). The urea-synthesizing periportal hepatocytes consume bicarbonate, which will acidify the cytosol and promote glutamine uptake (Bode, 2001) through a System N transporter, which we may now identify as periportally

located SNAT5 (and also relatively less abundant periportal SNAT3). It is therefore likely that hepatic plasma becomes depleted of glutamine as it progresses along the periportal region and is then replenished by glutamine release from perivenous cells. Intrahepatic glutamine concentration is also strongly related to external pH, increasing under alkaline conditions (Lenzen *et al.* 1987), as would be predicted on the basis of SNAT5/SNAT3 transport activities. The Na^+–H^+ exchange inhibitor amiloride abolishes any extra–intracellular pH gradient in perfused liver; this is associated with inhibition of hepatic glutamine uptake and loss of pH dependency for hepatic glutamine concentration ($2\,\mu$mol (g liver)$^{-1}$ under all conditions; Lenzen *et al.* 1987). In the intact liver, a shift from net glutamine uptake to net glutamine release (e.g. as occurs in plasma acidosis or after feeding a low-protein diet) appears to occur largely as a result of decreased periportal glutamine uptake and degradation with relatively constant perivenous glutamine synthesis and release (Almond *et al.* 1992; Watford, 2000). A relatively important role for SNAT5 in such processes may be inferred both from the periportal distribution of the transporter and its functional properties. For example, a reduced glutamine influx through hepatic SNAT5 during metabolic acidosis and consequent increased net glutamine efflux would help limit the substrate supply for urea synthesis and result in the diversion of glutamine to the kidneys, where it is used to drive increased ammonium (i.e. acid) excretion in the urine (Häussinger *et al.* 1985).

Many catabolic conditions result in increased intracellular Na^+ concentration, which may also contribute to changes in flows of glutamine during disease by increasing unidirectional (and hence net) efflux from SNAT5-expressing cells. The reported reduction in glutamine efflux from liver cells (Häussinger *et al.* 1985) (also astrocytes; Deitmer, Bröer & Bröer, 2003) in the absence of external Na^+ is likely to result from depletion of the intracellular Na^+ pool over lengthy experimental periods in Na^+-free buffer and a concomitant decrease in System N activity. This is not a significant problem for large cells such as oocytes over brief experimental time periods, under which conditions both we and others (Bröer *et al.* 2002) show at least transient stimulation of efflux when external Na^+ is removed.

SNAT3 and also possibly SNAT5 are also believed to be involved in glutamine–glutamate cycling within the brain (Mackenzie & Erickson, 2004). SNAT5 appears to differ from SNAT3 in being an effective transporter of serine; thus it is conceivable that net L-Ser efflux through SNAT5 may be a major route by which serine leaves astrocytes as part of the Gly–Ser shuttle within the brain (Verleysdonk & Hamprecht, 2000). Both SNAT3 and SNAT5 are expressed in the choroid plexus (Xiang *et al.* 2003), where their principal function may be removal of glutamine (and hence amino-N) from the brain.

The newly established role of the Na^+-dependent System N amino acid transporters SNAT5 and SNAT3 in bidirectional substrate transport contrasts with the traditional view that such carriers act as secondary active transporters. We detected no significant increase in efflux of glutamine from System A (SNAT2)-expressing oocytes, which indicates that slippage or reversal of this transport mechanism does not occur to any appreciable extent, consistent with the representation of SNAT2 as a classical rheogenic transporter that operates using the energy from the inwardly directed Na^+ gradient.

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