

## Identification of the multivalent PDZ domain protein PDZK1 as a binding partner of sodium-coupled monocarboxylate cotransporter 1 (SMCT1)

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Sodium-coupled monocarboxylate transporter 1 (SMCT1), belonging to the sodium/glucose cotransporter gene family (SLC5A8), is expressed in the colon, small intestine and kidney. It mediates the transport of short-chain fatty acid, nicotinate and lactate (1). Because URAT1 transports urate in exchange for intracellular organic anions such as lactate and nicotinate, SMCT1 is suggested to be the important factor that may modify the transport function of URAT1 (2). In addition, the C-terminal domain of SMCT1 is exposed to the cytoplasmic compartment and contains PDZ motif, one of the famous protein-protein interaction modules, suggesting that it may interact with PDZ proteins. In the present study, we have used the yeast two-hybrid screening to investigate the putative SMCT1-associated proteins in the kidney. Using the SMCT1 C-terminal tail (SMCT1-CT) as bait, we performed a yeast two-hybrid screen of a cDNA library constructed from human adult kidney. From a total of  $3.7 \times 10^6$  independent colonies

screened, 22 positive clones were obtained. Of these positive clones, 13 yielded an identical sequence encoding the gene for the multivalent PDZ domain-containing protein PDZK1. Deletion of the SMCT1 C-terminal PDZ motif abolished the interaction with PDZK1 in the yeast two-hybrid system. In addition, the first and third PDZ domains of PDZK1 associate strongly with the SMCT1-CT. The association of SMCT1 with PDZK1 enhanced [ $^3\text{H}$ ]nicotinate transport activities in HRPE cells (1.7-folds). The elucidation of these interactions may lead to the further understanding of the function and regulation of urate transport via monocarboxylate handling in human kidney

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## In human intestinal cells, short-term epidermal growth factor (EGF) increases ASCT2 surface expression and glutamine (GLN) transport activity by MAPK and PI3K dependent pathways

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Epidermal growth factor (EGF) is a major trophic factor for the intestine, and supports repair and adaptation after massive enterectomy. The decrease in Gln transport which occurs after small bowel resection (SBR) is reversed by a long-term combination of EGF plus growth hormone. This increase is in part due to elevated expression of ASCT2 protein. Transcytosis of many preformed transporters to the membrane is dependent on PI3K signal transduction pathways. We hypothesized that short-term treatment of human intestinal cells in culture with EGF alone will increase Gln transport due to transcytosis of preformed ASCT2 protein to the apical membrane in a PI3K dependent signal transduction pathway. Confluent monolayers of C2BBel1 (a bru-

sh border expressing subclone of Caco2) were subjected to treatment with EGF Receptor (EGFR) tyrosine kinase inhibitor (AG 1478), a PI3K inhibitor (wortmanin) and a MAPK inhibitor (PD98059) prior to 1 h incubation with EGF. Gln transport was determined using  $^3\text{H}$  labeled Gln in the presence or absence of sodium with and without the inhibitors MeAIB (5 mM, transported by A) and arginine (5 mM, transported by B0,+) individually and together. ASCT2 protein was measured in crude extracts (CE) and in isolated biotinylated surface proteins fraction (SP) by Western blot analysis. EGF almost doubled Gln transport activity mainly by increasing systems B0/ASCT2 activity. The increased transport activity was eliminated by AG 1478 and partially

abrogated by wortmanin and PD98059. There was no change in ASCT2 protein in CE under any of the conditions. In contrast, ASCT2 amount was doubled in SP. The increase in ASCT2 surface expression was eliminated by AG 1478 and

partially inhibited by wortmanin and PD98059. We conclude that in human intestinal cells, short-term EGF increases ASCT2 surface expression and glutamine (Gln) transport activity by MAPK and PI3K dependent pathways.

## Induction of CAT transporters for cationic amino acids by rapamycin in human endothelial cells

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In human endothelial cells L-arginine transport is referable to the activity of the leucine-resistant, NEM sensitive system  $\gamma^+$  (CAT transporters) and of the leucine inhibitable system  $\gamma^L$  ( $\gamma^L$  LAT transporters). While no regulatory mechanism of system  $\gamma^L$  has been identified thus far, it is known that CAT expression in endothelial cells is sensitive to several transduction pathways, such as PKC and NF- $\kappa$ B (1,2), that may lead to the modulation of Arg transport in response to various hormonal and inflammatory stimuli. However, no information is yet available on possible relationships between system  $\gamma^+$  and mTOR, a kinase involved in cell growth and protein synthesis control. We have here evaluated the effects of rapamycin, a specific mTOR inhibitor, on Arg transport in human endothelial cells of saphenous (HSVECs) or umbilical (HUVECs) derivation. The results obtained demonstrate that in HUVECs, but not in HSVECs, rapamycin alone stimulates system  $\gamma^+$ -mediated Arg transport. However, when used together with TNF $\alpha$ , rapamycin produces a marked, additive stimulation of Arg transport in both cell models. The kinetic analysis reveals that the simultaneous treatment with both compounds causes an increase of the  $V_{max}$  of high affinity Arg transport and the ap-

pearance of a low affinity component. These results are consistent with qRT-PCR data which show a hugely stimulated expression of the mRNAs for low affinity CAT2A and high affinity CAT2B transporters in cells treated with either compound. More marked increases are exhibited by cells exposed to both rapamycin and TNF $\cdot$ . Western Blot analysis indicates that also CAT2 protein(s) are more abundant under the same conditions. These data suggest that mTOR exerts a tonic, inhibitory effect on the expression of CAT2 transporters. The appearance of the low affinity, stress associated CAT2A isoform in endothelial cells treated with rapamycin in the presence of TNF $\alpha$  points to an important role of mTOR for endothelial welfare under conditions of inflammatory stress.

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## Electrophysiological analysis of the lysosomal cystine transporter, a protein defective in cystinosis

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Cystinosis is a rare autosomal recessive disease characterized by defective lysosomal efflux of cystine. The defective gene encodes a 7-transmembrane-domain protein, named cystinosin, which bears a tyrosine-based lysosomal sorting motif and shows distant relationship to bacterial rhodopsins.

We previously demonstrated its cystine transport activity by mutating the tyrosine-based motif and by applying radiolabelled substrates to whole cells at acidic extracellular pH.

In this study, we characterized in more detail the transport activity using two-electrode voltage clamp recording of

*Xenopus laevis oocytes*. When cystinosin was present at the cell surface, cystine elicited an inward current which increased at acidic pH. The electric charge was directly proportional to the number of translocated cystine molecules with a ratio of 1.18, thus suggesting a 1:1 H<sup>+</sup> symport mechanism. Application of other amino acids, including cysteine, did not evoke significant current, showing that cystinosin is highly selective for cystine in contrast to other lysosomal transporters which export several catabolites from the lysosome.

To gain insight into the transport mechanism, we measured the transport kinetic parameters between -150 and +50

mV and found that the K<sub>m</sub> for cystine strongly depends on membrane potential at pH 6 (with a low K<sub>m</sub> below -80 mV), but not at pH 5 (where K<sub>m</sub> is constantly low). These data suggest that cystine binding cooperatively interacts with protonation of a residue located within the electric field, a model confirmed by the detection of small transient currents elicited by cystine when the steady state current is negligible. Identification of this protonable residue should shed light on the H<sup>+</sup>-coupling mechanism. These experiments might also be useful to clarify the defect of pathogenic mutations with no obvious molecular phenotype.

## Comparative pharmacology at human and baboon sert using [<sup>3</sup>H]citalopram and [<sup>3</sup>H]DASB

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The Serotonin (5-HT) transporter (SERT) is a known molecular target for the treatment of psychiatric disorders with SSRIs being amongst the most clinically used drugs particularly in depression. PET studies using [<sup>11</sup>C]DASB in man have revealed that high SERT occupancy (>80%) appears to be crucial in achieving clinical efficacy of SSRIs (1). Recent reports have also suggested that [<sup>11</sup>C]DASB in baboons is a useful PET human surrogate (2). Classically however [<sup>3</sup>H]citalopram is the radiolabel of choice to determine the affinity of compounds acting at SERT in pre-clinical drug discovery programmes. The aim of this study was therefore to investigate the pharmacology of human and baboon SERT using both [<sup>3</sup>H]citalopram and [<sup>3</sup>H]DASB.

Radioligand filtration binding experiments using membranes derived from both recombinant human SERT

stably expressed in Lewis Lung Carcinoma Kidney Porcine cells (LLCPK) and recombinant baboon SERT stably expressed in Human Embryonic Kidney Fibroblast cells (HEK293-F) were conducted with [<sup>3</sup>H] Citalopram and [<sup>3</sup>H]DASB.

The rank order of affinities of the compounds tested (paroxetine > DASB > citalopram > 5-HT) is maintained across the two species (human and baboon) irrespective of the radiolabel used ([<sup>3</sup>H]citalopram and [<sup>3</sup>H]DASB).

Data represent mean pK<sub>i</sub> values determined from at least three independent experiments.

This is the first report of the pharmacological characterization of baboon SERT which also provides preliminary evidence that baboon SERT pharmacology is similar to human SERT pharmacology. This may also be predicted by the high degree of SERT sequence homology between the-

Table 1.

Compounds	[ <sup>3</sup> H] Citalopram Binding				[ <sup>3</sup> H] DASB Binding			
	Human SERT LLC PK		Baboon SERT HEK293F		Human SERT LLC PK		Baboon SERT HEK293F	
	pK <sub>i</sub>	s.e.m.	pK <sub>i</sub>	s.e.m.	pK <sub>i</sub>	s.e.m.	pK <sub>i</sub>	s.e.m.
Paroxetine	10.06	0.03	9.67	0.01	9.85	0.02	9.95	0.01
citalopram	8.79	0.02	8.61	0.03	8.55	0.01	8.63	0.02
DASB	9.62	0.04	9.36	0.05	9.45	0.05	9.52	0.04
5-HT	5.82	0.02	5.73	0.01	5.57	0.03	5.59	0.02

se species. The use of [<sup>3</sup>H]citalopram for measuring the affinity of compounds at SERT therefore appears to translate into baboon and human where current PET strategies utilize [<sup>11</sup>C]DASB as the radiotracer.

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## Osmosensitivity of SNAT2 expression: relationships with nutritional stress and functional significance

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SNAT2, the ubiquitous member of SLC38 family, accounts for the activity of transport system A for neutral amino acids in most mammalian tissues. Given that neutral amino acids represent a major fraction of cell organic osmolytes, SNAT2 expression and activity are sensitive to both nutritional and osmotic stress. Thus, both amino acid starvation and cell shrinkage induce SNAT2, although through distinct signalling pathways, since the former triggers the phosphorylation of eIF2alpha, while the latter is eIF2alpha independent (1). However, even in the presence of a clear cut eIF2alpha phosphorylation, the induction of SNAT2 mRNA and the increase in system A transport activity, promoted by amino acid starvation, are suppressed by the incubation under hypotonic conditions, suggesting that the response of SNAT2 to nutritional stress requires a threshold intracellular ion strength. The silencing of SNAT2 expression, obtained with anti-SNAT2 siRNAs, prevents the increase in system A transport activity caused by hypertonic treatment, lowers the intracellular amino acid pool, and significantly delays cell volu-

me recovery, thus demonstrating that the hypertonic increase of SNAT2 expression is needed for a fast hypertonic RVI. SNAT2-silenced cells have a viability significantly lower than controls after a 48h incubation under hypertonic conditions, although mechanisms of chronic adaptation to the hypertonic stress are intact. These results indicate that SNAT2 activity plays a role in the long term adaptation of mammalian cells to osmotic stress.

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## EAAT3 transporters are expressed only in subsets of C6 glioma cells and partially co-localize with actin cytoskeleton

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EAAT3 is a ubiquitous transporter for glutamate, whose expression in CNS is preferentially neuronal. However, one of the most widely used cell models for in vitro studies on EAAT3 regulation is represented by C6 glioma rat cells. In these cells we have recently observed that the membrane expression of the transporter is influenced by the or-

ganization status of the actin cytoskeleton (1). Here we investigate the relationships between EAAT3 and actin cytoskeleton with CLSM. In C6 cultures EAAT3 expression is extremely heterogeneous. The maximal expression is detected in "round-bipolar" cells (2), cells supplied of axon-like processes possibly committed to neuronal-type differentia-

tion, and the lowest in cells with a “flat” morphology, likely proceeding through a glial-type differentiation process. In round bipolar cells, EAAT3 is expressed both in discrete intracellular vesicular compartments and in axon-like cell processes in which it exhibits a partial co-localization with actin. Moreover, a pool of EAAT3 transporters is co-localized with  $\alpha$ -adducin, a cytoskeletal protein involved in actin filament capping. Treatment of C6 cells with phorbol esters, a condition that stimulates EAAT3 activity, causes both an increase of EAAT3 expression on plasma membrane and actin remodeling to membrane ruffles. Under these conditions, the abundance of adducin is lowered and its co-localization with EAAT3 suppressed. These results point to the

existence of an adducin-mediated, PKC-sensitive interaction between an intracellular pool of EAAT3 transporters and actin cytoskeleton.

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## ABC1-transporter mediates IL1-beta release from microglia cells

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The presence of proinflammatory IL-1 beta in acute/chronic neurodegenerative diseases suggests an important role for this cytokine in many neuronal pathologies. Because microglia has been indicated as the major source of IL-1beta in the brain, it's of crucial importance to define the molecular mechanisms mediating its release from these cells. IL1 beta is a leaderless protein (no conventional secretory peptide) which is not released through the classical ER- to-Golgi pathway. Different mechanisms of release have been shown to be involved, from exocytosis of endolysosome-related vesicles to shedding of microvesicles from the plasma membrane, and they are known to be induced by ATP stimulation.

Aim of the present study was to investigate the mechanism of IL1 beta release from microglia. We found that both exogenous or endogenous ATP, released from astrocytes, induces formation and shedding of annexin-positive microvesicles from microglia. The isolation and biochemical characterization of shed vesicles reveals the presence of IL

1- beta inside these organelles. The cytokine is then released in a P2X7- and calcium- dependent mechanism. How the cytokine is able to cross the plasma membrane is still a topic of debate. Given a recent report demonstrated that Schwann cells release IL1-beta by means of the ATP binding cassette transporter ABC-1, we investigated whether this mechanism also operates at the CNS level. WB analysis demonstrated that shed vesicles contain ABC-1 transporter; furthermore, IL 1-beta determination by ELISA assay indicated that the ABC-1 inhibitor glibenclamide strongly prevents IL1 beta release from the vesicles. These observations identify a crucial role for the ABC-1 transporter in the release of IL1 beta from vesicles shed from activated microglia. Given the importance of IL1 beta in the onset of neuroinflammation, pharmacological modulation of the ABC-1 transporter could be taken into consideration as a mean to reduce the inflammatory event leading to neuronal degeneration.

## Functional characterization of sugar transport in the intestinal epithelium of an insect (*Aphidius ervi*, Hymenoptera)

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*Aphidius ervi* is an entomophagous wasp, widely used in biological control because adult females lay the eggs in the haemocoel of several aphid species. After hatching, *A. ervi* larvae develop in the living host feeding on its haemolymph and, just before metamorphosis, kill the host by devouring its internal tissues and pupating inside the body wall, from which the adult will finally emerge. Like many other parasitoids, *A. ervi* finely redirects host metabolism in order to optimize haemolymph nutrient contents to support larval specific needs.

In aphid haemolymph, sugar titre is extremely high, reaching a concentration of 129 and 60 mM for fructose and glucose respectively. The peculiar sugar-rich environment in which *A. ervi* larvae develop, suggestive of a critical role of these nutrients for the insect survival, stimulated us to identify the transporters involved in glucose and fructose

intestinal absorption, especially considering that information on sugar transport in insect midgut is scanty.

The study performed in larval *A. ervi* intestine, isolated and incubated in vitro, allowed us to detect, with functional, immunohistochemical and immunoblotting approaches, the transport proteins responsible for glucose and fructose uptake and to characterise their basic properties. The results unequivocally show that the cellular model for sugar transport in the larval stage of this insect is surprisingly similar to that conventionally described for mammals (i.e. SGLT1-like and GLUT5-like transporters on the apical membrane of the intestinal epithelial cell, and GLUT2-like transporters on the basolateral one), with the expression, apparently constitutive, of GLUT2 transporters also in the apical membrane, in agreement with the model for the transcellular absorption of hexoses recently proposed (Kellett, J. Physiol. 531, 585, 2001).

## Characterization of the synergistic collectrin effect on B<sup>0</sup>AT1 function *in vitro*

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A homolog to ACE2 lacking the catalytic domain, termed Collectrin, has been identified and shown to be expressed apically in mouse renal proximal tubule cells. Based on molecular interaction studies, Collectrin has been recently shown to associate with multiple apical transporters, in particular with the broad range sodium dependent amino acid transporter, B<sup>0</sup>AT1. B<sup>0</sup>AT1 is a member of the Na<sup>+</sup> (Cl<sup>-</sup>) dependent amino acid and neurotransmitter transporter family (SLC6A). When expressed in *X. laevis* oocytes, mouse B<sup>0</sup>AT1 in combination with mouse Collectrin transported 3-fold more L-Ile (600 pmol/oocyte/hour) than when expressed alone (150 pmol/oocyte/hour) after 2 days of expression. This synergistic effect of Collectrin on B<sup>0</sup>AT1 function was also seen in MDCK cells stably transfected with B<sup>0</sup>AT1 and Collectrin that transported 7-fold more L-Ile (55.1±9.4 nmol/3'/9.6 cm<sup>2</sup> dish) than cells expressing

B<sup>0</sup>AT1 (7.8±4.2 nmol/3'/9.6 cm<sup>2</sup> dish) or Collectrin alone (0.5±2.3 nmol/3'/9.6 cm<sup>2</sup> dish). The increase in transport rate, at least in oocytes, was not due a change in substrate affinity that remained unchanged (0.61±0.03 mM versus 0.75±0.07 mM alone). The interaction of these two membrane proteins, B<sup>0</sup>AT1 and Collectrin, is now being studied with the modified yeast two hybrid method, named split ubiquitin. When mouse collectrin was used as bait, interaction with the prey mouse B<sup>0</sup>AT1 was observed. The mechanism of this interaction and the significance of it to the transport function still have to be clarified. These data identify Collectrin as a key regulator of B<sup>0</sup>AT1 function. Since B<sup>0</sup>AT1 is a major player in the renal re-absorption of neutral amino acids, this effect of Collectrin on B<sup>0</sup>AT1 expression and function plays a major physiological role in amino acids homeostasis.

## Role of the transporter hCNT1 (SLC28A1) in 5'DFUR cytotoxicity in the breast carcinoma cell line MCF7

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Nucleoside analogs are used in the treatment of a variety of tumors. Their transport across the plasma membrane may determine their cytotoxicity. Two gene families have been identified as responsible for the uptake of natural nucleosides, Concentrative Nucleoside Transporters (CNTs, SLC28) and Equilibrative Nucleoside Transporters (ENTs, SLC29). We attempted to identify the role of concentrative transporter hCNT1 in the pharmacological action of 5'-deoxy-5-fluorouridine (5'DFUR), an intermediate metabolite of capecitabine and direct precursor of the cytostatic agent 5'-fluorouracil, in the breast carcinoma cell line MCF7. The heterologous expression of hCNT1 (MCF7-hCNT1) in MCF7 cells, whose nucleoside uptake was only mediated by equilibrative transporters, increased slightly cell sensitivity to 5'-DFUR treatment. The inhibition of equilibrative activity in MCF7 with dipyrindamole blocked the 5'DFUR cytotoxicity, but not in MCF7-

hCNT1. Moreover, under equilibrative transport inhibition, induction of some transcriptional targets of 5'-DFUR (p21, FAS, AQP3, RPL3, RRM2B and BAX) was blocked in MCF7, whereas dipyrindamole had no effect in the transcriptional response to 5'-DFUR treatment in MCF7-hCNT1 cells. To confirm the role of hCNT1 in 5'DFUR cytotoxicity, a panel of nucleoside derivatives suitable for hCNT1-inhibition was obtained. The molecule T-Ala inhibited hCNT1-mediated transport, even though it was not a substrate of the transporter. Furthermore, the cytotoxic action of 5'-DFUR as well as the transcriptional changes produced by this nucleoside analogue were partially inhibited by T-Ala in MCF7-hCNT1 cells. These results show a link between nucleoside transporter function and the pharmacogenomic response to nucleoside analogs and further support the hypothesis that pattern rather than single transporter determine the cytotoxic effect of nucleoside derivatives.

## Vitamin D dependent dietary adaption of the type IIc sodium phosphate cotransporter

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Vitamin D is suggested to stimulate proximal tubular phosphate reabsorption by increasing the amount of type IIa sodium dependent cotransporter (NaPi-IIa) in the brush border membrane. Recently a new member of the type II sodium-phosphate cotransporter family, NaPi-IIc, has been described. This protein is only expressed in the kidney where it colocalizes with NaPi-IIa. It has been initially proposed that NaPi-IIc is only important in weaning animals, but recently mutations have been found in patients with hereditary hypophosphatemic rickets with hypercalciuria suggesting an important role in man. Here we investigated the role of vitamin D in the adaption of NaPi-IIc to changes in dietary phosphate intake. For this we used two mouse models: VDR KO lacking the vitamin D receptor and mice deficient for 25-hydroxyvitamin-D<sub>3</sub>-1 $\alpha$ -hydroxylase (1 $\alpha$ OH KO), that lack the enzyme responsible for the final activation of vitamin D.

On a low Pi diet (0.1 %), all mice strains showed no si-

gnificant differences in urinary Pi excretion, while on a high Pi diet (1.2 %), urinary Pi excretion was reduced as compared to the respective controls. Western blot analysis showed that on a low Pi diet, both NaPi-IIc and NaPi-IIa proteins were up-regulated in VDR KO and 1 $\alpha$ OH KO mice and that both cotransporters were down-regulated after mice were fed a high phosphate containing diet. However, the abundance of NaPi-IIc protein was significantly reduced in VDR KO animals compared to wildtype both under low and high Pi diets whereas the type IIa cotransporter was not affected. 1 $\alpha$ OH KO mice showed a non significant reduction of NaPi-IIc protein compared to the wild type mice under the same conditions.

These data suggest that vitamin D may have a role in the regulation of the type IIc sodium-phosphate cotransporter by dietary phosphate intake and that the vitamin D receptor is indispensable for normal dietary adaption of IIc.

## Iron acquisition in *Leishmania infantum* amastigotes

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Iron is an essential element for all living systems and *Leishmania* is no exception. However, iron can also be very toxic, therefore, its acquisition and storage are very regulated processes. We have started looking at the process of iron acquisition in amastigotes. Here we present the first results of this work. We used 3 different sets of experiments. 1) we analysed amastigote replication under different iron sources, 2) we investigated the kinetics of hemin and hemoglobin binding and 3) we looked at three *Leishmania* proteins for its potential role as iron transporters.

Using axenic amastigotes we demonstrate that *L. in-*

*fantum* is able to acquire iron from different iron sources, namely, hemoglobin, hemin and inorganic iron. In our assays lactoferrin, transferrin and ferritin did not support parasite growth. Experiments performed to dissect the mechanism of hemin and hemoglobin internalization in amastigotes suggest the presence of specific but distinct receptors. Complementation experiments in *S. cerevisiae* were used as a strategy to analyse the possibility that three *L. infantum* proteins belonging to the iron-zinc family of transporters function in the uptake of inorganic iron. The results indicate this is indeed the case.

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## Endothelial dysfunction is programmed in human intrauterine growth restriction: evidence for the role of hypoxia and PKC in endothelium from the umbilical vein

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Intrauterine growth restriction (IUGR) is associated with hypoxia and vascular disorders later in life. In human umbilical vein endothelium (HUVEC) L-arginine transport is mediated by system y<sup>+</sup>/CATs (Cationic Amino acid Transporters). In HUVEC from IUGR we have shown that there is lower L-arginine transport mainly due to lower expression of CAT-1. Nitric oxide (NO) derives from the conversion of L-arginine to L-citrulline via endothelial NO synthase (eNOS). Objective. We studied the effect of hypoxia and the role of PKC on L-arginine transport and NO synthesis in HUVEC from fetuses with IUGR. Methods. HUVEC cultured in M-199 were exposed (0-24 h) to normoxia (5% O<sub>2</sub>, ~35 mmHg PO<sub>2</sub>) or hypoxia (2% O<sub>2</sub>, ~15 mmHg). L-Arginine transport was determined in presence or absence of PKC inhibitors. hCAT-1, hCAT-2B and eNOS mRNA were quantified by real-time PCR. eNOS activity was determined by L-[3H]citrulline formation, to-

tal and phosphorylated eNOS (Ser<sup>1177</sup>) and PKC ( $\alpha/\beta$ II) proteins were detected by Western blot. Results. Maximal transport velocity (V<sub>max</sub>) for L-arginine transport was reduced in IUGR (28%) and hypoxia (44%). Hypoxia effect was blocked by calphostin C (PKC inhibitor) in normal and IUGR cells. hCAT-1, hCAT-2B and eNOS mRNA levels were reduced in IUGR and hypoxia. However, total eNOS and PKC $\alpha$  protein levels were increased in IUGR (1.7-fold) and hypoxia (2.3-fold), while phosphorylated eNOS at Ser<sup>1177</sup> was reduced in normal and IUGR HUVEC under hypoxia. NOS activity was reduced in IUGR (67%) and hypoxia (80%). Conclusion: IUGR and hypoxia reduced L-arginine/NO pathway activity may result from lower expression and activity of hCAT-1, hCAT-2B and eNOS, involving a NO-dependent activation of PKC in HUVEC.

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## Interaction between lysine 102 and aspartate 338 in the insect amino acid cotransporter KAAT1

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KAAT1 is a lepidopteran neutral amino acid transporter belonging to the NSS super family, which has a special cation selectivity, being activated by Na<sup>+</sup>, K<sup>+</sup> and Li<sup>+</sup>. We have previously demonstrated that D338 is essential for KAAT1 K<sup>+</sup> activation and for the coupling of amino acid and driver ion fluxes. By means of sequence comparison, site-directed mutagenesis and expression in *Xenopus laevis* oocytes, we identified K102 as a residue interacting with D338. In comparison with the wild type, the single mutants K102V and D338E showed altered leucine uptake and transport-associated currents both in the presence of Na<sup>+</sup> and K<sup>+</sup>. The double mutant K102V/D338E showed increased leucine uptake in the presence of a Na<sup>+</sup> gradient, and uptake recovery in the presence of a K<sup>+</sup> gradient. No recovery was observed in leucine induced currents. Furthermore, in the presence of the oxidant Cu(II) (1,10-phenanthroline)<sub>3</sub>, we observed specific and reversible inhibition of the K102C/D338C mutant, thus indicating that these residues interact both structurally and

functionally although the normal transport cycle requires, in some step, their reciprocal movement. Since in the recently solved crystal structure of the NSS transporter LeuTAa (1), the residue corresponding to D338 has been located in the Na<sup>+</sup> binding site, our results indicate that K102, interacting with D338, could be part or at least contribute to the spatial organization of KAAT1 cation binding site.

We have therefore identified two residues, K102 located in TM II and D338 located in TM VII, which come into close proximity with each other and contribute to the substrate interaction and conformational transition characterizing KAAT1 function.

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## A PDZ target sequence controls the surface expression and recycling of the EAAC1/EAAT3 glutamate transporter

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The neuronal glutamate transporter EAAC1/EAAT3 (Excitatory Amino Acid Carrier-1) mediates the uptake of the excitatory neurotransmitter from the synaptic cleft. It is also expressed in epithelial cells where it provides the principal route of glutamate and aspartate absorption. The transporter activity and localization are modulated by auxiliary proteins that still have to be identified.

In the C-terminus of the EAAC1/EAAT3 transporter, we observed a consensus sequence (-S-Q-F) for interaction with class I PDZ domains and we investigated the role of this motif in the transporter localization and activity. Mutant transporters were generated and overexpressed in the CV1, COS and MDCK (Madin Darby canine kidney) cell lines, and their localization and activity were tested by means of immunofluorescence, biotinylation, and uptake experiments.

We found that removal of the PDZ-interacting sequence (T-S-Q-F) or substitution of the serine residue at -2 position with alanine or glutamate affected the cell surface stability of the transporter. Indeed, the steady state cell surface expression of mutant transporters was lower compared to wild type protein, but it was greatly increased by inhibition of the clathrin-dependent endocytosis (hyperosmotic stress). Double immunofluorescence experiments revealed that mutant transporters accumulated in an endocytic compartment which did not colocalize with transferrin, a marker of the recycling compartment. Instead, we found a partial colocalization with LAMP-2, a marker of the lysosomal compartment, after inhibition of lysosomal degradation by means of leupeptine treatment.

We suggest that the PDZ target sequence of EAAC1/EAAT3 transporter, and likely its interaction with

PDZ proteins, may control the surface stability and/or recycling of the transporter. In the absence of this interaction, the transporter reaches the plasma membrane but instead of being retained in- or recycled to- the cell surface, it

is internalized and degraded in a lysosomal compartment.

We are now in the process to identify PDZ proteins interacting with the EAAC1/EAAT3 transporter in epithelial and neuronal cells.

## Essential role for collectrin in renal amino acid transport

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Angiotensin-converting enzyme 2 (ACE2) is a regulator of the renin angiotensin system and plays a role in acute lung failure, cardiovascular functions, and SARS infections. The collectrin gene (transmembrane protein 27; *Tmem27*) is located next to the ACE2 locus on the X chromosome and encodes a type I transmembrane glycoprotein. The membrane proximal domain of Collectrin shares homology with ACE2. Earlier studies indicated that Collectrin is expressed in the kidney and in the  $\beta$ -cells of the pancreas. Here we report that Collectrin mRNA is expressed in kidney and pancreas, and to a lesser extent in intestine, liver, heart, and stomach. To determine the *in vivo* function, we generated collectrin<sup>-/-</sup> mutant mice. Blood and urine levels of electrolytes and urea, uric acid, and creatinine were all within the normal range in the knockout mice. Importantly, loss of Collectrin did not affect basal glucose levels. However collectrin<sup>-/-</sup> mice were found to exhibit a selective and severe defect in kidney amino acid transport. In wild-type mice Collectrin protein was found to be expressed at proximal tu-

bule brush border membranes and to co-localize with the neutral amino acid transporter, B<sup>0</sup>AT1. Furthermore in collectrin<sup>-/-</sup> mice proximal tubule expression of B<sup>0</sup>AT1 and of three other SLC6 family members (XT3s1/SIT1, XT2, and XT3) was dramatically down-regulated. The expression of the apical glutamate/aspartate transporter, EAAC1 was slightly decreased whereas expression of the apical exchanger for cystine, arginine, lysine and ornithine, B<sup>0+</sup>AT, and of the sodium/phosphate co-transporter, Na<sup>+</sup>/Pi IIa, and the basolateral amino acid exchanger, 4F2hc/LAT2 remained unchanged. B<sup>0</sup>AT1-type Na<sup>+</sup>-dependent neutral amino acid transport activity in collectrin<sup>-/-</sup> brush border membrane vesicles was decreased by ~50%. Finally, co-immunoprecipitation experiments demonstrated that Collectrin associates with B<sup>0</sup>AT1, XT2 and XT3 but not with B<sup>0+</sup>AT. Taken together the data indicates that Collectrin acts as a master regulator of renal amino acid uptake and may provide a molecular mechanism for amino acid loss associated with diseases such as Fanconi syndrome or diabetes.

## Identification of Endophilins 1 and 3 as selective binding partners for VGLUT1 and their co-localization in neocortical glutamatergic synapses: Implications for VGLUT trafficking and excitatory vesicle formation

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1. Selective protein-protein interactions between neurotransmitter transporters and their synaptic targets play important roles in regulating chemical neurotransmission. We screened a yeast two-hybrid library with bait containing the C-terminal amino acids of VGLUT1 and obtained clones that encode endophilin 1 and endophilin 3, proteins considered to play an integral role in glutamatergic vesicle formation.

2. Using a modified yeast plasmid vector to enable more cost-effective screens, we analyzed the selectivity and specificity of this interaction. Endophilins 1 and 3 selectively recognize only VGLUT1 as the C-terminus of VGLUT2 and VGLUT3 do not interact with either endophilin isoform. We mutagenized four conserved stretches of primary sequence in VGLUT1 that includes two poly-

proline motifs (Pro1, PPAPP, and Pro2, PPRPPPP), found only in VGLUT1, and two conserved stretches (SEEK, SYGAT), found also in VGLUT2 and VGLUT3. The absence of the VGLUT conserved regions does not affect VGLUT1–endophilin association. Of the two polyproline stretches, only one (Pro2) is required for binding specificity to both endophilin 1 and endophilin 3.

3. We also show that endophilin 1 and endophilin 3 co-

localize with VGLUT1 in synaptic terminals of differentiated rat neocortical neurons in primary culture. These results indicate that VGLUT1 and both endophilins are enriched in a class of excitatory synaptic terminals in cortical neurons and there, may interact to play an important role affecting the vesicular sequestration and synaptic release of glutamate.

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## Identification of amino acid substitutions required to convert the creatine transporter to a gaba transporter

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The creatine transporter (CRT) is a member of the Na<sup>+</sup>/Cl<sup>-</sup>-dependent neurotransmitter transporter family, also known as neurotransmitter sodium symporters (NSS) or solute carrier family 6 (SLC6) transporters. The CRT is closely related to the GABA transporter, GAT-1 yet the two transporters show no overlap in substrate specificity. Recently the publication of a high resolution (1.65 Å) crystal structure of the leucine transporter (LeuT<sub>AS</sub>) from *Aquifex aeolicus*, a bacterial homologue of the SLC6/ NSS transporter family, revealed details of substrate binding [Yamashita, A., S.K. Singh, T. Kawate, Y. Jin, and E. Gouaux (2005) *Crystal structure of a bacterial homologue of Na<sup>+</sup>/Cl<sup>-</sup>-dependent neurotransmitter transporters*. *Nature*, 437, 215–23]. The aliphatic side chain of leucine was found to interact with a hydrophobic pocket formed from the side chains of residues Val104, Tyr108 in TM3, Phe253 and Phe259 in TM6 and Ser355 and Ile359 in TM8. In agreement with this model, two corresponding residues in TM3 of the CRT, Cys144 and Tyr148, have been shown to be close to the substrate binding site and essential for CRT

activity, respectively [Dodd, J.R. and D.L. Christie (2005) *Substituted cysteine accessibility of the third transmembrane domain of the creatine transporter: defining a transport pathway*. *J Biol Chem*. 280, 32649–54]. We have now designed mutations, based on sequence comparisons of the CRT and GABA transporters and the LeuT<sub>AS</sub> structural template, to alter the substrate specificity of the CRT. Two or three amino acid substitutions within the predicted substrate binding site were sufficient to result in a complete loss of creatine transport activity and the gain of a specific GABA transport function. The K<sub>m</sub> values for GABA transport of mutants with three to four targeted mutations were similar to that of the wild-type CRT for creatine. Concluding statement: These studies confirm that the LeuT<sub>AS</sub> structure provides a good model for studies of the substrate and inhibitor specificity of eukaryotic SLC6 transporters. Our finding that substrate specificity can be changed through mutation of a few key amino acid residues suggests a mechanism for the evolution of the substrate specificity of SLC6 transporters.

## Role of CNT3 in the transepithelial flux of nucleosides and nucleoside-derived drugs

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The role of the concentrative nucleoside transporter CNT3 in the establishment of a transepithelial flux of both

natural nucleosides and pharmacologically active nucleoside-based drugs has been evaluated in two different renal

epithelial cell lines. Murine PCT cells show an endogenous CNT3 activity at their apical membrane that is responsible, when cells are grown in a transwell dish, for the sodium dependent transepithelial flux of both purine and pyrimidine nucleosides. Genetically engineered MDCK cells that express the human orthologue of CNT3 (hCNT3-MDCK cells) can direct it and insert it into the apical membrane and thus they can also generate a trans-epithelial flux of both nucleosides and nucleoside-derived drugs. In all cases, part of the transported nucleoside was retained and transformed in the cell and part of it was released to the opposite compartment. Most importantly, the metabolic modifications

suffered by the transported nucleoside were dependent on the carrier that had mediated its entry into the cell. Thus, when cytidine was taken up by CNT3, it was readily deaminated to uridine and then converted to uracil, both products being released. On the contrary, in the absence of any functional CNT3 carrier, cytidine was retained in a phosphorylated form or released to the opposite compartment unaltered. All these results underline a critical role of CNT3 in the renal reabsorption of nucleosides and their derivatives and point towards the existence of some metabolic "channeling" of nucleosides from their transporters to some key enzymes in their metabolism

## Adenosine reduces L-arginine transport and nitric oxide synthesis in human placental microvascular endothelium in preeclampsia

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Preeclampsia is associated with high adenosine fetal plasma level. A<sub>2A</sub> adenosine receptors activation increases nitric oxide (NO) synthesis and L-arginine transport (L-arginine/NO pathway) in human umbilical vein endothelium. We studied whether adenosine modulates L-arginine/NO pathway in human placental microvascular endothelial cells (hPMEC). hPMEC primary cultures were performed from normal and preeclamptic placentas. L-Arginine transport, L-[<sup>3</sup>H]citrulline formation, inducible (iNOS) and endothelial (eNOS) NO synthase proteins, and human cationic amino acid transporter 1 (hCAT-1) and hCAT-2B mRNA levels were determined in hPMEC primary cultures in absence or presence of A<sub>2A</sub> selective agonist CGS-21680 or antagonist ZM-241385. A higher V<sub>max</sub> (~2-fold) for L-arginine transport, L-

[<sup>3</sup>H]citrulline synthesis (~6-fold) and hCAT-2B mRNA level (~3-fold), but lower iNOS abundance (~80%) was exhibited in preeclampsia. eNOS was undetectable in hPMEC. CGS-21680 increased L-arginine transport, L-[<sup>3</sup>H]citrulline formation, and hCAT-2B mRNA levels in normal pregnancies. ZM-241385 blocked the effect of CGS-21680 on hCAT-2B mRNA level in normal pregnancies. CGS-21680 reduced L-arginine transport and L-[<sup>3</sup>H]citrulline formation, but did not alter hCAT-2B or hCAT-1 mRNA levels in preeclampsia. Adenosine-increased L-arginine/NO pathway could be a compensatory mechanism in hPMEC from preeclampsia.

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## SNAT2 determines levels of anabolic amino acids L-Gln and L-Leu and signals to protein metabolism through mTOR in L6 muscle cells

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Wasting of lean tissue as a consequence of metabolic acidosis is a serious clinical problem in patients with chronic renal failure. It has been attributed to inhibition by low

pH of the System A (SNAT2) transporter which carries the anabolic amino acid L-Gln into muscle cells. However SNAT2 is only one of several L-Gln transporters in muscle.

The aim of this study was therefore to determine the effect of selective SNAT2 inhibition on intracellular amino acid profiles and amino acid dependent signalling through mTOR in L6 skeletal muscle cells.

After 2h at an extracellular pH of 7.1 to model acidosis (which halves SNAT2 activity), intracellular L-Gln fell to  $20.2 \pm 0.2$  nmol/35mm well compared with  $38.6 \pm 0.9$  at control pH (7.4) ( $P < 0.05$ ). Complete inhibition of SNAT2 at pH 7.4 with the selective competitive substrate methylaminoisobutyrate (MeAIB) further depleted intracellular L-Gln ( $10.2 \pm 0.2$ ,  $P < 0.05$ ). The L-Gln gradient across the plasma membrane may also drive uptake of other anabolic amino acids, notably L-Leu. SNAT2 blockade with MeAIB strongly depleted L-Leu ( $0.91 \pm 0.02$  nmol/well, compared

with  $2.1 \pm 0.1$ ,  $P < 0.05$  in control cultures). With 100nM insulin, SNAT2 inhibition with low pH or MeAIB significantly impaired signalling through mTOR to p70S6 kinase, ribosomal protein S6, and 4E-BP1. Similar effects on amino acids and signalling were obtained by silencing SNAT2 expression with small interfering RNAs.

It is concluded that even though SNAT2 is only one of several L-Gln transporters in muscle, it has the dominant effect on active accumulation of L-Gln and other anabolic amino acids and hence on signals through mTOR. In view of the importance of such signals in regulating protein mass, nucleotide/nucleic acid metabolism and cell growth, SNAT2 is a key target for nutritional research to combat wasting illness.

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## **$\beta$ -estradiol rescues $\Delta F508$ -CFTR functional expression in CFBE41o- cells via NHERF1 up-regulation**

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Cystic fibrosis (CF) is a disease caused by mutations within the cystic fibrosis transmembrane conductance regulator (CFTR) gene. The most common mutation,  $\Delta F508$ , results in a protein that is defective in folding and trafficking to the cell surface but is functional when properly localized. We have demonstrated that over-expression of the PDZ protein NHERF1 induces both a redistribution of CFTR from the cytoplasm to the apical membrane and the PKA-dependent activation of CFTR-dependent chloride secretion (1). As it has been demonstrated that NHERF1 expression levels are increased upon estrogen stimulation (2), in the present study we determined if long term  $\beta$ -estradiol treatment can rescue  $\Delta F508$ -CFTR functional expression in cystic fibrosis cells via the up-regulation of NHERF1.

Methods: CFBE41o- cells are a human bronchial cell line derived from CF individuals homozygous for the  $\Delta F508$  mutation of CFTR. We performed functional measurements of PKA-dependent chloride efflux to analyze  $\Delta F508$ -CFTR activity in polarized CFBE41o- confluent cell monolayers grown on permeable filters upon six or twelve hours of  $\beta$ -estradiol treatment.

Results: We found that  $\beta$ -estradiol stimulation of CF cell monolayers increased both NHERF1 expression and, at the same time, rescued apical  $\Delta F508$ -CFTR activity. Importantly, down-regulation of NHERF1 expression by transfection with siRNA for NHERF1 blocked the estrogen-dependent rescue of apical  $\Delta F508$ -CFTR transport activity.

These results demonstrate that estrogen treatment rescues  $\Delta F508$ -CFTR functional expression via an increase in NHERF1 expression levels and lead us to suggest estrogen treatment as a potential therapeutic strategy for CF patients.

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## Modulation of MPP<sup>+</sup> uptake by procyanidins in Caco-2 cells: involvement of oxidation/reduction reactions

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It is becoming increasingly evident that the absorption of certain nutrients and drugs are largely influenced by the concomitant ingestion of other substances. As various xenobiotics belong to the class of organic cations, the aim of this work was to study the modulation of the intestinal apical uptake of organic cations by diet procyanidins. Five procyanidin fractions with different structural complexity were obtained after fractionation of a grape seed extract. The effect of these compounds on 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>) uptake was evaluated in Caco-2 cells. Apical uptake of <sup>3</sup>H-MPP<sup>+</sup> by Caco-2 cells was increased by a 60 min exposure to 600 µg/mL of procyanidin fractions, this increase being positively related with procyanidin structural complexity. <sup>3</sup>H-MPP<sup>+</sup> uptake increased with preincubation time. It was speculated that procyanidins were oxidized during preincubation, what could

interfere with transport activity. Use of oxidizing agents showed that the redox state of the transporter could affect its activity. Moreover, some series of Caco-2 cells were maintained in 25 mM glucose through at least 5 passages, with the purpose of altering cells oxidative state. 60 min preincubation of these cells with 600 µg/mL procyanidins increased <sup>3</sup>H-MPP<sup>+</sup> uptake to values similar to the ones obtained in normal cells but with oxidized procyanidins. When oxidized procyanidins were tested in these cells, <sup>3</sup>H-MPP<sup>+</sup> uptake was even higher. A relation between preincubation time and <sup>3</sup>H-MPP<sup>+</sup> uptake was also found. In conclusion, procyanidins are capable to modulate <sup>3</sup>H-MPP<sup>+</sup> apical uptake in Caco-2 cells, most probably through oxidation/reduction phenomena. Interactions between these compounds and drugs present in the diet may affect their bioavailability.

## Extracellular glucose concentration alters hEMT expression in Caco-2 cells

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Chronic exposure to high glucose concentration has been suggested to alter regulation and functional activity of proteins. The aim of this study was to assess the effect of high glucose concentration on organic cation transporter 1 (hOCT1) and extraneuronal monoamine transporter (hEMT) in Caco-2 cells.

Uptake of <sup>3</sup>H-MPP<sup>+</sup> (1-methyl-4-phenylpyridinium) by control (5.5 mM glucose in media) or high glucose (HG; 25 mM glucose) Caco-2 cells was evaluated. The effect of extracellular glucose on OCT1 and EMT expression was assessed using comparative RT-PCR.

A decrease in MPP<sup>+</sup> uptake was found in cells maintained in HG cells (0.739 ± 0.09 pmol/mg protein; n=6) relatively to control cells (1.2 ± 0.04 pmol/mg protein; n=6). Additionally two compounds known to inhibit <sup>3</sup>H-MPP<sup>+</sup> uptake in Caco-2 cells (1), corticosterone and clonidine, showed different effects on <sup>3</sup>H-MPP<sup>+</sup> uptake in cells cultured under the two conditions. Corticosterone (300 µM) inhibited uptake to 57.5 ± 10.9 % of control, n=6, in control cells, and to 77.9 ±

2.41; n=6, in HG cells; clonidine (50 µM) inhibited <sup>3</sup>H-MPP<sup>+</sup> uptake in control cells, but had no effect in HG cells. These results suggest that extracellular glucose concentration may alter transporters, possibly due to redox phenomena as has already been described (2).

As to kinetic parameters, a decrease of both Km and Vmax has been observed in HG cells. This means an increase of transport affinity and a decrease in the number of transporter units. Using comparative RT-PCR a decrease in hEMT expression was found in HG cells.

These results indicate that extracellular glucose concentration, possibly through alterations in cell oxidative state, influences MPP<sup>+</sup> uptake in Caco-2 cells.

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## Potential involvement of Sp1 on D-glucose modulation of human equilibrative nucleoside transporter 1 promoter activity in human umbilical vein endothelium

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Adenosine uptake is mediated via the human equilibrative nucleoside transporters 1 (hENT1) and hENT2 in Human Umbilical Vein Endothelial Cells (HUVEC). We recently reported that proximal SLC29A1 gene (for hENT1) promoter activity is increased in HUVEC from gestational diabetes mellitus. We now examined the potential role of Sp1 as modulator of SLC29A1 promoter activity in response to elevated extracellular D-glucose. Promoter activity was assayed in HUVEC primary cultures (passage 2) from normal pregnancies (Ethical committee approval and informed patient consent were obtained) exposed (24 hours) to 5 or 25 mM D-glucose. Sp1 protein levels were evaluated by western blot in nuclear and cytoplasmic sub-cellular fractions. SLC29A1 promoter specific chromatin immunoprecipitation (ChIP) was performed. Transcription-

nal activity of the reporter plasmid construct containing -1114 bp of SLC29A1 promoter and nuclear Sp1 protein abundance were increased in cells exposed to 25 mM D-glucose (1.45 and 1.9-fold, respectively). Specific Sp1 binding to the aggggGGACggggaa consensus sequence (between -815 and -801 bp upstream ATG of SLC29A1 gene) was increased by 1.7-fold in 25 mM D-glucose. However, 25 mM D-glucose reduced (~50%) hENT1-mediated adenosine transport. Thus, a stimulatory effect of Sp1 on SLC29A1 transcriptional regulation could be a compensatory mechanism for the reduced adenosine transport exhibited by human fetal endothelium in response to elevated extracellular D-glucose.

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## Localization of GLYT2 in lipid rafts: a new mechanism of glycine transport modulation

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The neuronal glycine transporter GLYT2 is a plasma membrane protein that removes the neurotransmitter glycine from the synaptic cleft, thereby aiding the termination of the glycinergic signal and the reloading of the presynaptic terminal. In this report, we show that GLYT2 is associated with cholesterol and sphingolipid-enriched lipid microdomains in the plasma membrane of brainstem neurons. The removal of cholesterol and sphingolipids from cultured cells and synaptosomes impairs both the association of GLYT2 with lipid rafts and its activity, suggesting that the

lipid environment may constitute a new mechanism to modulate GLYT2 transporter activity. We demonstrate that the down regulation exerted by 4 $\alpha$ -phorbol 12 myristate 13-acetate (PMA) on GLYT2 function and surface expression involves transporter removal from lipid rafts by lateral mobilization and internalization. GLYT2 inhibition by PMA can be partially prevented by cholesterol depleting agents, suggesting that PMA-induced internalization of GLYT2 might be mediated by lipid rafts. The possible mechanisms of PMA-induced endocytosis are being studied.

## Transient currents in the glycine neuronal cotransporter GLYT2

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Presteady-state (transient) currents elicited by voltage steps in the absence of organic substrate were investigated in *Xenopus laevis* oocytes expressing the neuronal glycine transporter GLYT2. These currents were abolished either by removal of Na<sup>+</sup> from the external solution, by addition of saturating (1 mM) amounts of glycine, or by incubation in the specific blocker ORG25543. Isolation of the transient currents by subtraction of the control traces from those obtained in any one of the above three conditions gave equivalent results, indicating that sodium ions are essential for their development. Integration of the transients confirmed the intramembrane nature of the charge movement underlying the process. Reduction of the external Na<sup>+</sup> concentration caused negative shifts in the voltage dependence of the transient currents, with some reduction in the maximal moveable charge, and a reduction of the transport-associated current. Similar effects were induced by reductions of external Cl<sup>-</sup> but, while

in zero sodium both transient and transport currents were completely abolished, these persisted in zero external chloride. In control conditions the time course of the transient currents was markedly bi-exponential at negative potentials, with the slower time constant in the order of tens of milliseconds. The apparent affinity for glycine was in the micromolar range and decreased at positive potentials. The main electrophysiological features of GLYT2 operation may be interpreted on the basis of a kinetic scheme including two sequential steps of fast and slow charge movement, and linking the amplitude of the transport-associated currents to the rate of the slow step. In analogy to the behaviour of other transporters, this scheme suggests a transformation, induced by the presence of the neurotransmitter, of the capacitive-like transient currents in resistive-like transport currents, in agreement with the observed appearance of a stationary transport-associated current when the transient currents are abolished.

## Comprehensive molecular profiling reveals novel phenotypic alterations in mice deficient for the high affinity peptide transporter PEPT2

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PEPT2 is an integral membrane protein in renal epithelial cells that operates as a rheogenic transporter for di- and tripeptides and structurally related drugs. PEPT2 is located in the apical membrane and its prime role is thought to provide an efficient route for the reabsorption of filtered di- and tripeptides originating from plasma. To further elucidate the physiological function of PEPT2 we have generated a knockout mouse line that lacks a functional PEPT2 protein. Animals lack an obvious phenotype. For a comprehensive characterization of metabolic changes in the kidney in response to loss of the peptide transporter PEPT2, kidney tissue samples of 5 PEPT2 null mice and wildtype control mice were submitted to transcriptome, proteome and metabolome profiling and analysed also urinary amino acid and peptide excretion rates. cDNA whole genome microarray analysis identified 147 transcripts with significantly altered expression in transporter-deficient animals. Proteome analysis from the same samples by 2D-PAGE and MALDI-TOF-MS identified 37 protein entities with altered

steady state levels in knockout animals. Metabolite profiling from the same tissue samples by GC-MS revealed prominent changes in levels of a variety of free amino acids and derivatives. Urinary excretion of amino acids demonstrated increased glycine and cysteine/cystine concentrations and loss of dipeptides in urine was assessed by amino acid analysis of urine samples prior and after *in vitro* dipeptidase digestion. Dipeptides constituted a noticeable fraction of urinary amino acids in *Pept2*<sup>-/-</sup> animals but only dipeptide-bound glycine and cystine were selectively increased. These findings were confirmed by a drastically increased concentration of cysteinyl-glycine (cys-gly) in urine of *Pept2*<sup>-/-</sup> mice. Urinary loss of cys-gly together with lower concentrations of cysteine, glycine and oxoproline in kidney tissue and altered expression of mRNA and proteins involved in metabolism of GSH suggests that PEPT2 is predominantly a system for reabsorption of plasma cys-gly originating from glutathione break-down, thus contributing to re-synthesis of GSH.

## Differential regulation of SNAT2-mediated amino acid transport by nutritional and osmotic stress

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Amino acid starvation lowers global protein synthesis via phosphorylation at Ser 51 of the  $\alpha$  subunit of the translation initiation factor eIF2 (eIF2 $\alpha$ ) and increases production of cell defense proteins. Part of this response is the induction of neutral amino acid transport through the ubiquitous transporter SNAT2 (system A) that leads to the recovery of cell volume and amino acid levels once extracellular amino acid are provided. Hypertonic stress also increases system A activity as a mechanism to promote cell volume recovery.

We demonstrate that, upon total amino acid starvation, MEF cells homozygous for a mutant eIF2 $\alpha$  (Ser51 $\rightarrow$ Ala), exhibited lower system A transport activity, decreased SNAT2 mRNA levels, and impaired cell viability compared with wild type cells. In contrast, the induction of system A activity and SNAT2 mRNA levels by hypertonic stress were independent of eIF2 $\alpha$  phosphorylation.

The translational control of the SNAT2 mRNA during amino acid starvation was also investigated. Transient transfections with dicistronic or hairpin-containing monocistronic vectors indicated that the SNAT2 5'-UTR contains an IRES that allows synthesis of the transporters under conditions of global protein synthesis inhibition. We also show that amino acid starvation increases mRNA and protein expression from a reporter construct containing both the SNAT2 intronic AARE and the SNAT2-5'UTR.

We conclude that induction of system A activity upon amino acid starvation requires eIF2 $\alpha$  phosphorylation, increased gene transcription, and IRES mediated translation. In contrast, osmotic induction of system A transport is independent of eIF2 $\alpha$  phosphorylation.

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## Essential role of SLC5A8/SLC5A12 in the renal reabsorption of lactate

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Lactate is present at significant levels in circulation. It is an important precursor for gluconeogenesis and also plays a vital role in the maintenance of brain function. This metabolite is reabsorbed in the kidney with great efficiency (>98% filtered load). This process involves two different Na<sup>+</sup>-coupled transport systems in the apical membrane and a Na<sup>+</sup>-independent transport system in the basolateral membrane of the proximal tubular cells. The basolateral membrane transport system is mediated by MCT1, a monocarboxylate transporter. The molecular identities of the apical membrane transport systems remained unknown. Recently, we cloned two transporters from the mouse kidney which function as Na<sup>+</sup>-coupled transporters for lactate, SMCT1 (slc5a8) as the high-affinity transporter and SMCT2 (slc5a12) as the low-affinity transporter. Here we report that the expression of slc5a8 and slc5a12 is markedly down-regulated in the kidney in mice with homozygous de-

letion for the transcription factor *c/ebp $\delta$* . The down-regulation is specific for these two transporters as the expression of several other transporters (MCT1, URAT1, OAT1, and NaDC3) is not affected. The effect seems to be kidney-specific as there is little or no effect on the expression of slc5a8 and slc5a12 in the brain and intestinal tract. Consequent to the down-regulation of these two lactate transporters in the kidney, there is marked increase in urinary excretion and decrease in blood levels of lactate in *c/ebp $\delta$ <sup>-/-</sup>* mice. This is also accompanied by increased excretion of urate, demonstrating tight coupling between lactate reabsorption and urate reabsorption. In vitro reporter assays demonstrate that the promoters of SLC5A8 and SLC5A12 respond specifically to C/EBP $\delta$  in HEK293 cells. These studies provide direct in vivo evidence for the obligatory role of slc5a8 and slc5a12 in the renal reabsorption of lactate and for the functional coupling between slc5a8/slc5a12 and URAT1.

## The role of serum- and glucocorticoid-induced kinase in aldosterone-mediated changes in chicken SGLT1 from distal intestine

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Chickens fed a diet with a low Na<sup>+</sup> content show a decrease in glucose transport across apical SGLT1 and basolateral GLUT2 in the ileum and rectum. These effects are the result of secondary hyperaldosteronism induced by low Na<sup>+</sup> diets (1). In addition, recent evidence suggests that serum- and glucocorticoid-regulated kinase (SGK) is induced by aldosterone and acts as a key mediator of aldosterone effects in sodium homeostasis (2). Here we report studies investigating the changes in the expression and activity of SGK in the jejunum, ileum and rectum of chickens fed wheat and barley with drinking water containing either 150 mM NaCl (HS) or 0.015 mM (LS) for 14 days. At day 14 mucosal scrapings from jejunum, ileum and rectum were collected for western blot analysis of SGK. In other experiments, intestinal segments were incubated (30 min; 25°C) with or without H89 agent, an SGK specific inhibitor. After the incubations, brush-border membrane vesicles (BBMV) were prepared in order to measure SGLT1 activity and specific phlorizin binding to SGLT1 (as an indicator of SGLT1 density). Western blot analysis of SGK shows that LS diet

enhances by more than 40% the kinase expression in the enterocytes from ileum and rectum, without any effect in the jejunum. In ileal and rectal BBMV obtained from LS chickens, the inhibition of SGK by H89 agent induces a recovery of SGLT1 activity and number, within the range of values characteristic of HS animals. We conclude that, in the chicken distal intestine, SGK may have a role in the aldosterone-mediated downregulation of brush-border membrane SGLT1 abundance and activity.

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## L-pipecolic acid is an inhibitor of the proton/amino acid transporter PAT2 (SLC36A2)

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The amino acid transporter PAT2 (SLC36A2), isolated from mouse and rat (1,2), functions as a Na<sup>+</sup>-independent pH-dependent H<sup>+</sup>-coupled transporter and has a more restricted substrate specificity and higher affinity than the related PAT1 (SLC36A1). Both PAT1 and PAT2 transport a variety of amino acids including those containing heterocyclic ring structures e.g. proline. When PAT function was determined by [<sup>3</sup>H]proline influx into PAT-expressing *Xenopus laevis* oocytes the six-membered ring piperidine carboxylates appeared to be low affinity substrates for PAT1 with L-pipecolic acid having a lower apparent affinity (IC<sub>50</sub> 16mM) than

nipecotic acid (IC<sub>50</sub> 4.1mM). L-Pipecolic acid was a more effective inhibitor (IC<sub>50</sub> 0.84mM) of [<sup>3</sup>H]proline uptake via rat PAT2 than nipecotic acid (IC<sub>50</sub> >20mM). The relative ability of L-pipecolic acid to inhibit [<sup>3</sup>H]proline uptake via rat PAT2 was similar to that observed with glycine (IC<sub>50</sub> 0.39mM) suggesting that these two substrates are recognised equally well by the transporter. However, when substrate transport through rat PAT2 was estimated by two-electrode voltage clamp measurement of substrate-induced current (using saturating substrate concentrations) the current observed following exposure to L-pipecolic acid was only 15% of that with glycine

suggesting that L-pipecolic acid acts as a non- or poorly-transported inhibitor of PAT2. Coexposure of PAT2-expressing oocytes to L-pipecolic acid and glycine confirmed this as a decrease in the glycine-induced current was observed. L-Pipecolic acid is thus a potential lead compound for the design of selective PAT2 inhibitors.

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## Homo- and heterooligomerisation between some members of the Na<sup>+</sup>/Cl<sup>-</sup>-dependent transporter family, KAA1, CAAT1 and rGAT1, seen as FRET

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Recent evidences indicates that NSS neurotransmitter sodium symporters form constitutive oligomers. Using the fluorescent proteins CFP and YFP together with the FRET approach we tested the possibility that the two highly homologous transporters KAA1 and CAAT1 could interact and potentially form homo and hetero oligomers. To better understand the domains involved in this phenomena we tested also the possibility of oligomer formation between KAA1 or CAAT1 and rGAT1. Different constructs with the two fluorescent proteins (CFP, YFP) located at the N or C terminus of the transporters have been tested for FRET on an epifluorescence set up, obtaining NFRET images. All the constructs have been transiently co-transfected in HEK 293 cells and observed 48 hours later. Accordingly with recent crystal structural models, higher NFRET values are obtained when both the FP's are positioned at the C terminus of the trans-

porters, suggesting the closeness of the C termini in the oligomer. Beside the evidence of homooligomer formation, heterooligomer arrangement is also possible between KAA1 and CAAT1. Low NFRET values have been obtained instead cotransfecting either KAA1 or CAAT1 together with rGAT1, suggesting no interaction. Heterooligomerisation of KAA1 and CAAT1 does not interfere with their transporter function (work in progress) as already shown in our lab for rGAT1: the functional unit appears therefore to be the monomer. These results suggest that heterooligomer formation could be reasonably possible only between proteins with high sequence identity, probably because the 3D structure plays a major role in regulating the assembly process. Further studies with other members of NSS transporter family are in progress in our lab in order to identify the determinants involved in oligomerisation

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## High D-glucose-increased L-arginine transport involves Sp-1 binding to the cationic amino acid transporter 1 promoter region in human fetal endothelium

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L-Arginine transport in human umbilical vein endothelial cells (HUVEC) is mediated mainly by system y<sup>+</sup>/hCAT-1. High extracellular D-glucose (25 mM) stimulates L-arginine uptake and increases hCAT-1 mRNA expres-

sion. We now studied the effect of high D-glucose on Sp1 expression and binding to a SLC7A1 (for hCAT-1) promoter region. HUVEC from normal pregnancies were cultured in 5 or 25 mM D-glucose (24 hours). Kinetics of L-[<sup>3</sup>H]ar-

ginine transport, hCAT-1 mRNA expression (real time PCR), Sp1 protein abundance at the cytoplasm and nucleus (western blot), and Sp1 binding to the promoter (chromatin immunoprecipitation) were determined. Sp1 nuclear abundance (~1.6-fold) and binding (~8.7-fold) to the GGGCGGG consensus sequence (between -350 and -216 bp of SLC7A1 promoter region) was increased by 25 mM D-glucose. In addition, incubation with 25 mM D-glucose

also increased the  $V_{max}$  for L-arginine transport (2.1-fold), with no significant changes in the apparent  $K_m$ . High D-glucose also increased hCAT-1 mRNA expression (~20-fold). Thus, the stimulatory effect of Sp1 on SLC7A1 transcriptional regulation could be responsible of D-glucose stimulation of L-arginine transport via system y<sup>+</sup>/hCAT-1 in HUVEC. Supported by FONDECYT 1030781/1030607. MG holds a CONICYT PhD fellowship.

## Effect of protons on the transport of nucleosides by the human cotransporter CNT3

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Human CNT3 is a high-affinity Na<sup>+</sup>/nucleoside cotransporter which transports both pyrimidine and purine nucleosides. Human CNT3 also functions as a H<sup>+</sup>/nucleoside symporter although it has been shown that it does not transport either guanosine or AZT in the presence of a proton gradient (1). In the present work we have compared the transport of a series of nucleosides by hCNT3 in the presence of Na<sup>+</sup> and H<sup>+</sup>. The transporter was cloned from human kidney and expressed in *Xenopus laevis* oocytes to obtain the kinetic parameters for the nucleosides using electrophysiological methods.

The  $K_{0.5}$  for uridine increased from 10  $\mu$ M in Na<sup>+</sup>-medium to 180  $\mu$ M in the absence of Na<sup>+</sup> at pH 5.5. The maximal current ( $I_{max}$ ) also increased by ~70% in the presence of H<sup>+</sup>. Surprisingly,  $I_{max}$  for 2'-deoxyuridine at acidic pH was ~50% higher than uridine  $I_{max}$ , although in Na<sup>+</sup>-medium  $I_{max}$  values were similar for both nucleosides. The kinetic parameters of cytidine showed similar behavior than uridine parameters, while the  $I_{max}$  was ~1.5 fold lower than that for uridine in the presence of either Na<sup>+</sup> or H<sup>+</sup>. The  $K_{0.5}$  for adenosine also in-

creased from 38  $\mu$ M in Na<sup>+</sup>-medium to 296  $\mu$ M in the presence of H<sup>+</sup>, whereas its  $I_{max}$  was similar to that for uridine in the presence of Na<sup>+</sup> and was not affected at pH 5.5. Unexpectedly, 1mM guanosine evoked inward currents at acidic pH that were half of that obtained in Na<sup>+</sup>-medium. Finally, the nucleoside analog N1-methyladenosine showed a  $K_{0.5}$  of 2-4 mM and produced ~30% of uridine maximal current in the presence of Na<sup>+</sup>, at 1 mM concentration. However, it did not induce any inward current in the presence of H<sup>+</sup>.

In summary, these results show different behavior of CNT3 for the transport of a specific nucleoside depending on the cation that is present in the medium.

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## Changes in amino acid transport in human SH-SY5Y neuroblastoma cells following retinoic acid-induced differentiation

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The capacity of neuroblastoma cells to undergo differentiation in response to retinoids is being targeted as the basis for development of novel retinoid-based therapeutic regimes for

the treatment of neuroblastoma disease (1). Relatively little is known about the changes in expression of membrane transport proteins in neuroblastoma cells following differentiation

but mapping these changes will be central to the development of novel treatments and therapies. Radiolabelled amino acid uptake (pH 7.4, 37°C) measurements were performed using human SH-SY5Y neuroblastoma cells cultured in the presence or absence of retinoic acid (RA) for 7 days. RA-induced differentiation had varying effects on uptake of a number of amino acids leading to a decrease in [<sup>3</sup>H]taurine, [<sup>3</sup>H]proline and [<sup>3</sup>H]glutamic acid uptake, an increase in [<sup>3</sup>H]lysine uptake but had no effect on [<sup>3</sup>H]GABA uptake. In undifferentiated cells, taurine uptake has many characteristics of the TauT transporter being a high affinity ( $K_m$  27 μM), Na<sup>+</sup>- and Cl<sup>-</sup>-dependent carrier that is inhibited by unlabelled taurine, β-alanine, GABA and β-ABA. Taurine uptake had similar charac-

teristics in differentiated cells. In conclusion, RA-induced differentiation of SH-SY5Y neuroblastoma cells is associated with a change in expression of the complement of amino acid transporters expressed at the plasma membrane which could be used to target novel therapeutics to neuroblastoma tissue. Supported by the MRC and Wellcome Trust. SAH holds an MRC postgraduate studentship.

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## Concentrations of excitatory amino acid transporter proteins in brain tissue

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The central nervous system expresses five different sodium-dependent glutamate (excitatory amino acid) transporters called EAAT1 (GLAST), EAAT2 (GLT), EAAT3 (EAAC), EAAT4 and EAAT5. EAAT1 and -2 are found throughout the brain and mainly localized in glial cell membranes. They are essential for maintaining low resting levels of extracellular glutamate, and for protecting neurons against excitotoxicity. The roles of the other three remain elusive. EAAT4 and EAAT5 are almost exclusively found in the cerebellar Purkinje cells and in the retina, respectively. EAAT3 is found in neurons in most brain re-

gions, but its contribution to extracellular glutamate clearance is unclear, and quantitative data on the concentrations of this transporter subtype have been missing. We have now determined the concentration of EAAT3 in rat brain tissue, and present an updated summary of our measurements of the tissue concentrations of EAAT1, -2, -3, and -4. By combining the tissue concentrations with immunocytochemical localization data and measurements of the membrane surfaces of the glial and neuronal cellular processes expressing the different transporter subtypes, we calculate the transporter membrane densities.

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## Consenting determinants of the bacterial and fungal purine uptake pathways in the nucleobase-ascorbate transporter signature motif

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Using a functional Cys-less background, we have performed Cys-scanning and site-directed mutagenesis at the nucleobase-ascorbate transporter (NAT) signature motif <sup>324</sup>QNGVIQMTG<sup>333</sup> of YgfO, a major xanthine-specific NAT/NCS2 homologue from *E. coli*. Analysis of an extensive series of mutants shows that (1) Gln324 is irreplaceable for high-affinity purine binding and uptake and Asn325 is strictly irreplaceable for active transport at any

substrate concentration tested; (2) Thr332 and Gly333 are crucial for specificity against different purine analogues and their replacement yields changes indicative of indirect, pleiotropic effects on substrate binding; (3) Bacterial and fungal NAT-motif determinants consent, as strikingly similar observations have been made, to a certain extent, with the major uric acid/xanthine-specific NAT/NCS2 homologue of the filamentous ascomycote *Aspergillus nidulans*; (4)

Conformation around the NAT motif is important: our data reveal a strip of residues (Ala323, Asn326, Gly327, Val328, Ile329, Thr332, Gly333 and Ser336) that form an N-ethylmaleimide-sensitive face of a short loop and a puta-

tive alpha-helix immediately downstream of the bacterial NAT motif. These residues may undergo essential conformational movements during turnover that are hindered by covalent attachment of the bulky N-ethylmaleimide.

## Comparative binding kinetics of [<sup>3</sup>H]citalopram and [<sup>3</sup>H]DASB at rat sert

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The Serotonin (5-HT) transporter (SERT) is a known molecular target for the treatment of psychiatric disorders. In PET studies using [<sup>11</sup>C]DASB in man, SSRIs need to occupy >80% SERT for clinical efficacy (1). In rodents, [<sup>3</sup>H]citalopram is the radiolabel of choice to determine *ex vivo* SERT occupancy. The aim of this study was therefore to characterise [<sup>3</sup>H]citalopram and [<sup>3</sup>H]DASB binding in rat brain cortex homogenates both in terms of pharmacology and kinetic profiles using classical and previously described methodologies (2).

Homologous competition experiments of citalopram and DASB gave pKD values of 9.29±0.05 and 9.78±0.06. DASB inhibited [<sup>3</sup>H]citalopram binding with a pKi value of 9.81±0.07 and citalopram inhibited [<sup>3</sup>H]DASB binding with a pKi value of 9.23±0.03. Additionally the pharmacology of standard SERT inhibitors displayed an identical rank order of affinity at these two binding sites whereby DASB > dapoxetine > citalopram > fluoxetine > imipramine > 5-HT. DASB and citalopram therefore bind to rat SERT at the same overlapping binding sites.

However, association (K<sup>+1</sup>) and dissociation (K<sup>-1</sup>) rates for [<sup>3</sup>H]citalopram and [<sup>3</sup>H]DASB binding were significant-

tly different. Methodologies (2) employed to measure the kinetics of unlabelled DASB and citalopram at the [<sup>3</sup>H]citalopram binding site, maintained such differences and predicted the classical kinetics of both [<sup>3</sup>H]citalopram and [<sup>3</sup>H]DASB approximating the pKDs determined from competition studies.

This is the first report to demonstrate that SERT inhibitors with kinetically different binding profiles, as assessed using [<sup>3</sup>H]citalopram and [<sup>3</sup>H]DASB binding, can be detected using a methodology (2) that investigates the effect of the presence of unlabelled compound (DASB and citalopram) on the association of [<sup>3</sup>H]citalopram binding to rat SERT. Such information may additionally be useful in not only determining comparative kinetics of other rat SERT inhibitors but also in refining translational PK/PD relationships in rodents and man.

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Table 1.

Kinetics	Classical methods		[ <sup>3</sup> H]Citalopram adapted method	
	[ <sup>3</sup> H]Citalopram	[ <sup>3</sup> H]DASB	Citalopram	DASB
K <sup>+1</sup> (M <sup>-1</sup> min <sup>-1</sup> ) (x107)	3.78±0.4	27.4±3.4*	5.7±0.2	35.8±4.7*
K <sup>-1</sup> (min <sup>-1</sup> )	0.01639±0.001	0.05547±0.01*	0.01352±0.001	0.03132±0.005*
pKD	9.31±0.05	9.63±0.05	9.63±0.02	10.06±0.04

## Progesterone inhibits folic acid transport in cultured human trophoblasts

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The aim of this work was to investigate the putative involvement of members of the ABC superfamily of transporters on folic acid (FA) cellular homeostasis in the human placenta. For this, the effect of selective inhibitors of MDR1, MRP and BCRP on <sup>3</sup>H-FA uptake and efflux was investigated in BeWo cells and primary cultures of human cytotrophoblasts.

<sup>3</sup>H-FA uptake and efflux in BeWo cells and in human cytotrophoblasts were unaffected or hardly affected by MDR1 inhibition (with verapamil), MRP inhibition (with probenecid) OR BCRP inhibition (with FTC). However, <sup>3</sup>H-FA uptake and efflux were inhibited by progesterone (200 μM) both in BeWo cells and human cytotrophoblasts. Inhibition of <sup>3</sup>H-FA uptake in BeWo cells by progesterone was concentration-dependent and seems to be very specific, since other tested steroids (β-estradiol, corticosterone, testosterone, aldosterone and estrone) were devoid of effect. However, the efflux was also inhibited by β-estradiol and

corticosterone and was stimulated by estrone, suggesting that there are distinct mechanisms mediating uptake and efflux of the vitamin in BeWo cells and that there may be an additional efflux mechanism sensitive to modulation by β-estradiol, corticosterone and estrone.

Moreover, we tested the effect of progesterone alone or in the presence of SITS, a known inhibitor of RFC1, upon the uptake of <sup>3</sup>H-FA BY BeWo cells. The level of inhibition was the same in both cases, suggesting that progesterone inhibits RFC1.

In conclusion, our results suggest that progesterone, a sterol produced by the placenta, may inhibit FA placental uptake in vivo.

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## Placental uptake of vitamin B<sub>9</sub>: effect of pathological conditions, drugs and drugs of abuse

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Vitamin B<sub>9</sub> (folic acid; FA) acts as a coenzyme in important cellular reactions being thus critically important for normal fetal development.

Pregnant women are exposed to several xenobiotics due to pharmacotherapy of mother's chronic or gestational disease or to lifestyle factors such as smoking, drug abuse and alcohol consumption. It is known that these conditions have deleterious effects on the fetus, but the cellular mechanisms involved are not completely understood.

Our aim was to study the effect of serotonin and hyperglycemia (markers for preeclampsia and diabetes, respectively), clonidine and insulin (drugs for therapy of hypertension and diabetes, respectively) and the drugs of abuse nicotine, cocaine, ethanol and its metabolite acetaldehyde on placental FA uptake. For this, we tested the effects of these compounds on the uptake of <sup>3</sup>H-FA by primary cultured human cytotrophoblasts.

Our results show that serotonin (0.1-300 μM), glucose (10-45 mM), clonidine (1-3000 nM) and insulin (1-13

μg/l) had no effect upon <sup>3</sup>H-FA uptake. Surprisingly, nicotine (0.1-1000 μM) increased <sup>3</sup>H-FA uptake at the higher concentrations tested, and cocaine (0.25-25 μM) was devoid of effect. Both ethanol and acetaldehyde (0.1-100 mM) tended to inhibit <sup>3</sup>H-FA uptake in a concentration dependent manner, although the inhibition was significant only for the highest concentration of acetaldehyde.

Our results are compatible with the harmlessness of clonidine and insulin therapy during pregnancy and with the known toxicity of ethanol consumption during this period. The results also suggest that the teratogenic or fetotoxic effects of serotonin, hyperglycemia, nicotine and cocaine are not associated with a deficient supply of FA to the placenta and the fetus.

This work was supported by FCT and Programa Ciência, Tecnologia e Inovação do Quadro Comunitário de Apoio (POCTI/SAU-FCF/59382/2004).

## Aminopeptidase inhibition uncovers large outward currents in dipeptide-injected *Xenopus* oocytes expressing PEPT1

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Cation-coupled nutrient transporters are often viewed as one-way machines, which serve exclusively the sodium (or proton) gradient-driven uptake of their substrates from the extracellular space into the cell. However, recent data demonstrate that several carriers can transport in the reverse direction when the overall driving force is reversed. Outward transport initiated by loading the cell via the transporter under investigation itself is limited by the driving forces which allow to build up only low substrate concentrations. The patch clamp technique overcomes this problem, but it is technically demanding. We here describe a simple method that allows the investigation of the reverse transport mode in a wide concentration range. Dipeptide substrates were injected into *Xenopus* oocytes expressing the peptide transporter PEPT1 to give a final concentration of  $\approx 25$  mM. The outward currents of most tested dipeptides declined after the injection very rapidly (seconds to minutes) to zero. This de-

cline was caused by the very fast hydrolysis of the dipeptides which could completely be inhibited by preincubation of the oocytes in the aminopeptidase-inhibitor bestatin. Comparison of the time course and height of outward currents in untreated (UO) and preincubated oocytes (PO) allowed to estimate the relative hydrolysis rate of the injected dipeptides and the transport capacity of PEPT1 in the outward (reverse) direction. Stepwise substrate injection allowed the estimation of substrate affinity for the reverse direction. The hydrolysis rate in UO was Ala-Ala $\approx$ Ala-Gly $\approx$ Gly-Phe>Gly-Gln>Gly-Leu>Gly-Gly-Gly $\approx$ Gly-Gly>>Gly-Sar. The outward current in PO reached its maximum after 4 min, remained stable over at least 10 min and was for Gly-Leu>Gly-Phe >Ala-Gly $\approx$ Gly-Sar $\approx$ Ala-Ala $\approx$ Gly-Gly>Gly-Gly>Gly-Gly-Gly. Unexpectedly, outward currents measured at +80 mV membrane potential were 4-5 times higher than inward currents at saturating substrate concentrations.

## Functional characterization of protein variants of HMRP4 (ABCC4) in *xenopus laevis* oocytes

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In comparison to metabolizing enzymes, the importance of genetic variants in transporters for drug responses is less well understood. We have undertaken a comprehensive search and functional characterization of protein variants of the multiple drug resistance protein (MRP) 4. This protein belongs to the C subfamily of the ATP-binding cassette (ABC) transporter superfamily and participates in the transport of diverse antiviral- and chemotherapeutic agents. Eight out of ten analysed missense variants (G187W, K304N, G487E, Y556C, E757K, V776I, R820I, V854F, I866V, T1142M) were identified in MRP4 by direct sequencing of population-derived DNA samples. The variant positions G487E and R820I were found at PharmGKP. When the MRP4/ABCC4 protein variants were evaluated by the SIFT and PolyPhen algorithm, G187W, G487E, Y556C, R820I, V854F, T1142M variants were predicted to have major deleterious effects. All MRP4 variants were cloned in-frame with the green fluorescent protein, to be used

as an expression marker. Vector-derived cRNAs were injected into oocytes and the protein expression assessed by western blot and fluorescence measurements. All MRP4 proteins (wild-type, variants) were found to be expressed predominantly in the oocyte membrane. Immunoblotting experiments showed that MRP4 mutant proteins Y556C, E757K, V776I and T1142M were expressed at lower levels compared to wild-type MRP4. Oocytes injected with transporter-coding MRP4 wild-type cRNA exhibited higher transport activity than control oocytes (water-injected), indicating the functionality of the expressed MRP4 protein. A detailed characterization of MRP4, and of their variants using  $^3\text{H}$  estradiol  $\beta$ -glucuronide,  $^3\text{H}$  9-(2-phosphonylmethoxyethyl)-adenine (PMEA) and  $^{14}\text{C}$  6-mercaptapurine, is in progress.

In conclusion, *Xenopus* oocytes are an experimental system well-suited to characterize the efflux transporter MRP4 protein and its protein variants

## Glutamate transporters in the developing medial nucleus of the trapezoid body (MNTB)

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Glutamate transporters in the central nervous system are essential for maintaining low resting levels of extracellular glutamate. Because of their high affinity, and because they are present at high concentrations, these transporters are also in a position to modulate extracellular glutamate diffusion. Data on the localizations and concentrations of transporters are therefore essential for the development of models of glutamatergic neurotransmission. The synapses made by the large nerve terminals called the calyces of Held, in the medial nucleus of the trapezoid body (MNTB) in the brain stem, is a frequently used model system for studies of glutamatergic transmission. The large size of these nerve

terminals makes it possible to perform electrophysiological recordings simultaneously from both the pre- and postsynaptic elements of the synapses. As a basis for development of mathematical models of glutamatergic neurotransmission at these synapses, we have investigated the protein expression of the different glutamate transporter subtypes in the MNTB of rats aged 9 days, 17 days, and 8 weeks. Immunocytochemical and immunoblot labelling intensities for EAAT1 and -3 were strongest at 9 days, and declined with age. EAAT2-labelling, on the other hand, was weak at 9 days, and increased with age, while EAAT4-labelling was low in the MNTB at all ages investigated.

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## Effects of flavonoids on p-glycoprotein expression in intestinal epithelial cells

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Flavonoids, abundant in food, interact with a number of efflux transporters, such as p-gp, which is expressed at the apical surface of epithelial cells of several organs. The overexpression of p-gp is a major cause for the failure of cancer chemotherapy in man. In vitro we studied the effects of 14 flavonoids on p-gp expression in human Caco-2 cells by western blot, FACS analysis and RealTime-PCR. Independent of the method, cultivation in media containing for example flavone, chrysin, quercetin or myricetin (10 µM) for four weeks increased p-gp protein expression up to 4,4 fold. In vivo the effect of flavone (400 mg/kg/day for 6 weeks) in mucosal tissue

of C57BL/6 mice was investigated by western blot. The occurrence of p-gp increased along the gastrointestinal tract with the highest expression found in the colon. The flavone treated animals showed an altered p-gp-expression in all investigated segments of the gut versus the corresponding segment of the vehicle control animals, whereupon the increase diminishes towards the colon (max. +56%). Due to the increasing popularity of supplements containing flavonoids, it is important to understand their effects on p-gp, since its modulation can lead to significant drug interference by affecting intestinal absorption, renal secretion, and biliary excretion.

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## Glycosylation affects membrane maturation of the OCTN2 carnitine/organic cation transporter

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Primary carnitine deficiency is disorder of fatty acid oxidation caused by mutations in the Na<sup>+</sup>-dependent

carnitine/organic cation transporter OCTN2. Most missense mutations identified in patients with primary carnitine defi-

ciency affect predicted transmembrane domains or intracellular loops of the transporter. One exception is R83L, located in an extracellular loop close to putative glycosylation sites (N57, N64, and N91) of OCTN2. Analysis by confocal microscopy indicated that R83L impaired maturation of the transporter to the plasma membrane. We tested whether glycosylation of OCTN2 was required for maturation to the plasma membrane. Substitution of the three putative glycosylation sites with glutamine (Q) decreased mildly carnitine transport when single sites were substituted. By contrast, simultaneous substitution of N57 and N64 cause a marked decline in carnitine transport that was fully abolished when all three glycosylation sites were substituted by glutamine (N57Q/N64Q/N91Q).

Analysis by confocal microscopy indicated that glutamine substitutions caused progressive retention of OCTN2 transporters in the cytoplasm, up to full retention (such as that observed with R83L) when all 3 glycosylation sites were substituted. Prolonged incubation with the glycosylation inhibitor tunicamycin decreased carnitine transport and retained OCTN2 transporters in the cytoplasm. Western blot analysis is testing the mobility of the mutant OCTN2 transporters with substitutions at glycosylation sites. These results indicate that glycosylation is essential for the maturation of OCTN2 carnitine transporters to the plasma membrane and suggest that R83L causes primary carnitine deficiency by impairing OCTN2 glycosylation.

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## Blood-brain barrier amino acid transporters

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Entry and efflux of amino acids through the blood-brain barrier (BBB) is mediated by specific transporters expressed at the luminal and/or abluminal membranes of brain microvascular endothelial cells (MVEC). The relatively low extracellular level of amino acids in brain is maintained to a large extent by this active barrier and plays an important role for many physiological processes such as neurotransmitters synthesis. Disturbances in brain amino acid levels have been implicated in diseases ranging from diabetes to various dementias. In addition, BBB amino acid transport is relevant for the central nervous system distribution of drugs and diagnostic markers such as tracers used for positron emission tomography (PET). One such promising new tracer which offers many practical advantages is O-(2-([<sup>18</sup>F-fluoroethyl] tyrosine (FET). To address at a molecular level the mRNA expression of BBB amino acid transporters in brain MVECs we used microarray technology on target RNA derived from three human sources: 1) primary cortex MVECs from normal human brain, 2) primary human brain endothelial cells from a com-

mercial source and 3) a transformed human brain MVE cell line. This first approach confirmed the previously described expression of the neutral amino acid exchanger LAT1-4F2hc (Slc7a5-Slc3a2) and suggested the expression of a number of other amino acid transporters. Based on these results, we are examining the protein expression and localization of amino acid transporters in mouse brain tissue sections using immunofluorescence. Further we are investigating the uptake of the FET isomers via various identified transporters of the blood-brain barrier expressed in *Xenopus* oocytes. Our results show that both FET isomers inhibit the uptake function of XPCT (Slc16a2) and PAT1 (Slc36a1), whereas only the L-isomer inhibits uptake by LAT1- (Slc7a5-) and LAT2-4F2hc (Slc7a8-Slc3a2). In summary, we are investigating the amino acid transport of the BBB endothelia by analyzing the mRNA expression of amino acid transporters via microarray and real time PCR in brain MVECs, combined with testing the protein expression, localization and function of identified gene products.

## Differential regulation of EAAC1 and GLT1 glutamate transporters trafficking by cyclosporine A

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In line with the roles of Na/K-dependent glutamate transporters in the termination of excitatory neurotransmission and in providing cells with glutamate for metabolic purposes, the transport activity and surface expression of neurotransmitter transporters are dynamically regulated, often through modulation of their intracellular trafficking (Vanoni et al., 2004, *J Cell Sci.* 117: 5417-26; Massari et al., 2005, *J. Biol. Chem.* 280: 7388-97).

The neuronal EAAC1 glutamate transporter is also expressed in renal cells, and loss of its function, in addition to seizures results in dicarboxylic aminoaciduria, whereas loss of glial GLT1 is involved in neurodegenerative disease such as amyotrophic lateral sclerosis (ALS). Many neurotransmitter transporters, including the EAAC1 and GLT-1, are regulated by protein kinase C (PKC) and these effects are associated with changes in cell surface expression. In order to study the molecular mechanisms of PKC regulation on EAAC1 and GLT1 we have analyzed the effects of kinases and phosphatases on their localization using as cellular model the renal epithelial polarized MDCK cell line. We

have found that PKC activation causes internalization through a clathrin-dependent pathways of EAAC1 and GLT1 in the common recycling endosomal compartment (CRE). However, the internalization of EAAC1 but not GLT1 was also dependent on the activity of the phosphatase calcineurin, as treatment with the specific inhibitor cyclosporin A prevented the PKC-induced internalization of EAAC1, without affecting GLT1 accumulation in the recycling compartment. We have also demonstrated that target of calcineurin is the C-terminal intracellular domain of EAAC1, because a GLT1 chimeric transporter having the C-terminal domain of EAAC1 remains at the cell surface after PKC activation when calcineurin was inhibited. These data indicate that isoform-specific mechanisms selectively modulate the trafficking of glutamate transporters and may explain the exclusive reduction of GLT1 in ALS. The differential regulation of EAAC1 and GLT1 could also lead to the development of therapies aimed at preventing or limiting neuronal damage without disruption of glutamate metabolism.

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## Localization of Asc-1 and ATA2/SNAT2 in rodent brains and their proposed roles as serine transporters

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D-serine is an endogenous modulator of NMDA/glutamate receptors, and L-serine is found to be essential for survival and development of neurons. Since neurons lack L-serine biosynthetic enzyme Phgdh, they must take up L-serine for their survival. We have recently identified asc-type amino acid transporter 1 (Asc-1) as a neuronal D-/L-serine transporter. Amino acid transporter A2 (ATA2/SNAT2) is also an L-serine transporter expressed in brain, although their physiological functions remain unclear. In this study, we performed immunohistochemical analysis of these serine transporters in the rodent brains, to investigate their roles as serine transporters. The Asc-1 immunoreactivity (Asc-1-ir) was detected in pyramidal neurons. It was clearly localized in dendrites as well as somata. The ATA2-ir was widely detected in neurons, whose

intracellular localization was similar to that of Asc-1-ir. Different from Asc-1-ir, ATA2-ir was also located in astrocytes and ependymal cells, especially around capillary blood vessels and ventricles. These findings suggest that Asc-1 could be important for synaptic clearance of D-serine via a postsynaptic uptake mechanism. In addition, both ATA2 and Asc-1 could be involved in the neuronal uptake of L-serine. Furthermore, ATA2 in astrocytes may also take up L-serine from the extracellular spaces including blood and CSF. These results suggest the significant contribution of Asc-1 and ATA2 to amino acid mobilization in the brain including the accumulation of L-serine in astrocytes as well as the neuronal uptake of L-serine that is used for survival of neurons.

## Single amino acid substitution changes substrate selectivity of the *manduca sexta* neutral amino acid transporter KAAT1

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The neutral amino acid transporter KAAT1 and its highly homologous CAATCH1, cloned from the midgut epithelium of the larva *Manduca sexta* have been classified as members of the Na<sup>+</sup>/Cl<sup>-</sup>-dependent transporter family. These transporters are able to couple the substrates translocation not only to sodium but also to potassium gradient. Recent evidence indicates that neurotransmitter transporters belonging to this family form constitutive oligomers but show monomeric functionality, and the data from the tertiary structure of the leucine-transporting bacterial homologue LeuT suggest the permeation pathway and recognize the amino acids involved in substrate and sodium binding. CAATCH1 and KAAT1 give rise to currents of different amplitude depending on the transported amino acid, the co-transported ion, pH and the membrane voltage. Transport-associated currents induced by different organic substrates are notably distinct between the two proteins; in particular in KAAT1, leucine is the amino acid that gives rise to the larger current in presence of potassium and is able to

generate a current also in presence of sodium, but in CAATCH1 this amino acid is not transported, and in sodium it also blocks the leakage current. In order to identify the determinants involved in these phenomena, we have mutated serine 308, the only residue that differs in the two transporters in the region forming the leucine binding site, according to the LeuT structure. This amino acid is conserved only in the members of the family able to transport leucine, and its substitution in KAAT1 with threonine, the residue present instead in CAATCH1, blocks the current induced by leucine in sodium, and causes a reduction of about 70% of the current in potassium. Surprisingly, the opposite mutation introduced in CAATCH1 changed only slightly its behaviour in presence of leucine and did not confer the ability to transport leucine. We are now investigating on the other amino acids that are different in the two proteins and located in domains involved in substrate and ions translocation, in order to better understand the substrate and ionic specificities in the other proteins of the family.

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## Human equilibrative nucleoside transporter 1 (hENT1) expression correlates with gemcitabine uptake and cytotoxicity in mantle cell lymphoma (MCL)

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Nucleoside transporters (NTs) might play a relevant role in the intracellular targeting of many nucleoside analogues used in anticancer therapy. Two gene families (SLC28 and SLC29) encode the two types of human NTs, Concentrative Nucleoside Transporter (CNT) and Equilibrative Nucleoside Transporter (ENT) proteins. We have previously described that chronic lymphocytic leukemia (CLL) cells express both SLC28- and SLC29-related mRNAs, although transport function seems to be mostly related to ENT-type transporters. In this study we have analyzed the role of ENTs in nucleoside-derived drug bioavailability and action in mantle cell

lymphoma (MCL) cells. We have analyzed the expression of hENT1 and hENT2 by real time RT-PCR and by Western Blot in five MCL cell lines and 20 primary MCL cells. High levels of hENT1 protein expression in MCL cells were detected in contrast to hENT1 expression in CLL cells, and a good correlation was found between protein and mRNA levels of hENT1, thus indirectly suggesting that hENT1 might be transcriptionally regulated in MCL cells. Uridine and gemcitabine transport significantly correlated with hENT1-related mRNA expression and protein levels, but no correlation was found for fludarabine uptake. Unless the doses of gemcitabine

to induce a cytotoxic effect in primary MCL cells ( $LD_{50} < 50 \mu\text{g/ml}$ ) were higher than those used in sensitive MCL cell lines ( $LD_{50} < 3 \mu\text{g/ml}$ ), these doses were lower than doses of fludarabine tested in the same cases. More importantly, a significant correlation between hENT1 mRNA and protein amounts, drug uptake and sensitivity to gemcitabine was also

observed. These results further support the hypothesis that nucleoside transporters are implicated in the therapeutic response to nucleoside analogues, suggesting that hENT1 expression might be useful to predict response to nucleoside analogues known to be taken up via ENT1 carriers, such as gemcitabine in MCL patients.

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## Nutritional regulation of the L-proline transport system in renal epithelial cells

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Efficient reabsorption of amino acids in renal proximal tubules is central to whole body nitrogen homeostasis and occurs by the concerted action of a spectrum of amino acid transporting systems. Reabsorption of proline/prolyl-derivatives is mediated by SIT1 (Sodium/Imino-acid Transporter, SLC6A20), a member of the  $\text{Na}^+$ - and  $\text{Cl}^-$ -dependent neurotransmitter family. It was identified as a candidate for the “classical System IMINO” in kidney. We have characterized SIT1 expression in renal OK cells by PCR and its function by tracer uptake experiments.

L-proline transport in OK cells is  $\text{Na}^+$ - and  $\text{Cl}^-$ -dependent and shows high affinity ( $K_m \sim 150 \mu\text{M}$ ). In competition assays, uptake was dose dependently reduced by non-labelled SIT1-substrates, e.g. N-methylproline and pipicolate. From our functional analysis we conclude that the L-proline transport in OK cells is in all respects reminiscent of SIT1. Amino acid depletion experiments in OK cells sug-

gested that the proline-transporting system is subject to a fast regulation of activity. When cells were cultured in an amino acid deprived medium for up to 8 hours, we could detect an up to 3-fold stimulation of [ $^3\text{H}$ ]-L-proline uptake. This increased activity was blocked when cells were treated with inhibitors of de novo protein synthesis. Amino acid levels in starved cells showed drastic reductions with the exception of taurine and sarcosine. Supplying individual amino acids or mixtures of distinct amino acids could partially prevent the upregulation of [ $^3\text{H}$ ]-L-proline transport during starvation.

In conclusion, the transport protein SIT1 is expressed in OK cells and L-proline transport shows all properties of System IMINO. Furthermore, proline uptake shows a fast adaptive upregulation during amino acid deprivation which makes the cell model suitable to further investigate the nutritional control of gene expression and transporter regulation.

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## Clarifying the role of arginine282 in the rabbit proton-coupled peptide transporter PepT1

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The majority of protein uptake from the diet is widely accepted to be via the proton-coupled di- and tri-peptide transporter PepT1. When arginine282 is mutated to a glutamate (R282E), dipeptide transport is no longer proton-stimulated or concentrative, suggested to be due to uncoupling of the transport of protons and peptides (1). Here we present results which clarify the role of arginine282.

Mutant PepT1 constructs were expressed in *Xenopus* oocytes, and function assessed by [ $^3\text{H}$ ]-D-Phe-L-Gln up-

take, as summarised in the table. For pH stimulation of peptide uptake, the residue at position 282 had to be positive (R, K) or titratable over the pH range studied (H). Only R282E failed to accumulate substrate above the equilibrium after 24hr incubation at  $\text{pH}_{\text{out}} 5.5$ , indicating that uptake was energised by the proton electrochemical gradient, even for the conservative anionic substitution, R282D.

These findings suggest that 1) proton stimulation is distinct from proton cotransport, as non-pH stimulated

constructs can be concentrative; and 2) the failure of R282E to concentrate substrate is more likely due to the non-specific cation conductance (1) collapsing the membrane potential than the uncoupling of proton-peptide co-transport.

Table 1.

PepT1 construct	pH stimulation	Accumulation
R282 (wildtype)	Yes	Yes
R282E	No	No
R282D	No	Yes
R282K	Yes	Yes
R282H	Yes	Yes
R282A	No	Yes
R282Q	No	Yes

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## Effect of 5-aminoimidazole-4-carboxamide riboside (AICAR) on the peptide transport systems of Caco-2 cells

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Activation of the AMP-protein kinase, AMPK, during cellular metabolic stress, promotes the cellular uptake of fuel sources such as glucose and fatty acids to promote ATP generation (1). In this study, we examined the effects of AICAR, an AMPK activator, in regulating peptide transport in the intestinal Caco-2 cell model. Transport was assessed by uptake and flux studies of the radiolabelled dipeptide [ $^3\text{H}$ ]-D-Phe-L-Gln, with [ $^{14}\text{C}$ ]-mannitol used as a marker for paracellular transepithelial flux.

Pretreatment of the cells for 24 hours with 1mM AICAR resulted in significantly increased PepT1 mediated [ $^3\text{H}$ ]-D-Phe-L-Gln apical influx compared to control cells ( $312 \pm 40$  vs.  $193 \pm 33 \text{fmol.cm}^{-2} \cdot 30\text{min}^{-1}$  respectively,  $n=5$ ,  $p < 0.05$ ) and concurrent decreased mediated rate of apical to basal flux ( $2.57 \pm 0.62$  vs.  $5.61 \pm 0.56 \text{fmol.cm}^{-2} \cdot \text{min}^{-1}$  respectively,  $n=5$ ,  $p < 0.01$ ). AICAR had no effect on the non-mediated pathway, as when 20mM Gly-L-Gln was added in the apical side of control or AICAR pretreated cells uptake was  $12.5 \pm 2.0$  vs.  $12.5 \pm 1.4 \text{fmol.cm}^{-2} \cdot 30\text{min}^{-1}$  respectively ( $n=5$ ).

Our results show that AICAR significantly increases PepT1 mediated peptide entry at the apical membrane. Additionally, the reduced rate of transepithelial transport seen in the AICAR pretreated cells, even though apical peptide entry was increased, strongly suggests that the drug has an inhibitory effect on the as yet unidentified basolateral peptide transporter. The molecular mechanisms behind this effect are currently unknown, but the lack of an AMPK recognition consensus in PepT1 would indicate an indirect route of action. Nevertheless, these results provide evidence for a link between the cellular metabolic state and the apical and basal peptide transport systems of the enterocyte.

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## B<sup>0</sup>AT1 expression in hypertension

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B<sup>0</sup>AT1 is a novel member of the Na<sup>+</sup> dependent neurotransmitter transporter family (SLC6), which mediates epithelial resorption of neutral amino acids across the apical membrane in the kidney and intestine. Rat B<sup>0</sup>AT1 gene is located on chromosome 1, within a QTL that accounts for salt-loading induced variance of blood pressure.

This study reports the expression of the Na<sup>+</sup>-dependent amino acid transporter B<sup>0</sup>AT1 in the kidney and intestine during the development of hypertension in the SHR and the effect of high salt intake.

Animals of 4 and 12 weeks of age were fed normal (NS) or high (HS - 1% saline as drinking water) salt diet for 24 hours. Tissue samples were collected, frozen in liquid N<sub>2</sub>, and stored at -80 degrees C before analysis for mRNA abundance. By expression/cloning a cDNA sequence of rat B<sup>0</sup>AT1 was obtained. Quantitative real-time PCR was performed to evaluate the abundance of B<sup>0</sup>AT1 transcript.

A final cDNA fragment of 2033 bp containing 1905 bp

of ORF was obtained. The predicted amino acid sequence of this rat B<sup>0</sup>AT1 cDNA encodes for a protein of 634 amino acid residues. This rat B<sup>0</sup>AT1 protein shows an amino acid sequence identity of 95% with mouse counterparts and 85% identity with human B<sup>0</sup>AT1. Renal abundance of the B<sup>0</sup>AT1 transcript was lower in SHR than WKY at both 4 and 12 weeks of age. In contrast, no significant differences between strains were observed in intestine in terms of the expression of B<sup>0</sup>AT1. HS intake produced a significant increase (~ 45% augment) in renal B<sup>0</sup>AT1 mRNA of SHR of 12 weeks of age, which was accompanied by increases in renal dopamine, a natriuretic hormone.

It is concluded that Na<sup>+</sup> dependent B<sup>0</sup>AT1 amino acid transporter is under-expressed in the SHR and this is organ specific and precedes the onset of hypertension. Overexpression of the Na<sup>+</sup>-dependent B<sup>0</sup>AT1 at the kidney level during HS intake in adult SHR reveals their inability for adequate sodium handling when hypertension is well established.

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## Reassignment of transmembrane domain 1 (TM1) in the rabbit proton-coupled peptide transporter PepT1

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The majority of uptake of protein from the diet is widely accepted to be via the proton-coupled di- and tri-peptide transporter PepT1. Hydrophathy plots of the PepT1 sequence (1) suggested a 12 transmembrane domain (TM) protein, but subsequent EE epitope tag mapping was unable to resolve the N terminal TMs (2). Here we present data from studies re-examining the assignment of PepT1 TM1.

The program MEMSAT3 (<http://bioinf.cs.ucl.ac.uk/psipred/>) predicts that TM1 is formed from amino acids (aa) 28-45, not aa7-25 as previously suggested (1); the other 11 TMs are the same. This could explain why there was no protein expression when an EE epitope tag was inserted at aa39 (2), ie in the middle of the 'new' TM1 sequence. We have already shown that insertion of a FLAG tag at amino acid aa108 gave a functionally normal PepT1 protein, indicating that this region comprises the extracellular loop, presumably between TMs 3&4.

Expression of PepT1 with a FLAG epitope tag inserted at aa49 (in the proposed extracellular loop between

TM1&2) gives a functional transporter, as measured by [<sup>3</sup>H]-D-Phe-L-Gln uptake into expressing *Xenopus* oocytes. Truncating PepT1 by removing all of the protein up to the start of TM2 (PepT1<sub>Δ1-55</sub>) resulted in an expressed but non-functional protein. Less radical truncations are currently being constructed to establish the minimum N-terminal required for a functional transporter.

Taken together, these data support the new TM1 assignment from aa28-45, and would explain the failure of protein expression in the initial epitope insertion study (2). This information is essential for computer modelling the as yet uncrystallised PepT1 molecule.

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## Expression and function of Glycine Transporters GLYT1 on GABAergic Neurons and GLYT2 on Mouse Spinal Cord Astrocytes

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It is widely accepted that glycine transporters of the GLYT1 type are situated on astrocytes whereas GLYT2 are present on glycinergic neuronal terminals where they mediate glycine uptake. We here used purified preparations of mouse spinal cord nerve terminals (synaptosomes) and of astrocyte-derived subcellular particles (gliosomes) to characterize functionally and morphologically the glial vs. neuronal distribution of GLYT1 and GLYT2. Both gliosomes and synaptosomes accumulated [<sup>3</sup>H]GABA through GAT1 transporters and, when exposed to glycine in superfusion conditions, they released the radioactive amino acid in a receptor-independent manner, as a consequence of glycine penetration through its selective transporters. The glycine-evoked release of [<sup>3</sup>H]GABA was exocytotic from synaptosomes but GAT1 carrier-mediated

from gliosomes. Based on the sensitivity of the glycine effects to selective GLYT1 and GLYT2 blockers, the two transporters contributed equally to evoke [<sup>3</sup>H]GABA release from GABAergic synaptosomes; even more surprising, the neuronal GLYT2 contributed more efficiently than the glial GLYT1 to mediate glycine potentiation in [<sup>3</sup>H]GABA-releasing gliosomes. These functional results were largely confirmed by confocal microscopy analysis showing abundant co-expression of GAT1 and GLYT2 in GFAP-positive gliosomes and of GAT1 and GLYT1 in MAP2-positive synaptosomes. To conclude, functional GLYT1 are present on neuronal axon terminals and functional GLYT2 are expressed on astrocytes, indicating not complete selectivity of glycine transporters in their glial vs. neuronal localization in the spinal cord.

## T-type amino acid transporter-1 (SLC6A10) controls amino acid efflux via heterodimeric exchanger LAT2-4F2hc

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The best characterized basolateral amino acid transporters of the proximal kidney tubule and small intestine are the obligatory exchangers  $\gamma$ -LAT-4F2hc and LAT2-4F2hc (SLC7/SLC3 family of amino acid transporters), that cannot contribute to net amino acid (re)absorption [1]. Basolaterally localized aromatic amino acid transporter TAT1 is expressed in the kidney proximal tubular cells and functions as a facilitated diffusion pathway with symmetrical properties in terms of selectivity and apparent affinity. HPLC analysis of the free amino acid content of *Xenopus* oocytes expressing TAT1 showed that its net transport rate changes as a function of the transmembrane aromatic amino acid concentration difference, an important property in the context of epithelial cells that face a variable apical influx of neutral amino acids [2]. We hypothesize that TAT1 can supply the parallel exchangers with recycling uptake substrates that could drive the efflux of other amino acids. Here we demonstrate such a functional cooperation between TAT1 and LAT2-4F2hc in mediating the efflux of L-Gln, using *Xenopus* oocytes as an expression system. The efflux of LAT2-4F2hc

substrates upon expression of TAT1 was confirmed by HPLC. The functional cooperation between TAT1 and LAT2-4F2hc does not require their physical interaction, as shown by the negative coimmunoprecipitation and crosslinking experiments. Their cooperation is probably confined to an unstirred layer which surrounds the surface of the *Xenopus* oocyte, as it does in the basolateral membrane of epithelial cells, representing an area of different amino acid concentrations, as compared to the bulk volume. The fact that LAT2 can be replaced by LAT1, another obligatory exchanger, and TAT1 by LAT4, another facilitated diffusion pathway, confirms that this functional cooperation is made possible by the unstirred layer.

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## Fast Fluorometric Method for measuring Pendrin Transport Activity

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The SLC26A4 protein (Pendrin), is mainly expressed in the inner ear, kidney and thyroid where it acts as a chloride/anion exchanger. In the thyroid gland Pendrin transports I<sup>-</sup> towards the follicular lumen, and in the inner ear contributes to the conditioning of the endolymphatic fluid. Mutations of SLC26A4 gene cause the Pendred Syndrome (PS), a syndromic deafness characterized by severe or profound sensorineural hearing loss and thyroid dysfunction (1) and more than 150 different mutations are described in humans so far.

We aimed to assess function of wild type Pendrin and some mutant isoforms using a fast fluorometric method that exploits the EYFP (enhanced yellow fluorescent protein) sensitivity to halide intracellular concentration and based on a previously described procedure used to measure the chloride transport of the CFTR ion channel (2). HEK293Phoenix cells transfected with EYFP were used for the titration of the system, either for chloride or iodide, and the observed values were fitted with a simple exponential equation. Thereafter, using the experimental parameters obtained in the titration experiments, we measured the *in vivo* fluorescence variations due to Cl<sup>-</sup> or I<sup>-</sup> at different concentrations in the extracellular medium. Results were in agreement with those we obtained with <sup>36</sup>Cl<sup>-</sup> uptake studies confirming that

wild type pendrin is able to transport iodide and chloride. Otherwise the mutant S28R, which we previously described in a patient with sensorineural hearing loss and goiter, showed markedly impaired I<sup>-</sup> and Cl<sup>-</sup> transport capability, though its cellular distribution is indistinguishable from wild type. We also tested a novel mutation, consisting in a 11 bp duplication (1561\_1571CTTGGGAATGGC), found in a family showing a high intrafamilial phenotypic variability, that displayed an impaired chloride and iodide transport.

Our approach will permit to discriminate between wild-type and mutant Pendrin isoforms functions thus helping to elucidate the mechanism underlying the PS clinical phenotype.

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## Evidences for expression and function of SLC15 transporters in rat thyroid gland and in the rat thyroid cell line PC CL3

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In mammalian cells, transport of small peptides is mediated by members of the Solute Carrier 15 (SLC15) gene family. In the present study, we have investigated peptide transport activity and expression of SLC15 peptide transporters in both thyroid gland and a rat thyroid cell line (i.e. PC CL3 cells), that retain *in vitro* many typical markers of the differentiated thyroid follicular cells, such as thyroglobulin (Tg) synthesis and secretion, I<sup>-</sup> uptake and dependence on TSH for growth.

The fluorescent tracer-dipeptide  $\beta$ -Ala-Lys-N $\epsilon$ -7-amino-4-methyl-coumarin-3-acetic acid (Ala-Lys-AMCA) was used to study peptide transport in both intact rat thyroid slices and in cultured PC CL3 cells. In addition, RT-PCR analysis was employed to detect SLC15 specific transcripts in both thyroid and PC CL3 cells.

Fluorescence microscopy revealed that Ala-Lys-AMCA specifically accumulated in follicular cells of the thyroid gland as

well as in PC Cl3 cells. This accumulation was inhibited by the dipeptide carnosine, a typical substrate for the SLC15 transporters. In particular, in PC Cl3 cells, the dipeptide-derivative Ala-Lys-AMCA was taken up by a saturable, high affinity transport process. RT-PCR analysis allowed detection of SLC15A2(PEPT2)- and SLC15A4(PHT1)-related transcripts in total RNA extracted from both thyroid and PC Cl3

cells. In contrast, SLC15A1(PEPT1)- and SLC15A3(PHT2)-related transcripts could not be detected.

Our result constitute the first evidence for occurrence of members of the SLC15 transporters family in thyroid follicular cells. SLC15 transporters could contribute to recycling of small peptides derived from extracellular and lysosomal Tg proteolysis, which is an essential step for thyroid hormone synthesis.

## PepT1 in a coldwater marine teleost larvae- atlantic cod: cloning and preliminary studies of expression and phylogeny

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The functional characteristics of the simple digestive tract in fish larvae at the onset of exogenous feeding have long been a subject for discussion. This world-wide interest relates to large problems in aquaculture that appears to be related to the digestion and absorption of live and formulated diets. While many studies have described the ontogeny of the digestive enzymes, virtually nothing is known about the mechanisms responsible for nutrient absorption. Due to their critical roles as substrates for protein synthesis and energy catabolism in larval fish our research is focusing on amino acids. Cloning and functional description of these carrier systems in fish is still in its infancy, but recently PepT1 was characterizing in zebrafish (1) an agastric species adopted to warm freshwater.

The aim of this work was to characterize the ontogeny of the peptide transport system in the digestive tract of a marine cold water teleost- the Atlantic cod, *Gadus morhua* L. Atlantic cod is a commercially important fish and our main

model to study the ontogeny of digestive function in teleosts.

As part of this work we have cloned and sequenced the PepT1 in cod following data base mining and sequence comparison. Degenerated primers were constructed based on those analysis and corresponding cDNAs amplified by RT-PCR and cloned. Identification was confirmed by DNA sequence analysis. In a preliminary study total RNA was prepared from two groups (fed and starved) of cultured cod from onset of exogenous feeding. The temporal expression of PepT1 in the larvae was determined using PCR suggesting that PepT1 gene was present at first feeding. The phylogenetic relationship will be discussed.

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## Inhibition of amino acid transport by dinitrosyl iron complexes (DNIC)

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Low molecular mass DNICs are nitrosating agents found to play a role in the regulation of protein function, i.e., ion channel activity. Endogenously, DNICs are formed by the reaction between NO<sup>\*</sup>, free cellular iron, and cysteine or glutathione, particularly in cells expressing high levels of the inducible NO synthase. We used the model substance DNIC-thiosulfate to study the effects of low molecular mass DNICs on amino acid transport in human cells. To-

tal arginine uptake was reduced in a concentration dependent manner in U373MG glioblastoma cells. This was mainly due to the inhibition of leucine-sensitive arginine transport (system y<sup>L</sup>-mediated). In contrast, reduction of the leucine-insensitive component (system y<sup>+</sup>) was only 35% and did not show a strict concentration dependence. Similarly, hCAT-1 was inhibited to a comparable extent in U373MG cells overexpressing this system y<sup>+</sup> transporter.

This inhibition was accompanied by depolarization of the membrane potential by 15 mV suggesting an unspecific effect of DNIC-thiosulfate on system  $y^+$  activity in these cells. These data indicate that DNICs inhibit specifically systems  $y^L$  and not system  $y^+$  transporters. In line with system  $y^L$  inhibition, DNIC-thiosulfate also inhibited argi-

nine-sensitive leucine uptake. In addition, the arginine-insensitive leucine uptake (system L) was reduced in a similar way.

Conclusions: DNICs inhibit the 4F2hc-associated transporters  $y^L$  and LAT, but not the CAT proteins that do not require 4F2hc for proper function.

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## Human alveolar macrophages from normal subject do not transport arginine through system $y^+$ : possible relationship with low NO production

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iNOS-dependent NO production by rodent alveolar macrophages (AM) is associated to arginine transport through system  $y^+$  and, in particular, CAT2B transporter. No information is yet available on arginine transport in human AM that, at variance with rodent models, have a low NO production. Here we have characterized Arg transport in human AM obtained from normal subjects. The experiments were performed either in freshly isolated AM or in cells cultured for 6d with GM-CSF, both in the absence and in the presence of LPS. Under all conditions most of Arg influx was inhibitable by Leu in the presence, but not in the absence of  $Na^+$ , pointing to the operation of system  $y^L$ . The addition of Lys did not increase further the inhibition of Arg transport by Leu, indicating that the role of system  $y^+$  was, at best, marginal. However, both  $y^L$ -related genes

(SLC7A7/ $y^L$ LAT1 and SLC7A6/ $y^L$ LAT2) and the  $y^+$ -related gene SLC7A1/CAT1 were found expressed by human AM. Upon treatment with LPS a marked induction of another system  $y^+$ -related gene, SLC7A2 for CAT2B, was also observed. Even under these conditions, system  $y^+$  activity was not detected while NO production and iNOS expression remained at low values. We conclude that, at variance with rodent AM, human AM do not transport Arg through system  $y^+$ . Lack of system  $y^+$  activity may limit Arg availability for NOS and contribute to maintain a low NO output from AM. Moreover, since mutations of SLC7A7/ $y^L$ LAT1 cause Lysinuric Protein Intolerance, a disease often associated with alveolar proteinosis, these results suggest that a defect in system  $y^L$  activity in AM is responsible for lung alterations of LPI patients.

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## Expression of cystine/glutamate transporter under pathological conditions and analysis of cystine/glutamate transporter-deficient mice

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In mammalian cultured cells, we have characterized an anionic amino acid transport system, designated system  $x_c^-$ , which mediates cystine influx coupled with the efflux of intracellular glutamate. System  $x_c^-$  is composed of two proteins, xCT and 4F2 heavy chain, and xCT is thought to mediate the transport activity. System  $x_c^-$  plays an important role in

maintaining intracellular GSH and cystine/cysteine redox balance out of the cell in vitro. It has been demonstrated that xCT is strongly induced by various stimuli, including oxidative stress, electrophilic agents, bacterial lipopolysaccharide (LPS), and food-derived polyphenols. xCT mRNA was constitutively expressed in brain, thymus, and spleen, and it was

enhanced by intraperitoneal injection of sublethal dose of LPS, which is an in vivo model for Gram-negative sepsis. In addition to these tissues, xCT mRNA was induced in the bronchial epithelium of lung by the administration of lethal dose of LPS. While the peripheral blood neutrophils did not express xCT mRNA, the neutrophils elicited into the peritoneal cavity by inflammatory stimulus expressed xCT mRNA and exhibited the activity of system x<sub>c</sub><sup>-</sup>. To further investigate the role of this transporter in vivo, we have made xCT-deficient mice and characterized the mice and the cells. The xCT-deficient mice were healthy in appearance and fertile.

However, cystine concentration in plasma was significantly higher in the xCT-deficient mice, compared with that in the littermate wild-type mice, whereas there was no significant difference in plasma cysteine concentration. Thus, plasma cystine/cysteine redox balance in xCT-deficient mice was significantly oxidized, indicating that system x<sub>c</sub><sup>-</sup> contributes to maintaining the plasma redox balance at least in part in vivo. Plasma redox imbalance is thought to relate to aging and some diseases. The present results suggest that system x<sub>c</sub><sup>-</sup> is involved in the defense against oxidative shift in plasma under pathological conditions.

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## A novel isoform of organic cation transporter OCT3

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The catecholamines epinephrine, norepinephrine (NE), dopamine and serotonin serve as neurotransmitters in the central and/or the peripheral nervous system. Changes in synaptic concentrations of monoamines are associated e.g. with mental dysfunction and neuropsychiatric disorders, and are key mechanisms mediating drug addiction.

Extracellular NE is transported back into presynaptic axonal varicosities by a high-affinity sodium dependent reuptake system, originally called *uptake 1*, now known as norepinephrine transporter NET. An additional low affinity and sodium independent transport system called *uptake 2* has been proposed for several years. Its identity was independently disclosed by two groups who cloned OCT3 from placenta and a human glioblastoma cell line and named it *extraneuronal transporter for monoamine transmitters (EMT)* or *organic cation transporter 3 (OCT3)*, respectively. OCT3 is a member of the solute carrier (SLC) superfamily and the SLC22 subfamily, with the human gene name SLC22A3.

Mouse OCT3 is most abundantly expressed in placenta, ovary and uterus, but can be found at low levels in most tissues, such as heart, lung, ileum and the brain. [<sup>3</sup>H]MPP<sup>+</sup> sensitive uptake<sub>2</sub> was found in astrocytes, though OCT3 mRNA and function has also been described in neurons. Our group, for example, could show that OCT3 also occurs in neurons of the superior cervical ganglion.

Here we present data indicating that neuronal tissue expresses a novel isoform of OCT3, which has not been reported so far. Our northern blot experiments as well as our RT-PCR-studies indicate that the brain-specific OCT3 isoform differs from placenta in the aminoterminal region of the protein. Western-blot experiments using different antibodies further support these findings and indicate the expression of a shorter OCT3 isoform in the brain and the sympathetic nervous system. Results of a 5'RACE performed to identify the exact sequence of this novel variant will be presented.

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## Characterization of the tritium labeled analog of L-threo-beta-benzyloxyaspartate (tboa) binding to glutamate transporters

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L-Glutamate acts as a major excitatory neurotransmitter in mammalian central nervous systems. Excitatory amino acid transporters (EAATs) play important roles in maintaining the extracellular glutamate concentration at low levels to limit the activation of the glutamate receptors

and to protect neurons from excitotoxicity. In order to identify the physiological roles of each subtype, subtype selective EAAT ligands are required (1). In this study, we developed a binding assay system to characterize EAAT ligands for all EAAT subtypes. Recently, we synthesized

novel analogs of threo- $\beta$ -benzyloxyaspartate (TBOA) and reported that they block glutamate uptake by EAATs1-5 much more potently than TBOA (2). The strong inhibitory activity of the TBOA analogs suggested that they would be suitable to use as radioisotope labeled ligands, and we therefore synthesized a tritium labeled (2S,3S)-3-{3-[4-ethylbenzoylamino]benzyloxy}aspartate ( $[^3\text{H}]\text{ETB-TBOA}$ ).  $[^3\text{H}]\text{ETB-TBOA}$  showed significant specific binding to EAAT-transfected COS-1 cell membranes with high affinity for all EAAT subtypes. The binding was  $\text{Na}^+$ -dependent and was displaced by known EAAT substrates and blockers. The rank order of inhibition by these compounds was consistent with the previously reported

glutamate uptake assay results. Thus, the  $[^3\text{H}]\text{ETB-TBOA}$  binding assay will be useful to screen novel EAAT ligands for all subtypes of EAATs.

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## Increased ADMA plasma levels after administration of basic amino acids to a patient with coronary spasm

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A 37 year old male patient presented at the clinic with frequent coronary spasm that were largely resistant to classical treatment regimen. The first measurement of plasma amino acids revealed low arginine (Arg, 40  $\mu\text{M}$ ) and elevated ornithine (Orn, 120  $\mu\text{M}$ ) levels. The patient responded well to oral administration of Arg (18g per day) so that all other treatments could be stopped. Several weeks later, Arg and Orn levels were normal, even when pausing the Arg treatment for several days. Arg resorption and urea levels in plasma and urine of the patient were similar as in healthy subjects. However, the plasma level of asymmetrical dimethyl arginine (ADMA), a competitive inhibitor of nitric oxide synthase (NOS), was largely increased after administration of a single dose of either Arg or Orn (9g each) compared to controls receiving the same dose, respectively. This

suggested that ADMA had accumulated intracellularly and that basic amino acids facilitated its export that was impaired otherwise in the patient. In line with this hypothesis, the expression of two transporters for basic amino acids (CAT-1 and  $\gamma$ -LAT1) was reduced in peripheral blood mononuclear cells of the patient. The patient also exhibited elevated plasma levels of glutamine, glutamate, leucine and isoleucine that were either unchanged (Gln, Glu) or decreased after ornithine administration (Leu, Ile). Furthermore, Orn and Pro levels increased far above the levels in healthy subjects after Orn administration. Conclusions: An impairment of ADMA efflux may cause accumulation of this NOS inhibitor in endothelial cells and thus lead to vasoconstrictions. The elevated Gln levels observed in the patient may amplify this effect.

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## Amino acid homeostasis in *Caenorhabditis elegans* lacking the intestinal peptide transporter PEP-2

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The *C. elegans* di- and tripeptide transporter PEP-2, a homologue of the mammalian PEPT1, acts in parallel to amino acid transporters in the intestinal epithelium. *pep-2(lg601)* mutant animals are not capable of taking up intact

di- and tripeptides from the gut lumen. The lack of intestinal peptide transport reduces the availability of dietary amino acids and consequently growth and development of the nematode is severely impaired and animals show a decrea-

sed brood size, retarded postembryonic development and reduced body size [1]. We here show, that the lack of peptide transport into epithelial cells is partially compensated by an increased intestinal uptake of amino acids in *pep-2(lg601)* animals. Real time RT-PCR is employed to identify the underlying amino acid transporters with altered mRNA expression levels that could help to understand regulation of amino acid homeostasis in the *pep-2* knockout background. In parallel, the mRNA level of TOR (*C. elegans let-363*), the main sensor of amino acids in the cell, is determined in the *pep-2* mutant background.

*pep-2(lg601)* animals are also more resistant to heat and oxidative stress than wild type worms. Analysis of glutathione levels revealed that *pep-2* animals have 2-3 fold higher cellular concentrations of glutathione. To understand

this compensatory regulation, key enzymes of the glutathione synthesis pathway such as the gamma-glutamylcysteine synthetase *gcs-1* were assessed for transcriptional regulation in the mutant background and controls. While *gcs-1* shows no regulation, there is a 1.7 fold increase of the GSSG reductase (C46F11.2a) mRNA in the *pep-2(lg601)* animals, indicating an increased potential for GSSG reduction to GSH in *pep-2* animals.

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## Disruption of SLC7A7 gene compromises the embryonic growth of the mouse model of lysinuric protein intolerance by IGF1 down-regulation

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Lysinuric protein intolerance (LPI, MIM 222700) is an autosomal recessive defect of cationic amino acid (CAA) transport at the basolateral membrane of epithelial cells of intestine and kidney, caused by mutations of SLC7A7 gene. A null mouse of *Slc7a7* was generated by random insertional mutagenesis in ES cells (Lexicon Genetics Incorporated, The Woodlands, Texas). More than 400 *Slc7a7<sup>-/-</sup>* intercrosses led to only two *Slc7a7<sup>-/-</sup>* live animals. At E16.5 stage, the proportions of *Slc7a7* genotypes were found as expected for an autosomal recessive transmission and the *Slc7a7<sup>-/-</sup>* embryos were already smaller than controls. Most of *Slc7a7<sup>-/-</sup>* pups were lost at birth because of cannibalism. None of the null embryos showed gross morphological abnormalities.

To study the gene dysregulation due to *Slc7a7* loss of function, we compared the gene expression profiles of adult

*Slc7a7<sup>-/-</sup>* mice and control mice by using DNA microarrays of liver and intestine. Results from liver microarray indicated up-regulation of genes encoding enzymes of the urea cycle (arginase 1, argininosuccinate lyase, and glutamate dehydrogenase) with the exception of ornithine transcarbamylase. Within the SLC7 family, *Slc7a2* was up-regulated in liver and *Slc7a9* was down-regulated in intestine, respectively. All *Slc7a7<sup>-/-</sup>* embryos showed intrauterine growth retardation (IUGR) as seen at E16.5 stage. To elucidate the pathogenesis of this IUGR, we tested the expression of *Igf1*, *Igf2* and *Igfbps* by Real-Time PCR. Liver *Igf1* and *Igf2* mRNA levels were much lower than controls. Composite results were found for *Igfbps*: down-regulation of *Igfb1*, up-regulation of *Igfbp2* and *Igfbp6*. This is the first observation which links CAA metabolism and intrauterine growth mediated by the *Igf* pathway.

## Solute and Water Transporter Expression in ARPKD: Implications for Hepatic Cystogenesis

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**Background/Aims:** In Autosomal Recessive Polycystic Kidney Disease (ARPKD), hepatic cysts progressively grow and expand over time, likely because of disturbances in cell proliferation, apoptosis, and water and solute transport. We recently reported that in the PCK rat, an animal model of ARPKD, liver cysts are derived from cholangiocytes, the epithelial cells that line bile ducts. We also showed that in bile duct explants isolated from both normal and PCK rats, when grown in 3-D culture, both formed cystic structures that progressively expand over time; however, cysts derived from PCK bile ducts expanded at a 3-fold greater rate than those derived from normal rats. In addition, we have also shown that cholangiocytes contain an organelle that sequesters functionally related transport proteins, (i.e., the water channel protein aquaporin 1 (AQP1), the chloride channel CFTR, and the anion exchanger AE2), that can account for

ion-driven water transport. Since abnormalities in water transport may play an important role in progressive cyst expansion in cystic liver disease, our Aim was to determine the relative expression of AQP1, CFTR, and AE2, in normal and PCK rats. **Methods:** Expression of AQP1, CFTR, and AE2 was assessed by quantitative RT-PCR and Western blot using freshly isolated bile duct explants from both normal and PCK rats. **Results:** In explants from PCK rats, mRNA levels of AQP1, CFTR and AE2 were increased by 3.8, 36.8 and 39.3 fold ( $p < 0.05$ ), respectively, compared to normal bile ducts. Similarly, as revealed by quantitative western blots, protein expression of all three transporters was increased in the PCK rat: AQP1 2.5 fold; CFTR 2.0 fold; and AE2 2.7 fold ( $p < 0.05$ ) over normal. **Conclusion:** The data suggest that elevated expression of AQP1, CFTR and AE2 contribute to the progressive expansion of hepatic cysts in ARPKD.

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## Functional and structural characterization of a prokaryotic peptide transporter with similar features as mammalian PEPT1

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The *ydgR* gene of *Escherichia coli* encodes a protein of the proton-dependent oligopeptide transporter (POT) family. We cloned YdgR and overexpressed the His-tagged fusion protein in *E. coli* BL21 cells. Bacterial growth inhibition in the presence of the toxic phosphonopeptide alafosfalin established YdgR functionality. Transport was completely abolished in the presence of the proton ionophore CCCP, suggesting a proton-coupled cotransport mechanism. YdgR transports selectively only di- and tripeptides and structurally related peptidomimetics such as aminocephalosporins with a substrate recognition pattern almost identical to the mam-

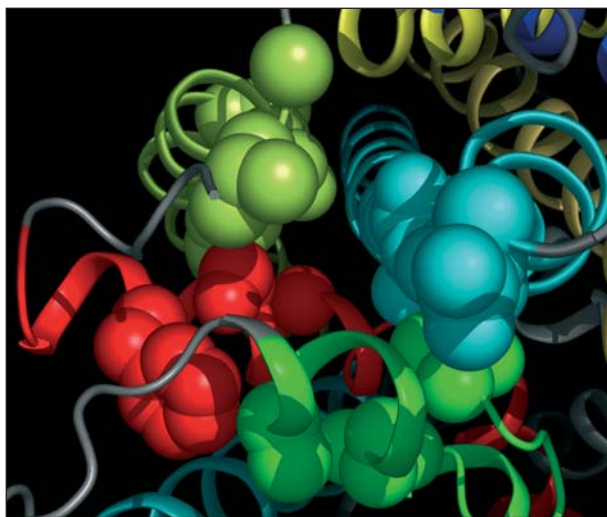
malian peptide transporter PEPT1. The YdgR protein was purified to homogeneity from *E. coli* membranes and functionally reconstituted into proteoliposomes. Blue native-polyacrylamide gel electrophoresis and transmission electron microscopy (TEM) of detergent-solubilized protein suggests YdgR to exist in monomeric form. TEM revealed a crown-like structure with a diameter of ~8 nm and a central density. These are the first structural data obtained from a proton-dependent peptide transporter and YdgR seems an excellent model to study protein-ligand interaction as well as the molecular architecture of cell membrane peptide transporters.

## The cytoplasmic substrate permeation pathway of serotonin transporter

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Serotonin transporter (SERT) catalyzes reuptake of the neurotransmitter serotonin (5-HT) and is a target for antidepressant drugs and psychostimulants. It is a member of a large family of neurotransmitter and amino acid transporters. SERT is phosphorylated and activated by cGMP-dependent protein kinase. Site-directed cysteine modification of the region around the phosphorylation site identified a helical region of the transporter with high accessibility to the cytopla-



sm [1]. This helical region corresponds to the cytoplasmic half of the fifth transmembrane domain in the high resolution structure of LeuT, a prokaryotic homologue of SERT [2]. The accessibility of these positions depends on conformational changes corresponding to interconversion of SERT between two forms that face the extracellular medium and the cytoplasm, respectively. Binding of the extracellular inhibitor cocaine decreased accessibility at these positions, while 5-HT, the transported substrate, increased it. The effect of 5-HT required the simultaneous presence of  $\text{Na}^+$  and  $\text{Cl}^-$ , which are transported into the cell together (symported) with 5-HT. In light of the LeuT structure, these results begin to define the pathway through which 5-HT diffuses between its binding site and the cytoplasm. They also confirm a prediction of the alternating access model for transport, namely that all symported substrates must bind together before translocation.

### References

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