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Monitoring rat brain lactate concentration *in vivo* by NMR shows rapid, reversible, increases induced by brief infusions of ammonium

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Hyperammonemia-induced increases in brain lactate have been studied mainly with protocols designed to mimic hepatic encephalopathy, but it has been suggested that there is a physiological flux of ammonium from neurons to astrocytes as part of glutamate-glutamine shuttling (1). In vitro, ammonium activates enzymes of glycolysis and inhibits the TCA cycle, and so tends to increase lactate production. Also, NH_4^+ is avidly taken up by astrocytes (2,3) and there is evidence for a metabolic flux through lactate in the brain during normal activity (4). Minimum conditions for a possible role of ammonium in regulating lactate production are that ammonium-induced increases in lactate be rapid and reversible. We have used localized ^{1}H NMR spectroscopy at 7 Tesla to monitor changes in the concentrations of brain metabolites. Rats were anæsthetised with 5% isofluorane and maintained with mechanical ventilation with 1–1.5% isofluorane in air enriched with O_2 to 35%. A 4 x 4 x 4 mm^3 voxel was centred on the striatum, the echo time was 136 ms, and spectra were acquired over 3 min 12 s. We measured the areas of peaks corresponding to choline compounds (tCho), creatine /creatineP (tCr), N-acetyl aspartate (NAA) and lactate. In Fig. 1, when 1M NaCl was infused through a femoral vein over 4 min at a rate that gave a total quantity of 2.5 mmol/(kg body wt) the lactate signal did not change. A second infusion, identical except that NaCl was replaced by NH_4Cl, caused lactate to increase 5-fold. All but about 2.4% of the ammonium had been cleared from the blood by 1 min after the end of the infusion. On average, control infusions of NaCl caused no change in the signals for lactate or other metabolites except for tCr (–3.07%, s.e.m. = 0.81%, 5 infusions in 5 rats, $P < 0.02$, all Ps by Student's two-tailed *t* test). With NH_4Cl, the lactate signal started to increase during the infusion and reached a peak 3.10 ± 0.35 times baseline ($P < 0.0001$) at 13.2 ± 2.1 min after the infusion (9 infusions in 5 rats). It then recovered halfway to baseline by 31.2 ± 5.7 min after the infusion. tCr and tCho signals were unchanged. The NAA signal increased by 3.52 ± 0.68% ($P < 0.0006$). In parallel experiments, changes in cerebral blood flow were measured by inverting the magnetization of water protons in the carotid arteries and detecting the ensuing signal changes in the brain (5). After ammonium infusion, cerebral blood flow increased by a factor of 2.15 ± 0.16 (5 infusions in 3 rats, $P = 0.0002$), suggesting that the lactate increase was not caused by hypoxia. Ammonium-induced increases in lactate signal could be observed at least three times in one experiment.

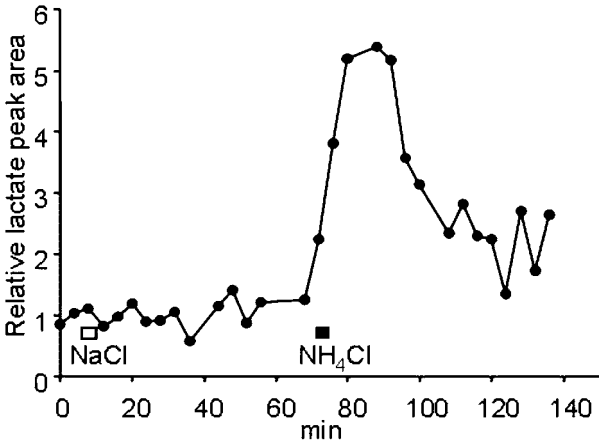

Figure 1. Time course of changes in the area of the lactate peak in an illustrative experiment. Infusion of NaCl over 4 min caused no change, but NH_4Cl did. Data acquisition lasting 3 min 12 s was started simultaneously with the beginning of the NH_4Cl infusion, and this point already showed an increase.

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Where applicable, the authors confirm that the experiments described here conform with the Physiological Society ethical requirements.

C20

Identification and cloning of alternative N termini of human NBCn1

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The electroneutral Na-HCO_3 cotransporter NBCn1 (SLC4A7) is located at the basolateral membrane of the thick ascending limb, where the 1:1 uptake of Na^+ and HCO_3^- into the cell neutralizes the intracellular acid load caused by the apical uptake of NH_4^+. NBCn1 also plays an important role in intracellular pH regulation in vascular smooth muscle cells (see Romero et al. 2004 for review). Human NBCn1 has an unusually long N terminus (Nt) that is about half the length of the entire protein, and is reported to begin with the amino-acid sequence Met-Glu-Arg-Phe, MERF. The unusual length of the Nt is due to a unique 124-residue cassette (Exon 7) and a 13-residue cassette A at the extreme end of exon 6. Rat NBCn1 has been reported to begin with a different Nt (Met-Glu-Ala-Asp, MEAD), and may have exon 7 spliced in or out as a cassette. (Rat NBCn1 also contains a cassette B, which consists of 36 residues in the C terminus.) The different Nt of the human NBCn1-A MERF vs the rat NBCn1-B through -E MEAD have been thought to represent species differences. However, we now report four new full-length human cDNA clones, two beginning with MERF and two beginning with MEAD. The two beginning with MERF were found in

skeletal muscle and kidney but not liver libraries; they differ in a potential polymorphism in exon 7. The two beginning with MEAD were found in skeletal muscle, liver, kidney and heart libraries; they differ in the presence vs absence of exon 7. Examination of the human genome indicates that MERF and MEAD indeed represent alterative N-termini originating from different exons, exon 1 in the case of MERF (5 kb upstream from exon 2 and encoding 11 amino acids) and what we shall term exon 0 in the case of MEAD (30 kb upstream from exon 2 and encoding a different 16 amino acids). The same holds true for the mouse genome, where previously only MEAD was though to exist. To date, NBCn1 is the most diverse Na^+-coupled SLC4, with at least three cassettes that may be spliced in or out, as well as two alternative N termini. Our results could impact our understanding of NBCn1 physiology in the following ways: (1) detection of NBCn1 using only an antibody directed against MERF would fail to detect NBCn1 splice variants beginning with MEAD. Although one might contemplate developing an antibody against MEAD, MEAD closely resembles the Nt of another SLC4 family member, NCBE (SLC4A10). (2) Different tissues, as we have seen, may exhibit differential expression of the new NBCn1 splice variants. (3) The choice of N termini and the presence or absence of particular cassettes presumably affects NBCn1 physiology via binding partners and/or effects on the function of the transmembrane domain.

Romero et al. (2004). Pflügers Arch 447, 495-509.

Where applicable, the authors confirm that the experiments described here conform with the Physiological Society ethical requirements.

C21

Transplanted rat metanephroi: evidence of an immature urine concentrating mechanism

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Transplantation of embryonic kidneys has been proposed as a potential solution to the problem of kidney donor shortage. We have reported previously that rat metanephroi transplanted into a host of the same species have glomerular filtration rates comparable with those achieved by dialysis [1]. Here we estimate nephron number and examine the expression of key transporters central to the concentration of urine in order to determine the maturity of transplanted tissue. Metanephroi from embryonic day 15 (E15) Lewis rat embryos were transplanted adjacent to the abdominal aorta of adult female Lewis rats under isoflurane anaesthesia (1 l/min O_2, 2.5% isoflurane). Post-operative checks on these rats were made following recovery from anaesthesia. Metanephroi were explanted between 21 days and 3 months later and fixed in 4% paraformaldehyde or snap frozen. Glomeruli were counted in transplanted metanephroi at varying ages post-transplant and compared with embryonic day 21 (E21) and post-natal day 1 (PN1) Lewis rat kidneys using a non-biased stereological counting technique involving counting glomeruli within paired serial sections of kidneys using a modified version of the dissector method [1]. Aquaporin 1 and 2 (AQP 1 and 2) and urea transporters A-1,2 and 3 (UT-A1,2 and 3) were localised by immunohistochemistry. Relative gene expression of these transporters was carried out by quantitative PCR (qPCR). Statistical analysis was by one-way ANOVA; all values are expressed as mean ± S.E.M. Number of glomeruli in transplanted metanephroi (4399 ± 216, $n = 9$) were significantly lower compared to PN1 kidneys (7023 ± 587, $n = 5$, $P < 0.05$) but not E21 kidneys (2578 ± 358, $n = 5$, $P > 0.05$). Most metanephroi expressed AQP1 (5/6), AQP2 (4/5) but not UT-A1,2 or 3. This pattern of expression was also observed in E21 and PN1 Lewis rat kidneys. qPCR compared levels of gene expression in transplanted metanephroi, E21 and PN1 samples to adult kidney tissue (given an arbitrary expression of 1). AQP 1 gene expression in transplanted metanephroi (0.10 ± 0.02, $n = 5$) was not significantly different compared to either E21 (0.20 ± 0.08, $n = 6$, $P > 0.05$) or PN1 kidneys (0.30 ± 0.12, $n = 7$, $P > 0.05$). AQP2 gene expression in transplanted metanephroi (0.10 ± 0.04, $n = 6$) was significantly lower compared to both E21 (0.24 ± 0.04, $n = 7$, $P < 0.001$) and PN1 kidneys (0.24 ± 0.05, $n = 7$, $P < 0.001$). Gene expression for UT-A1/2 was very low in transplanted metanephroi (0.03 ± 0.01, $n = 6$) but not significantly different from E21 (0.03 ± 0.01, $n = 7$, $P > 0.05$) or PN1 kidneys (0.10 ± 0.03, $n = 7$, $P > 0.05$). UT-A1/3 gene expression was undetectable in transplanted metanephroi (n = 6) but E21 kidneys (0.02 ± 0.00, $n = 7$) had significantly lower expression than PN1 kidneys (0.04 ± 0.01, $n = 7$, $P < 0.05$). These results provide evidence that transplanted metanephroi, up to 3 months following transplantation, are at a stage of nephrogenesis comparable with kidneys at around the time of birth in the rat.

Marshall D *et al.* (2005). *Trans Proc* **37**, 194-197.

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SA13

Ammonium transport mediated by human Rh glycoproteins

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The Rhesus (Rh) family is composed of RhD and RhCE proteins, and of RhAG, RhCG and RhBG glycoproteins. Sequence analysis shows that this family belongs to the superfamily of ammonium transporters, which includes AMT (AMmonium Transporters) and MEP (MEthyl Permeases), which are families of proteins from plants and yeasts. A first experimental study that gave evidence that RhAG (the Rh glycoprotein specifically expressed in erythroid cells) and RhCG (an epithelial Rh glycoprotein, particularly expressed in the distal nephron) are also ammonium transporters was obtained in yeast: RhAG and RhCG transfection in MEP-deficient yeasts restore their growth in ammonium-containing medium (Marini et al. 2000). From this study was raised the question Rh glycoproteins acting as ammonium transporters in mammals, whereas it was it was accepted that transmembrane NH4+ transport is mainly mediated by K+-transporting systems, and that non-ionic diffusion is responsible for NH3 transport through biological membranes (despite a few challenging reports, Cooper et al. 2002). In this context, several laboratories began to investigate the functional role of Rh glycoproteins in vetebrate cells: are they ammonium transporters? If so, what is the transported species (NH3 or NH4+)? Up to now, functional results converge to conclude that Rh glycoproteins are indeed ammonium transporters. But, as it is the case for the various AMT and MEP proteins, the transported species (and the mode of transport) is still debated: NH3 transport, NH4+ transport, NH3 and NH4+ transport, or NH4+ and H+ exchange.

In our laboratory, we have studied the functional role of human RhAG and RhCG after their heterologous expression in two different expression systems: HeLa cells and Xenopus laevis oocytes. Changes in intracellular pH (pHi) during exposure to an ammonium-containing solution (NH4Cl in the millimolar range) were monitored using video-imaging system coupled to the fluorescence of the pH-sensitive probe BCECF or using pH-selective microelectrodes. Analysis of ammonium-induced pHi changes indicates that these cells differed in their endogenous membrane NH3 and NH4+permeabilities: whereas HeLa cells exhibit a sudden huge alkalinization (related to NH3 influx into the cell, and its protonation) followed by a teeny tendency of pHi to recover (secondary acidification related to NH4+ influx into the cell, and its partial dissociation), X. laevis oocytes exhibit a barely visible increase in pHi, rapidly blunted by a large cell acidification. These results are consistent with a large NH3 but a low NH4+ membrane permeability in HeLa cells, but the opposite in X. laevis oocyte. By measuring ammonium-induced pHi changes in transfected cells, we observed that expressing RhAG and RhCG enhance both initial alkalinization and secondary acidification induced by ammonium exposure, consistent with the enhancement of ammonium influx (NH3 + NH4+) in a non-synchronic way (NH3 then NH4+ influx). Interestingly, sub-millimolar (100 to 1000 microM) ammonium concentrations induced inward currents in voltage-clamped (Vc = - 50 mV) RhAG- and in RhCG- expressing oocytes. Further analysis of the ammonium-induced current is consistent with NH4+-related current, depending on Rh-induced NH3 transport. Taken together, these results show not only that RhAG and RhCG are ammonium transporters, but also that these human proteins are promoting the transmembrane transport of NH3 and of NH4+ (Bakouh et al. 2004; Benjelloun et al. 2005).

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SA14

Phylogenetics of the Amt/Mep/Rh superfamily: insights into the functional diversification and organismal physiology of gas channels

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The Amt/Mep/Rh superfamily is a unique branch of the major facilitator superfamily with hundreds of members from a vast array of organisms spread in the three domains of life [1]. It is further divided into Amt/Mep subfamily as represented by E. coli Amt (ammonium transporter) and S. cerevisiae Mep (methylammonium permeases) and Rh subfamily by Rh antigen-related proteins of animal red cells and epithelial tissues. Model Amt/Mep members of prokaryotes, eukaryotic microbes and plants have been extensively studied owing to their importance for nitrogen uptake and assimilation. Amt/Mep were thought to act like active transporters for the charged NH4+ specie, but more recent studies yielded key physiological and structural evidence showing that they are gas channels for NH3 and not NH4+ transporters [2,3]. These studies have identified Amt/Mep as the first biological gas channels and established the structural principle for gas conductance function.

While ammonium as the transport specificity of Amt/Mep has been confirmed, the substrate for the Rh subfamily remains uncertain. Rh proteins were proposed as Amt/Mep functional equivalents given their limited sequence similarity and apparent distant relationship. Subsequent studies of Rh homologues in different organisms yielded controversial results with regard to substrate identification. Physiological studies by the Berkeley group explored the substrate for Rh proteins in the unicellular green alga, indicating that the substrate for Rh proteins is the gas CO2 [2,4]. Other studies used mammalian Rh proteins for transient expression in yeast Δmep mutant or frog oocytes to assay ammonium or methylammonium uptake. Although the latter studies have not reached on the agreement of which species (gas vs cation) was involved and whether uptake was electrogenic or neutral, they all indicate that Rh proteins transport ammonium and/or

methylammonium [2,5]. One caveat in these assays was that the concentrations of ammonium or methylammonium used were overwhelmingly higher than that under homeostatic physiological conditions.

Here we assembled a large dataset of the Amt/Mep/Rh superfamily to explore what selective forces might govern the evolutionary relatedness of the two subfamilies and how their members would differ from one another in terms of their primary sequence relationship, transmembrane topologic organization and molecular phylogenetic patterns. We further analyzed and compared the sequence divergence and codon composition for potential occurrence of adaptive evolution in each subfamily separately. Our studies revealed the following observations that are consistent with the view that Amt/Mep and Rh subfamilies are sequence-related yet substrate-distinct biological gas channels.

(1) Amt/Mep genes and Rh genes differ in organismal distribution. The former are ubiquitous in eubacteria, fungi and plants, are scattered in archaea and invertebrates, and are absent in vertebrates. In contrast, Rh genes are rare in prokaryotes and absent in plants, but show an increasing occurrence in microbial eukaryotes (except fungi) and become ubiquitous in metazoan with a major expansion in vertebrates. (2) Rh genes and Amt/Mep genes coexist in a diverse spectrum of species including microbial eukaryotes and certain invertebrates, signifying their paralogous relationship over a long period of evolutionary time. (3) Although Amt/Mep and Rh proteins are related to one another by marginal sequence relatedness, they are subject to independent evolution after their early isolation into two paralogous branches apparently in primitive eukaryotic microbes. (4) Members of the Amt/Mep subfamily are highly divergent internally, which could be shaped by environmental adaptation, and they are divided into three major clusters where archaeal Amt are dispersed with prokaryotic and eukaryotic members. (5) Amt/Mep proteins and Rh proteins share a limited set of conserved amino acids most of which (15/17 residues) are hydrophobic ones or the small glycine residues that can be assigned to the interval transmembrane segments. (6) Amt/Mep subfamily genes and Rh subfamily genes are highly different in the usage of the second codon positions, which identified a major driving force that discriminates the two subfamilies. (7) Amt/Mep proteins and Rh proteins strikingly differ in charge distribution: the former essentially lack membrane-embedded negative charged amino acids, in sharp contrast with the latter. (8) Along with the evolution of vertebrates, the erythrocyte Rh proteins have been subject to strong positive selection, particularly in the lineage from rodents to higher mammals. We discuss the implications of these findings in the light of gas channel function and organismal physiology.

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SA15

Different transport mechanisms in plant and human AMT/Rh-type ammonium transporters

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The conserved family of AMT/Rh proteins facilitates ammonium transport across animal, plant and microbial membranes. A bacterial homolog, AmtB, forms a channel-like structure and appears to function as a NH3 gas channel. To evaluate the function of eukaryotic homologs, human RhCG glycoproteins and plant ammonium transporters were expressed and compared in Xenopus oocytes and yeast. Rh glycoproteins mediated saturable electroneutral transport of methylammonium (MeA). Transport was strongly favored by increasing the pHo and was inhibited by ammonium. Ammonium induced rapid cytosolic alkalinization in Rh glycoprotein-expressing oocytes. In contrast, expression of plant AMTs induced pHo-independent MeA+ uptake and specific NH4+- and MeA+-currents. These currents were distinct from endogenous currents. The different mechanisms of transport were also observed after heterologous expression in appropriate yeast strains. Thus, homologous AMT/Rh-type proteins function in a distinct manner: while plant AMTs specifically transport NH4+, or co-transport NH3/H+, Rh glycoproteins mediate electroneutral NH3 transport.

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SA16

An ammonium flux from neurons to glial cells

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The majority of neurotransmitters mediating fast neurotransmission in the central nervous system are amino acids (glutamate, GABA and glycine). Their metabolism and exchange between cells suggest there might be high fluxes of ammonium (NH_4^+/NH_3) in the brain. The recent reports of membrane transporters which can transport ammonium selectively have increased interest in the role of ammonium as a potential intercellular messenger in normal brain function.

An intercellular flux of ammonium has been described in the bee retina. In this tissue, exogenous glucose is not significantly taken up by neurons (the photoreceptors), which rely on adjacent glial cells for supply of an amino acid, alanine, as an energetic substrate. When photoreceptors are activated, ammonium is released, presumably as a result of the metabolic oxidation of alanine. Ammonium is then avidly taken up into glial cells through a selective route, a NH_4-Cl cotransporter. This ammonium flux has been recently monitored in real time with ammonium-sensitive microelectrodes in retinal slices, showing that it is controlled by the glial NH_4-Cl cotransporter.

In mammals, about 90% of central synapses are glutamatergic. Following glutamate release into the extracellular space, gluta-

mate is taken up through transporters, mainly into glial cells (Marcaggi & Attwell, 2004). In order to recycle glutamate to the nerve terminals, a flux of glutamine in the opposite direction has been suggested because of the preferential localization of glutamine synthetase (converting glutamate and ammonium into glutamine) in glial cells and glutaminase (converting glutamine into glutamate and ammonium) in neurons (Marcaggi & Coles, 2001). This recycling, called glutamate-glutamine cycle, implies a production of ammonium by neurons and use of ammonium by glial cells, and thus requires a nitrogen flux from neurons to glial cells. Alanine has recently been proposed as the nitrogen carrier mediating this nitrogen flux. However, this proposal is not supported by the recent characterization and localization of putative alanine transporters (Marcaggi & Attwell, 2004). I therefore hypothesize that ammonium is mediating the nitrogen flux from neurons to glial cells. This flux might be quantitatively as important as the flux of synaptic glutamate release, which was estimated to be as high as the flux of brain glucose consumption (Sibson et al. 1998).

To date, ammonium release as a result of neuronal activity has only been shown in the frog sciatic nerve and the bee retina. However, it can be predicted that ammonium will be released during synaptic activity for the following two reasons. First, the production of presynaptic glutamate and GABA from precursor glutamine will produce ammonium in glutamatergic and GABAergic terminals. Second, exogenous NH_4Cl neutralizes the acid pH of synaptic vesicles (Li et al., 2005), implying a vesicular membrane permeability to NH_3. $[NH_3]$ in vesicles can therefore be expected to equilibrate with $[NH_3]$ in the surrounding cytoplasm, and a NH_4^+ gradient will be established due to the pH difference (ΔpH) between the cytoplasm and the lumen of the vesicles (typically ~ 1.5), with $[NH_4^+]$vesicle/$[NH_4^+]$cytoplasm = $10^{\Delta pH}$. Thus, ammonium will be concentrated in vesicles and might be expected to be released during synaptic transmission. Cultured astrocytes have been reported to transport NH_4^+ through non-selective routes, driven by the membrane potential and Cl^- gradient (Marcaggi & Coles, 2001). Although this remains to be confirmed for glial cells in intact nervous tissue, such transport would support a flux of ammonium from neurons to glial cells as described in the bee retina. Ammonium can activate three enzymes of glycolysis: hexokinase, phosphofructokinase, and pyruvate kinase (Muntz & Hurwitz, 1951). Ammonium is thus a good candidate messenger to modulate glial metabolism locally in response to synaptic transmission and neuronal activity.

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