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ORIGINAL ARTICLE

Delivery of glutamine synthetase gene by baculovirus vectors: a proof of concept for the treatment of acute hyperammonemia

MA Torres-Vega[1], RY Vargas-Jerónimo[1], AG Montiel-Martínez[1], RM Muñoz-Fuentes[1], A Zamorano-Carrillo[2], AR Pastor[3] and LA Palomares[3]

Hyperammonemia, a condition present in patients with urea cycle disorders (UCDs) or liver diseases, can cause neuropsychiatric complications, which in the worst cases result in brain damage, coma or death. Diverse treatments exist for the treatment of hyperammonemia, but they have limited efficacy, adverse effects and elevated cost. Gene therapy is a promising alternative that is explored here. A baculovirus, termed Bac-GS, containing the glutamine synthetase (GS) gene was constructed for the *in vitro* and *in vivo* treatment of hyperammonemia. Transduction of MA104 epithelial or L6 myoblast/myotubes cells with Bac-GS resulted in a high expression of the GS gene, an increase in GS concentration, and a reduction of almost half of exogenously added ammonia. When Bac-GS was tested in an acute hyperammonemia rat model by intramuscularly injecting the rear legs, the concentration of ammonia in blood decreased 351 μM, in comparison with controls. A high GS concentration was detected in gastrocnemius muscles from the rats transduced with Bac-GS. These results show that gene delivery for overexpressing GS in muscle tissue is a promising alternative for the treatment of hyperammonemia in patients with acute or chronic liver diseases and hepatic encephalopathy or UCD.

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INTRODUCTION

Hyperammonemia, caused mainly by acute or chronic liver failure or urea cycle disorders (UCDs), provokes a variety of neuropsychiatric abnormalities that are related to the mental and motor status of the patient. In its more severe manifestation, it includes brain edema and intracranial hypertension with consequent death.[1,2] In case of liver failure, all the spectrum of neuropsychiatric changes is encompassed in the term hepatic encephalopathy (HE).[1] There are subtle alterations in learning and motor coordination that are only detected with psychometric tests that are called minimal HE (MHE).[3] Since the first studies in dogs over one century ago, ammonia had been considered the main cause of MHE or overt HE.[4–10] The mechanisms triggered by an elevated ammonia concentration that lead to an altered communication and function in the brain have started to be revealed.[2,3,10]

During development of cerebral disturbances, precipitating factors, such as the inflammatory response and diazepam, have a synergistic relationship with ammonia.[11–13] All these factors induce *in vitro* astrocyte swelling through formation of reactive nitrogen and oxidative species.[14] Current treatments for HE were designed to reduce the ammonia levels in blood and brain by administering non-absorbable disaccharides (lactulose and lactitol) or antibiotics (Rifaximin), which have significantly diminished several manifestations of the illness in a few well-controlled clinical trials with HE patients.[15–20] However, these therapies often cause diverse adverse effects, like headaches, constipation, diarrhea, flatulence, vomiting, anorexia, abdominal pain and urticarial skin reactions. Furthermore, the cost of the HE therapy with these agents is expensive, and it includes hospitalizations and emergency room visits of patients due to frequent relapses.[21,22] Thus, there is a need to develop more effective approaches for decreasing morbidity and mortality in individuals with hyperammonemia, such as the gene therapy approach we propose here. Gene transfer has been tested to avoid elevated ammonia levels in animal models of UCD.[23] However, there are no reports of a gene therapy for directly reducing the excessive circulating ammonia concentration to interrupt MHE or overt HE triggering and progression.

For reducing hyperammonemia, in this work we propose the delivery of the glutamine synthetase (GS) gene to the skeletal muscle. GS in an enzyme that utilizes ammonia for the synthesis of glutamine,[24] with a subsequent decrease of the concentration of the toxic metabolite. Some studies have shown that ammonia is metabolized in several organs besides the liver, such as the brain, kidney and muscle. It has been rationalized that increasing the removal of ammonia in skeletal muscle can ameliorate HE in patients with liver failure.[25–27] For this purpose, we used the baculovirus Bac-GS as a vector for delivery of the GS gene. The baculovirus has several advantages for gene delivery to mammalian cells. It is able to efficiently transduce dividing and non-dividing mammalian cells *in vitro* and *in vivo*, but it cannot replicate in them; it possesses a large genome with high capacity for insertion of one or multiple foreign genes and regulatory elements, it triggers a mild host immune response and it can be easily produced to high titers and large scale in insect cells.[28–30] The baculovirus-mediated expression of reporter genes in vertebrate animals has been performed, and now this vector is

[1]Departamento de Gastroenterología, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Ciudad de México, México; [2]Departamento de Biomedicina Molecular, Escuela Nacional de Medicina y Homeopatía, Instituto Politécnico Nacional, Ciudad de México, México and [3]Departamento de Medicina Molecular y Bioprocesos, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, México. Correspondence: Dr MA Torres-Vega, Departamento de Gastroenterología, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Vasco de Quiroga No. 15, Col. Sección XVI, Delegación Tlalpan, Ciudad de México, C.P. 14000, México.
E-mail: m1torres@prodigy.net.mx
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being tested for gene therapy, mainly to attack cancer and for regenerative medicine.[29,31–33]

Here we show that Bac-GS efficiently transduced MA104 and L6 myoblast/myotube cells and rat skeletal muscle, and mediated the overexpression of the GS protein. Furthermore, transduction with Bac-GS was able to significantly reduce ammonia levels in the culture medium of MA104 cells and in the blood of rats with acute hyperammonemia. Thus, the baculovirus Bac-GS is a promising gene therapy for controlling hyperammonemia in patients with liver dysfunction or UCD.

RESULTS

Transposition of GS cDNA in the baculovirus genome and production of recombinant viral particles with the transgene (Bac-GS)

Rat liver GS complementary DNA (cDNA) was cloned in a plasmid containing an expression cassette controlled by the cytomegalovirus promoter; the cassette was flanked by the internal terminal repeats of the adeno-associated virus serotype 2 (Figure 1a). It has been reported that adeno-associated virus internal terminal repeats enhance the expression of therapeutic genes delivered by baculovirus.[33] This transfer vector was called pFBCGS-C2, which was the base for generation of the recombinant baculovirus Bac-GS, as it is described in Materials and methods section. The presence of GS cDNA in the genome (bacmid) of amplified baculovirus Bac-GS particles, which had titers of 10^8–10^9 plaque forming units (p.f.u.) ml^{-1}, was confirmed by PCR with specific primers for rat liver GS. PCR on the bacmid DNA and the transfer vector pFBCGS-C2 generated a 1.15-kb double-stranded DNA amplicon, which is in agreement with the expected size of the GS cDNA (Figure 1b). This 1.15-kb band was not obtained in the DNA isolated from the control baculovirus particles (Bac-control), which contained the same modified expression cassette than Bac-GS, but without the GS cDNA.

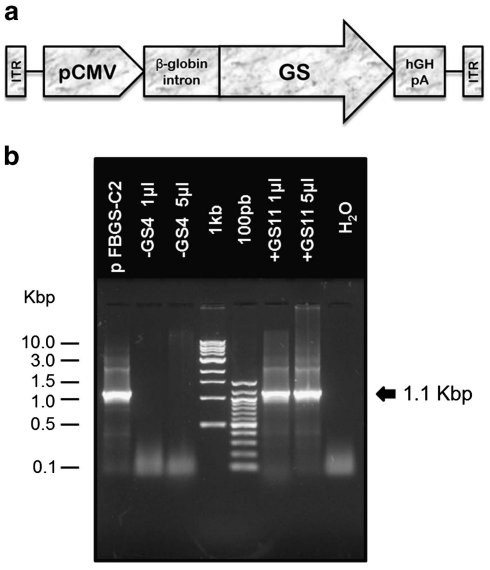

Figure 1. Generation of recombinant baculovirus Bac-GS and Bac-control. (**a**) Map of the expression cassette inserted in the genome of the baculovirus Bac-GS. ITR, adeno-associated virus internal terminal repeats; pCMV, cytomegalovirus promoter; GS, rat liver GS cDNA; hGH pA, polyadenylation signal from the human growth hormone. The genome of the baculovirus Bac-control has the same cassette than Bac-GS, but without the GS cDNA. (**b**) Characterization of the baculovirus stock carrying the GS therapeutic gene by PCR. Plaques of Bac-GS and Bac-control were isolated and amplified in Sf9 cells. Baculovirus stocks with titers of 10^8–10^9 p.f.u. ml^{-1} were obtained. One or 5 µl of genomic DNA purified from the Bac-GS stock (+GS11) was used as template for PCR with specific primers for GS. DNA fragments of 1.1 kbp corresponding to the expected size of the GS cDNA (lines 6 and 7), were obtained. A band with the same size was amplified in a sample from the transfer plasmid pFBGS-C2 (line 1), but neither from the DNA from Bac-control (−GS4, lines 2 and 3), nor from water (line 8).

Quantification of baculovirus-mediated GS mRNA expression in MA104 cells

To determine if Bac-GS was able to transduce mammalian cells with the GS therapeutic gene, it was incubated with epithelial MA104 kidney cells from *Macaca mulatta* (10 p.f.u. per cell). Cells were also incubated with Bac-control and Bac-green fluorescent protein (GFP) at the same p.f.u. per cell ratio. Around 80% of MA104 cells were fluorescent after transduction with Bac-GFP (results not shown). Two days post-incubation, total RNA was isolated from Bac-GS and Bac-control-transduced cells and GS expression was detected by reverse transcriptase real-time PCR (Figure 2a). The messenger RNA (mRNA) coding for GS was seven times more abundant in MA104 cells incubated with Bac-GS than in cells incubated with Bac-control (0.82 ± 0.16 versus 0.10 ± 0.05, $P < 0.05$; Figure 2b). The reverse transcriptase real-time PCR signal obtained from the samples transduced with Bac-GS originated from the expressed GS mRNA, not from a possible contaminant bacmid DNA containing the GS cDNA, as no significant expression signal was registered in a control reaction without the reverse transcriptase enzyme (Bac-GS-noRT; Figure 2b).

Diminishing ammonia levels in the medium of MA104 cells by the baculovirus Bac-GS

To evaluate the potential use of Bac-GS in the therapy of HE, we developed a model of hyperammonemia *in vitro*. MA104 cells transduced 1 day before with Bac-GS, Bac-control (10 p.f.u. per cell) or not transduced were incubated in medium with 100 µM NH_4Cl and 40h later the ammonia levels were quantified. Incubation with Bac-GS significantly reduced the ammonia concentration to 65.3 ± 7.3 µM, which corresponds to a reduction of 43.8 and 45.9%, in comparison with the concentration in medium from cells incubated with NH_4Cl and transduced with Bac-control (10 p.f.u. per cell, 116.2 ± 4.5 µM) or not transduced (120.7 ± 4.1 µM), respectively ($P < 0.05$ in both cases; Figure 3a). Western blot analysis of lysates from these cells with an anti-GS antibody showed a band of 44 kDa in all samples. The band was 3.2 times more abundant in lysates from cells transduced with Bac-GS (Figure 3b). The band detected in cells not transduced or transduced with Bac-control is possibly the endogenous GS, whereas in cells transduced with Bac-GS the band represents the accumulation of the endogenous GS plus the baculovirus-mediated overexpressed GS.

Baculovirus delivery of therapeutic GS gene in L6 myoblast/myotubes

In order to determine if Bac-GS is able to transduce muscle cells, L6 myoblast cells were partially differentiated into myotubes and used as a model.[34] It has been reported that recombinant baculovirus can transduce C2–7 myoblast/myotubes.[31] L6 cells during incipient differentiation (some tubules formed after 5 days of incubation with 1% fetal bovine serum (FBS)) were transduced with Bac-GS or with Bac-control at 100 p.f.u. per cell or not transduced. Two days later, lysates of these cells were prepared and western blot was performed to detect GS. L6 myoblast/myotubes transduced with Bac-GS expressed a high amount of GS enzyme, which was not detected in lysates from similar cells not transduced or transduced with Bac-control (Figure 4). The 44-kDa band in lysates from L6 myoblast/myotubes transduced with Bac-GS migrated at the same size that one of the bands present in the

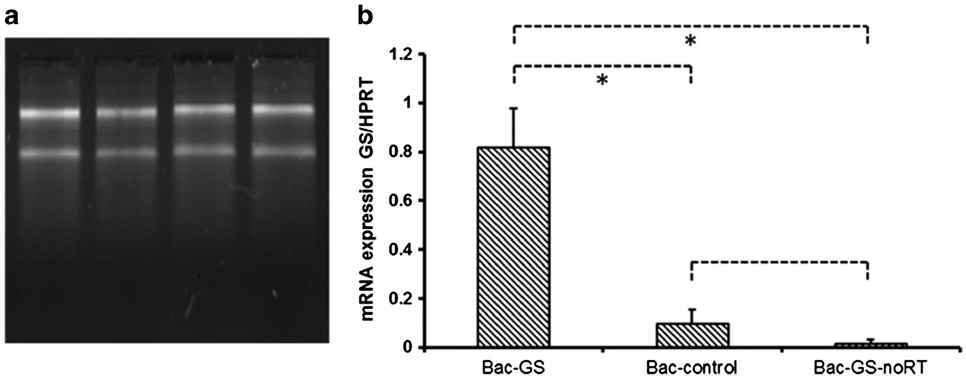

Figure 2. Baculovirus-mediated GS mRNA expression in MA104 cells. (**a**) Total RNA isolated from baculovirus-transduced MA104 cells (four samples). (**b**) Expression of GS mRNA by real-time reverse transcriptase-PCR in MA104 cells transduced with baculovirus Bac-GS or Bac-control (10 p.f.u. per cell). Expression was normalized with the house keeping gene hypoxanthine phosphoribosyltransferase 1 (HPRT1). The label Bac-GS-noRT is a control where the reverse transcriptase was omitted during RT of total RNA from Bac-GS-transduced MA104 cells. Mean + s.d. from six independent experiments are shown. $*P < 0.05$.

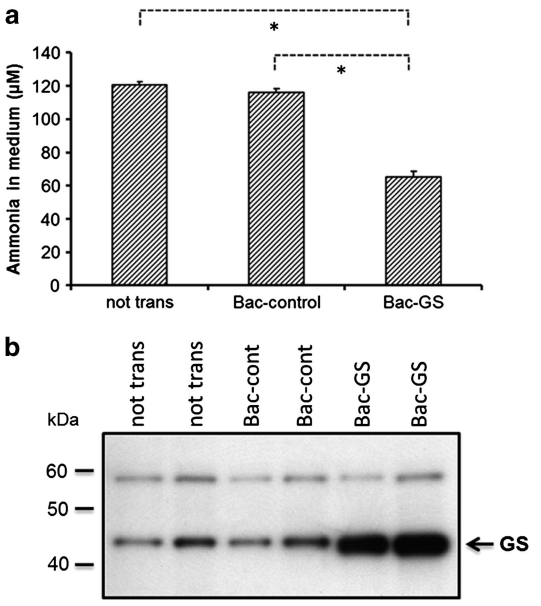

Figure 3. Effect of Bac-GS on the hyperammonemia model in MA104 cells. (**a**) MA104 cells previously transduced with Bac-GS or Bac-control (10 p.f.u. per cell) or not transduced (not trans) were incubated with medium containing 100 μM NH_4Cl. After 40 h of incubation, an aliquot of the medium was taken to quantify the concentration of ammonia. Mean+s.d. from six independent experiments are shown. $*P < 0.05$. (**b**) Western blot analysis of the GS protein expressed in samples from each experimental condition. Each condition was tested in duplicate. The nonspecific signal of a 58 kDa protein shows that a similar protein amount was loaded in all lanes.

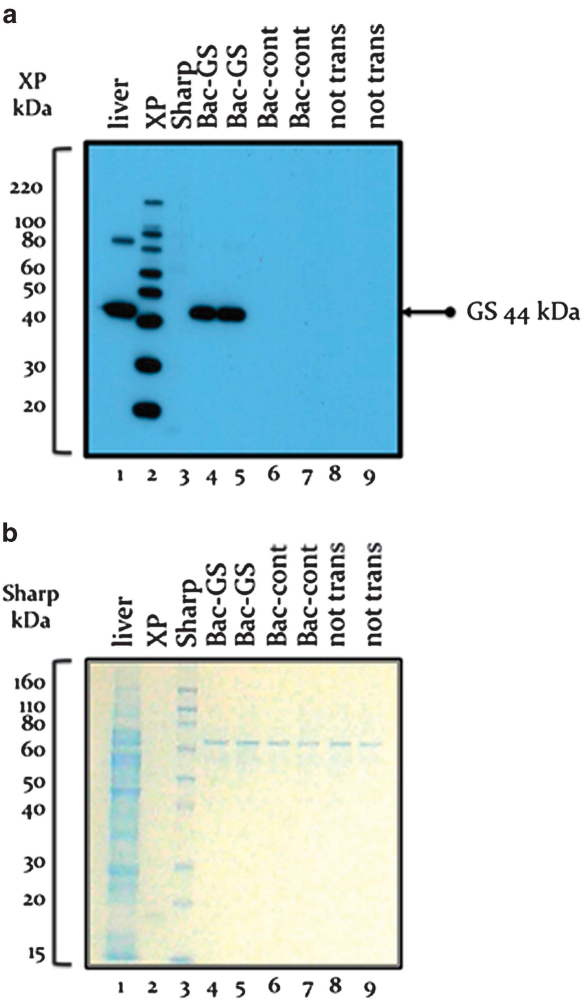

Figure 4. Expression of GS protein in L6 cells by transduction with the baculovirus Bac-GS. (**a**) Western blot of lysates from L6 cells transduced with Bac-GS (100 p.f.u. per cell), showing the GS protein as a sharp 44-kDa band (lanes 4 and 5). Lysates from rat liver (lane 1), L6 cells transduced with Bac-control (Bac-cont, 100 p.f.u. per cell, lanes 6 and 7) or not transduced cells (not trans, lanes 8 and 9) were also analyzed. Lane 1, 5 μg; lanes 4–9, 1 μg. XP: MagicMark XP Western MW (lane 2); Sharp: Novex Sharp pre-stained MW (lane 3). (**b**) Coomassie blue staining of a polyacrylamide gel showing similar protein loading of the samples in **a**.

rat liver lysate, most likely endogenous GS. The other band in the liver lysate is close to 90 kDa and possibly corresponds to a dimer of GS, which has a similar molecular weight (Figure 4).[24]

The baculovirus Bac-GS decreases ammonia concentration in the blood circulation of rats with acute hyperammonemia

The baculovirus Bac-GS was tested in an acute hyperammonemia rat model, modified from one reported in the literature,[35,36] to determine its efficacy for reducing ammonia levels in blood circulation. Mice in a similar model showed several progressive symptoms of HE, like passivity, lethargy and ataxia with myoclonus.[36] Bac-GS or Bac-GFP particles (1.5×10^7 p.f.u.) were

intramuscularly injected in both hind legs of 6–7 rats. Three days later, these rats were injected intraperitoneally with ammonium acetate (200 mg kg^{-1} body weight) to produce acute hyperammonemia. After 15 min, blood from the femoral artery was sampled to quantify the ammonia concentration.[36] The plasma from rats injected with Bac-GS was 66% less hyperammonemic than the plasma from rats that did not receive virus (181.4 ± 74.1 versus 532.8 ± 196.8 μM, $P < 0.05$; Figure 5a), which corresponds to an ammonia level reduction of 351 μM in blood circulation. Hyperammonemic rats injected with Bac-GFP had a high ammonia concentration in plasma (784.4 ± 124.2 μM). GFP was immunodetected in transduced gastrocnemius muscles, and showed that myotubes transduction was efficient (Figure 5b). It was not possible to directly measure GFP fluorescence, as the background signal was too high. Western blot analysis of protein lysates from gastrocnemius muscles belonging to rats transduced with Bac-GS showed at least two times more GS protein than lysates from rats transduced with Bac-GFP (Figure 5c). It is worth to mention that rats injected with Bac-GS did not exhibit any behavioral nor physical alteration compared with controls.

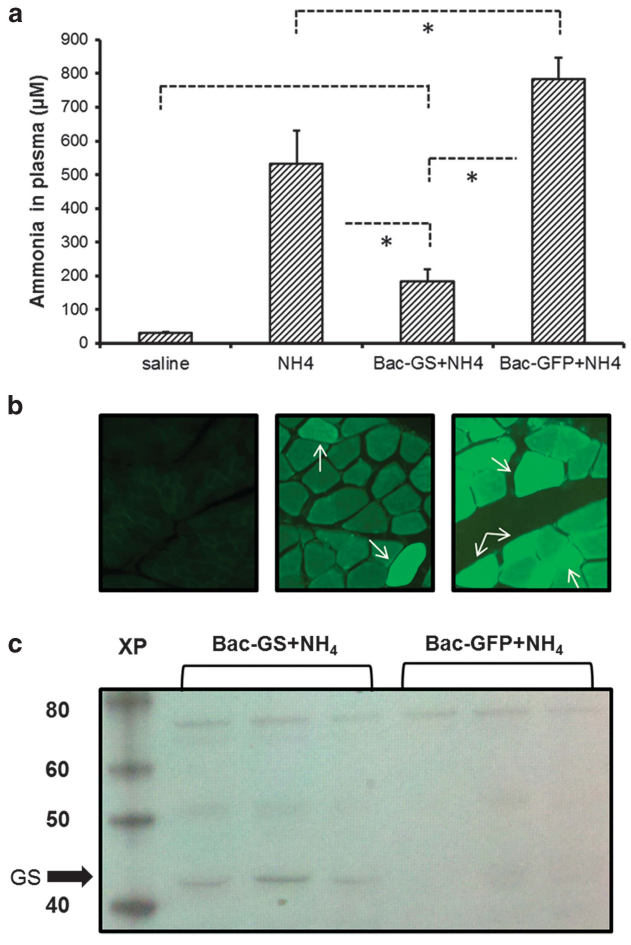

Figure 5. Lowering ammonia in circulating blood of hyperammonemic rats by treatment with the baculovirus Bac-GS. (**a**) Rats were made hyperammonemic by intraperitoneal administration of ammonium acetate (200 mg kg^{-1} body weight). Some of them were previously intramuscularly injected in the two rear legs with Bac-GS (Bac-GS+NH_4, $n = 7$). Fifteen min after ammonium overdose, levels of this toxin were quantified in blood of these rats. Quantification of ammonia was done also in hyperammonemic rats not receiving virus (NH_4, $n = 5$), receiving Bac-GFP (Bac-GFP + NH_4, $n = 6$) or not hyperammonemics (saline, $n = 6$). Mean+s.d.; *$P < 0.05$. (**b**) Indirect immunofluorescence to visualize GFP in slices of gastrocnemius muscles from rats injected with either saline (left) or Bac-GFP (center and right). Myotubes with a strong GFP expression are indicated with arrows. (**c**) Gastrocnemius muscles protein lysates were prepared from hyperammonemic rats transduced with Bac-GS ($n = 3$) or Bac-GFP ($n = 3$) and analyzed by western blot to detect GS. The nonspecific signal of a 72 kDa protein shows that a similar protein amount was loaded in all lanes. XP: MagicMark XP Western MW (lane 1).

DISCUSSION

Ammonia is one of the causes of brain dysfunction and death in liver diseases.[4–7,9,11,14] Developing an effective gene therapy for diminishing hyperammonemia in HE can solve some of the problems encountered during its treatment with disaccharides and antibiotics, such as undesirable side effects, failure in decreasing the ammonia levels and elevated costs.[22,27] For example, it has been estimated that the total-life-time combined cost of care for the HE treatment with the best cost per quality-adjusted life-year-gained index (Rifaximin provided as second-line therapy to patients failing an initial trial with lactulose, 'Rifaximin salvage') is $ 59 282 US.[21] On the road of establishing a more efficient and effective treatment for reduction of elevated ammonia levels in MHE and overt HE, which can be also used in UCD or other hyperammonemic medical conditions, in this work we present a gene transfer approach through the recombinant baculovirus Bac-GS that delivers a cDNA codifying for the GS enzyme, which gives transduced cells the *in vitro* and *in vivo* ability of detoxifying ammonia in the ambiance.

Transduction of MA104 cells with Bac-GS reduced the concentration of added ammonia 45%. Previously, the Enosawa and Chen research groups genetically modified HepG2 cells for overexpressing GS with the purpose of building bioartificial livers for treatment of hyperammonemia.[37,38] They observed an ammonia removal of 0.049 μM h^{-1} per 10^9 cells.[37] In comparison, we reduced around 66 times more the ammonia concentration, considering our conditions, such as the number of cells and length of the assay. This improvement in the *in vitro* ammonia detoxification with Bac-GS can be related to the efficient transduction of cells with the baculovirus, the cell type and the regulatory elements included in the expression cassette, like the adeno-associated viruses internal terminal repeats.[29,33]

It is worth to mention that transduction with Bac-GS increased 7 times the amount of GS mRNA expressed in non-human primate MA104 cells (Figure 2b), whereas the GS protein only augmented 3.2 times (Figure 3b), possibly due to posttranscriptional or posttranslational regulation for the GS enzyme in this cell type. Actually, it has been reported that glutamine decreases the activity of GS by a feedback regulation through a mechanism involving the degradation of the enzyme by the proteasome.[34,39–41] According with this mechanism, the accumulation of glutamine in MA104 cells because of the activity of overexpressed GS enzyme could explain a lesser increase of GS protein concentration than that observed for its corresponding mRNA.

It has been proposed that therapies designed with the aim of increasing the ammonia removal in tissues like skeletal muscle can contribute to diminish hyperammonemia in patients with MHE and overt HE.[25,26,27] Such a proposal is based on experiments with a skeletal muscle-specific GS-knock out mouse, where it was observed that the muscle GS was needed for detoxifying an overload of ammonia.[42] Moreover, an increase in GS concentration and activity has been observed in muscles of cirrhotic patients or in chronic and acute liver failure rat models,[43–46] possibly as an adaptation of the organism to compensate for a reduction in detoxification of ammonia in the liver and to maintain the glutamine levels. However, in most patients ammonia removal in

muscle by the overexpressed endogenous GS is not enough and hyperammonemia is triggered.

As an approach for augmenting ammonia detoxification in skeletal muscle, we first tested if the baculovirus Bac-GS was able to transduce myoblast/myotubes L6, which are cells with similar characteristics to muscle fibers.[34] The myoblast/myotubes L6 were effectively transduced with this recombinant virus and the amount of Bac-GS-mediated GS protein overexpressed in these cells was almost four times superior than endogenous GS protein in rat liver, as it is shown in our Results section (Figure 4). The level of endogenous GS protein in several mouse tissues has been measured, and it was found that muscle has 16 times less GS protein than liver.[47] According with these results, we consider that there is a high potential for over-producing GS in skeletal muscle by transducing it with Bac-GS. This would have a major impact for detoxifying ammonia in patients with hyperammonemia.

We moved forward by injecting Bac-GS directly in skeletal muscle of rats, which 3 days later received an overload of ammonia. We got a Bac-GS-mediated 351 μM plasma ammonia reduction and significant overexpression of GS in gastrocnemius muscles (Figure 5). In the rat model of HE in cirrhosis developed by Jover *et al.*,[6] rats with HE manifestations had circulating ammonia levels around 170 μM, in comparison with control (pair feed sham) rats, which had values around 95 μM and no HE signs. Hence, it can be expected that therapy with Bac-GS of Jover´s rats will be enough for reducing plasma ammonia to basal levels. Furthermore, we hypothesize that treatment with Bac-GS can also effectively eliminate hyperammonemia in other rat models, according to the ammonia levels typical in them, such as rats with cirrhosis by bile duct ligation, rats with acute liver failure by hepatic devascularization, and rats with portacaval anastomosis and infusion of blood into the duodenum.[48–50] For example, therapy of rats with CCl_4-induced cirrhosis with ascites plus portal vein occlusion using Rifaximin, which is the preferred drug in clinic for treatment of patients with HE, lowered the plasma ammonia level only 79 μM.[51] In our acute hyperammonemia rat model, Bac-GS decreased the ammonia levels over four times more than Rifaximin. Therefore, we consider that the gene therapy proposed here is a viable alternative to treat patients with various levels of hyperammonemia. The results with Bac-GS suggest that GS overexpression in skeletal muscle and possibly in other tissues effectively increased the capacity of removing an overload of ammonia in the body, as can be predicted by studies of inter-organ ammonia metabolism.[26]

For patients with UCD, gene therapy has been used in some UCD animal models, showing a decrease in hyperammonemia, an increase of survival and a conservation of brain morphology and behavior; however, the decrease of ammonia concentration does not persist completely from neonates to adults.[23,52,53] Considering these reports, the baculovirus-mediated GS overexpression in skeletal muscle is an attractive alternative for helping in the reduction of hyperammonemia in UCD patients. Interestingly, the delivery of GS gene by baculovirus can be applied for treatment of hyperammonemia developed in all urea cycle enzymes deficiencies.

It has been shown that the duration of expression of transgenes delivered by baculovirus varies from 1 week to several months *in vivo*.[30,31] Therefore, the possible application of Bac-GS in gene therapy protocols should focus for care of patients during the peaks of hyperammonemia on acute or chronic liver failure. For treatment of chronic hyperammonemia, readministration of Bac-GS is not recommended, as baculovirus triggers innate and adaptative immune responses. Humoral and cellular responses provoke a reduction of the expression of the transgene after a second administration of the vector.[30]

In conclusion, the reduction of ammonia concentration in the medium of MA104 cells or in the circulation of rats transduced with the baculovirus Bac-GS serves as a proof of concept and brings a new light regarding the use of this recombinant vector for gene therapy of acute episodes of hyperammonemia in MHE, overt HE, UCD or other illness. The virus delivery of the GS gene in conjunction with other genes for long overexpression of proteins participating in ammonia metabolism like transporters[54] and/or ameliorating inflammatory response in targets like skeletal muscle, surely will overcome the efficacy of the current HE treatments, by eliminating the undesirable side effects, decreasing the expenses because of pharmaceuticals and visits to the hospital and improving the quality of life in patients with this neuropsychiatric disorder.

MATERIALS AND METHODS

Construction of the transfer vector pFBCGS-C2

A pBluescript plasmid with the rat liver GS cDNA was kindly provided by Dr WH Lamers (The Netherlands). The complete GS cDNA was amplified from this plasmid with the specific GS primers forward 5′-AAAAAAG CTTGCCACCATGGCCACCTCAGC-3′ and reverse 5′-GGTGCTCGAGTTAG TTCTTGTATTGGAAGGG-3′. The amplified DNA fragment was inserted into the plasmid cytomegalovirus promoter-MCS (Stratagene, La Jolla, CA, USA), between the *Hind*III and *Xho*I restriction sites. The cassette with the GS cDNA was isolated from this construct by digesting with *Not*I. Then, the cassette was subcloned between the internal terminal repeats of adeno-associated virus serotype 2, by substitution of the *Not*I-*Not*I fragment from the pFBGFPR plasmid, which was kindly supplied by Dr RM Kotin (Bethesda, MD, USA) and has been described elsewhere.[55] The final construct was called pFBCGS-C2. To obtain a control empty plasmid, the GS cDNA from pFBCGS-C2 was eliminated by digestion with *Bam*HI and *Xho*I restriction enzymes, and ligating the plasmid backbone.

Production of recombinant baculovirus

The Baculovirus Expression System with Gateway Technology was used, following the instructions of the manufacturer (Invitrogen, Carlsbad, CA, USA). Briefly, to obtain the transposition of the cassette with the transgene into the genome of the baculovirus, the transfer plasmids were transformed in *Escherichia coli* DH10Bac. The screening of the positive colonies was done by PCR with the same primers described above. Recombinant bacmid DNA from the positive colonies was purified using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). One microgram of the purified DNA was transfected in Sf9 insect cells with Flyfectin (OZ Biosciences, Marseille, France). After 96-h posttransfection, the supernatant was recovered and the resulting baculovirus were plaqued for their purification. To produce stocks of purified recombinant baculovirus, viral plaques were selected to infect Sf9 cells with 100 μl of the baculovirus plaque suspension obtained, and finally two stocks were generated: +GS11 and –GS4 baculovirus passage 1. Viral stocks were tittered as described in Mena *et al.*[56] 125 ml of *Sf9* cells in 500 ml shaker flasks were infected with these stocks at a multiplicity of infection of 0.1 p.f.u. ml^{-1}. Supernatants and cells were harvested 96-h postinfection. Amplified Bac-GS (+GS11) and Bac-control (– GS4) baculovirus had a titer of 10^8–10^9 p.f.u. ml^{-1}; both were stored at 4 °C protected from light. The baculovirus Bac-GFP was produced following the same method described above.

PCR analysis in genomic DNA from baculovirus particles

Genomic DNA from the recombinant baculovirus particles was isolated using the Easy-DNA Kit (Invitrogen, Carlsbad, CA, USA), following the instructions of the manufacturer (protocol #7). To verify that the rat GS cDNA was inserted in the purified DNA, 1 and 5 μl of this DNA (167–1090 ng) was analyzed by PCR with the same specific primers described above. As a template for a positive control, also 0.5 ng of the plasmid pFBCGS-C2 was analyzed by PCR.

Transduction of mammalian cells with the recombinant baculovirus and hyperammonemia *in vitro* model

MA104 and L6 cells (ATCC, Manassas, VA, USA) were routinely cultured in Dulbecco's modified Eagle's medium high glucose (Gibco-Invitrogen, Grand Island, NY, USA) containing 10% FBS (Gibco-Invitrogen) and antibiotics. To induce the differentiation to myotubes, the medium of a 100% confluent L6 myoblast culture was replaced with medium containing only 1% FBS.[34] To transduce cells with the recombinant baculovirus, we

followed the procedure described in Keil *et al.*[57] Briefly, medium in six-well plates with confluent L6 or MA104 cells was replaced with 1.5 ml Dulbecco's modified Eagle's medium without serum or antibiotics, and the baculovirus particles were added (10 or 100 p.f.u. per cell). Plates were centrifuged at 600 × *g* for 1 h at 26 °C. Thereafter, for L6 cells, the medium was substituted by 2 ml of minimum essential medium (Thermo Scientific HyClone, Logan, UT, USA) pH 7.4 supplemented with 1% FBS and 5 mM sodium butyrate (Sigma, St Louis, MO, USA). When MA104 cells were used, the medium was changed for minimum essential medium pH 7.4, 0.2 mM glutamine, 2% FBS and 5 mM sodium butyrate. For the establishment of the hyperammonemia *in vitro* model, 24 h later, MA104 cells were incubated with 3 ml of minimum essential medium pH 7.4, 0.2 mM glutamine, 2 mM acid L-glutamic, 1 mM sodium pyruvate, 2% FBS and 0.1 mM NH_4Cl. After 40 h of incubation, an aliquot of medium (0.5 ml) was taken from the wells to quantify the ammonium concentration immediately.

Isolation of RNA from cells and quantitative reverse transcriptase-PCR

Total RNA was extracted from MA104 cells seeded on six-well plates using 1 ml of Trizol Reagent (Invitrogen, Carlsbad, CA, USA) per well, following the instructions of the manufacturer. A second round of RNA purification to eliminate contaminant DNA was done with the High Pure RNA Tissue Kit (Roche, Indianapolis, IN, USA). The RNA integrity was verified in 1.0% agarose gels. The cDNA was synthetized using the Transcriptor First Strand Synthesis Kit (Roche) with random primers. Real-time PCR was performed with the LightCycler Taqman Master Kit (Roche) on a LightCycler 2.0 Instrument (Roche). The quantitation of GS was made with the specific primers 5′-AGTCTGAAGGCTCCAACAGC-3′ and 5′-AAGGGGTCTCGAAACATGG-3′ that were designed using the software from the Universal ProbeLibrary Assay Design Center (Roche Applied Science, Penzberg, Germany), and the probe # 58 from the Universal ProbeLibrary Set, human (Roche). As housekeeping gene, the *Homo sapiens* hypoxanthine phosphoribosyltransferase 1 was quantified using the primers 5′-TGACCTTGATTTATTTTGCATACC-3′ and 5′-CGAGCAAGACGTTCAGTCCT-3′, which were designed with the same software listed above, and the probe # 73 from the same library.

Determination of ammonia

Quantification of ammonia in the medium of MA104 or in the plasma from rats was done with the Roche Cobas NH3 Ammonia assay (kit 11877984 Roche Diagnostics, Indianapolis, IN, USA) and the Roche Hitachi 912 chemistry analyzer, following the instructions of the manufacturer. The method is based on ammonia reaction with α-ketoglutarate and reduced nicotinamide adenine dinucleotide phosphate (NADPH) to form glutamate and NADP+. The reaction is catalyzed by glutamate dehydrogenase. The amount of the NADP+ is equivalent to the level of ammonia in the sample, which is spectrophotometrically measured.

Western blot analysis

Cells were lysed in Complete Lysis-M Reagent (Roche), following the instructions of the manufacturer. Tissues were broken by homogenization with polytron using the same Complete reagent listed above. Protein concentrations were measured by the bicinchoninic acid method (Pierce-Thermo, Rockford, IL, USA). Lysates were mixed with NuPAGE LDS Sample Buffer and NuPAGE Reducing Agent (Invitrogen, Carlsbad, CA, USA) and heated at 70 °C for 10 min. The samples were electrophoresed in NuPAGE 10% Bis-Tris Gels, which were run in NuPAGE MOPS SDS running buffer containing NuPAGE antioxidant (Invitrogen, Carlsbad, CA, USA). Five microliters of Novex Sharp pre-stained and MagicMark XP Western protein standards (Invitrogen, Carlsbad, CA, USA) were simultaneously run. Proteins were transferred from gels to nitrocellulose membranes using the NuPAGE transfer buffer and the XCell II Blot Module (Invitrogen, Carlsbad, CA, USA). The proteins were detected with a rabbit anti-GS antibody (1:2000, Sigma), followed by a goat anti-rabbit immunoglobulin G (whole molecule) peroxidase-conjugate (1:2000, Sigma) antibody, and developed with the SuperSignal West Pico chemiluminiscent substrate (Pierce-Thermo). Densitometric analysis was made with a ChemiDoc XRS+ Imaging System (Bio-Rad, Hercules, CA, USA).

Hyperammonemia rat model and virus administration

The experiments with rats were approved by the Animal Committee from the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán. Wistar male rats, 180–250 g body weight, were anesthetized intraperitoneally with a combination of Xylazine (Xilazina Aranda, Querétaro, México) and Ketamine (Anesket Pisa Agropecuaria, Atitalaquia, México). Vectors Bac-GS or Bac-GFP (10^8 p.f.u. ml^{-1}) were injected intramuscularly into the two rear legs at a total volume of 150 µl per leg at a speed of 7 µl min^{-1} using a infusion pump. In a modified version of the methods described in Hilgier and Olson[35] and Rangroo *et al.*,[36] 3 days after gene transfer, the animals were anesthetized again and intraperitoneally injected with 200 mg kg^{-1} body weight of ammonium acetate (Sigma) to induce hyperammonemia. Fifteen min later, blood was collected from the femoral artery and ammonia concentration was quantified in plasma.[36]

Indirect immunofluorescence

Fragments of gastrocnemius muscle from rats were fixed in 4% paraformaldehyde (Sigma) in phosphate-buffered saline (PBS) for 1–2 h. After PBS washes, the muscle fragments were layered over a 30% sucrose/PBS (Sigma) cushion at 4 °C overnight. The pieces of tissue were immersed in embedding medium (Tissue-Tek O. C. T. Compound, Sakura Inc., Torrance, CA, USA) and frozen on alcohol/dry ice. Twenty-micrometer slices of muscle were cut using a Cryostat (Hyrax C60, Zeiss, Jena, Germany). Slices were permeabilized for 10 min in PBS with 1% Triton (Sigma) and blocked with blocking buffer (1% albumin/PBS) for 2h. Later, slices were incubated with a rabbit anti-GFP antibody (1:500 in blocking buffer, Millipore, Temecula, CA, USA) at 4 °C overnight, followed by PBS washes and incubation with an Alexa Fluor 488 goat anti-rabbit immunoglobulin G antibody (1:100 in blocking buffer, Invitrogen, Eugene, OR, USA) at room temperature in dark during 1 h. After PBS washes, slices were mounted on glass slides with liquid mountant (Prolong Gold Antifade Mountant whit DAPI, Invitrogen, Eugene, OR, USA) and analyzed by fluorescent microscopy using an Axio Imager.A2 microscope (Zeiss, Göttingen, Germany) with an AxioCam MRc r3.1 camera (Zeiss, Göttingen, Germany) and the Axiovision Rel. 4.8.1 software (Zeiss, Göttingen, Germany).

Statistical analysis

Analysis of data was made with the analysis of variance–Tukey test. $P < 0.05$ was considered statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

1 Ferenci P, Lockwood A, Mullen K, Tarter R, Weissenborn K, Blei AT. Hepatic encephalopathy--definition, nomenclature, diagnosis, and quantification: final report of the working party at the 11th World Congresses of Gastroenterology, Vienna, 1998. *Hepatology* 2002; **35**: 716–721.
2 Walker V. Ammonia toxicity and its prevention in inherited defects of the urea cycle. *Diabetes Obes Metab* 2009; **11**: 823–835.
3 Felipo V. Hepatic encephalopathy: effects of liver failure on brain function. *Nat Rev Neurosci* 2013; **14**: 851–858.
4 Clemmesen JO, Larsen FS, Kondrup J, Hansen BA, Ott P. Cerebral herniation in patients with acute liver failure is correlated with arterial ammonia concentration. *Hepatology* 1999; **29**: 648–653.
5 Ong JP, Aggarwal A, Krieger D, Easley KA, Karafa MT, Van Lente F *et al.* Correlation between ammonia levels and the severity of hepatic encephalopathy. *Am J Med* 2003; **114**: 188–193.
6 Jover R, Rodrigo R, Felipo V, Insausti R, Sáez-Valero J, García-Ayllón MS *et al.* Brain edema and inflammatory activation in bile duct ligated rats with diet-induced hyperammonemia: a model of hepatic encephalopathy in cirrhosis. *Hepatology* 2006; **43**: 1257–1266.
7 Bernal W, Hall C, Karvellas CJ, Auzinger G, Sizer E, Wendon J. Arterial ammonia and clinical risk factors for encephalopathy and intracranial hypertension in acute liver failure. *Hepatology* 2007; **46**: 1844–1852.

8 Seyan AS, Hughes RD, Shawcross DL. Changing face of hepatic encephalopathy: role of inflammation and oxidative stress. *World J Gastroenterol* 2010; **16**: 3347–3357.
9 Felipo V, Urios A, Montesinos E, Molina I, Garcia-Torres ML, Civera M *et al.* Contribution of hyperammonemia and inflammatory factors to cognitive impairment in minimal hepatic encephalopathy. *Metab Brain Dis* 2012; **27**: 51–58.
10 Llansola M, Montoliu C, Cauli O, Hernández-Rabaza V, Agustí A, Cabrera-Pastor A *et al.* Chronic hyperammonemia, glutamatergic neurotransmission and neurological alterations. *Metab Brain Dis* 2013; **28**: 151–154.
11 Rama Rao KV, Jayakumar AR, Tong X, Alvarez VM, Norenberg MD. Marked potentiation of cell swelling by cytokines in ammonia-sensitized cultured astrocytes. *J Neuroinflammation* 2010; **7**: 66.
12 Rodrigo R, Cauli O, Gomez-Pinedo U, Agusti A, Hernandez-Rabaza V, Garcia-Verdugo JM *et al.* Hyperammonemia induces neuroinflammation that contributes to cognitive impairment in rats with hepatic encephalopathy. *Gastroenterology* 2010; **139**: 675–684.
13 Häussinger D, Sies H. Hepatic encephalopathy: clinical aspects and pathogenetic concept. *Arch Biochem Biophys* 2013; **536**: 97–100.
14 Lachmann V, Görg B, Bidmon HJ, Keitel V, Häussinger D. Precipitants of hepatic encephalopathy induce rapid astrocyte swelling in an oxidative stress dependent manner. *Arch Biochem Biophys* 2013; **536**: 143–151.
15 Mas A, Rodés J, Sunyer L, Rodrigo L, Planas R, Vargas V *et al.* Comparison of rifaximin and lactitol in the treatment of acute hepatic encephalopathy: results of a randomized, double-blind, double-dummy, controlled clinical trial. *J Hepatol* 2003; **38**: 51–58.
16 Sharma P, Sharma BC, Puri V, Sarin SK. An open-label randomized controlled trial of lactulose and probiotics in the treatment of minimal hepatic encephalopathy. *Eur J Gastroenterol Hepatol* 2008; **20**: 506–511.
17 Sharma BC, Sharma P, Agrawal A, Sarin SK. Secondary prophylaxis of hepatic encephalopathy: an open-label randomized controlled trial of lactulose versus placebo. *Gastroenterology* 2009; **137**: 885–891.
18 Bass NM, Mullen KD, Sanyal A, Poordad F, Neff G, Leevy CB *et al.* Rifaximin treatment in hepatic encephalopathy. *N Engl J Med* 2010; **362**: 1071–1081.
19 Agrawal A, Sharma BC, Sharma P, Sarin SK. Secondary prophylaxis of hepatic encephalopathy in cirrhosis: an open-label, randomized controlled trial of lactulose, probiotics, and no therapy. *Am J Gastroenterol* 2012; **107**: 1043–1050.
20 Sharma P, Sharma BC, Agrawal A, Sarin SK. Primary prophylaxis of overt hepatic encephalopathy in patients with cirrhosis: an open labeled randomized controlled trial of lactulose versus no lactulose. *J Gastroenterol Hepatol* 2012; **27**: 1329–1335.
21 Huang E, Esrailian E, Spiegel BM. The cost-effectiveness and budget impact of competing therapies in hepatic encephalopathy—a decision analysis. *Aliment Pharmacol Ther* 2007; **26**: 1147–1161.
22 Phongsamran PV, Kim JW, Cupo Abbott J, Rosenblatt A. Pharmacotherapy for hepatic encephalopathy. *Drugs* 2010; **70**: 1131–1148.
23 Alexander IE, Kok C, Dane AP, Cunningham SC. Gene therapy for metabolic disorders: an overview with a focus on urea cycle disorders. *J Inherit Metab Dis* 2012; **35**: 641–645.
24 Krajewski WW, Collins R, Holmberg-Schiavone L, Jones TA, Karlberg T, Mowbray SL. Crystal structures of mammalian glutamine synthetases illustrate substrate-induced conformational changes and provide opportunities for drug and herbicide design. *J Mol Biol* 2008; **375**: 217–228.
25 Morgan MY, Blei A, Grüngreiff K, Jalan R, Kircheis G, Marchesini G *et al.* The treatment of hepatic encephalopathy. *Metab Brain Dis* 2007; **22**: 389–405.
26 Wright G, Noiret L, Olde Damink SW, Jalan R. Interorgan ammonia metabolism in liver failure: the basis of current and future therapies. *Liver Int* 2011; **31**: 163–175.
27 Rose CF. Ammonia-lowering strategies for the treatment of hepatic encephalopathy. *Clin Pharmacol Ther* 2012; **92**: 321–331.
28 Kost TA, Condreay JP, Jarvis DL. Baculovirus as versatile vectors for protein expression in insect and mammalian cells. *Nat Biotechnol* 2005; **23**: 567–575.
29 Airenne KJ, Hu YC, Kost TA, Smith RH, Kotin RM, Ono C *et al.* Baculovirus: an insect-derived vector for diverse gene transfer applications. *Mol Ther* 2013; **21**: 739–749.
30 Luo WY, Lin SY, Lo KW, Lu CH, Hung CL, Chen CY *et al.* Adaptive immune responses elicited by baculovirus and impacts on subsequent transgene expression *in vivo*. *J Virol* 2013; **87**: 4965–4973.
31 Pieroni L, Maione D, La Monica N. *In vivo* gene transfer in mouse skeletal muscle mediated by baculovirus vectors. *Hum Gene Ther* 2001; **12**: 871–881.
32 Lin CY, Lu CH, Luo WY, Chang YH, Sung LY, Chiu HY *et al.* Baculovirus as a gene delivery vector for cartilage and bone tissue engineering. *Curr Gene Ther* 2010; **10**: 242–254.
33 Luo WY, Shih YS, Lo WH, Chen HR, Wang SC, Wang CH *et al.* Baculovirus vectors for antiangiogenesis-based cancer gene therapy. *Cancer Gene Ther* 2011; **18**: 637–645.
34 Feng B, Shiber SK, Max SR. Glutamine regulates glutamine synthetase expression in skeletal muscle cells in culture. *J Cell Physiol* 1990; **145**: 376–380.
35 Hilgier W, Olson JE. Brain ion and amino acid contents during edema development in hepatic encephalopathy. *J Neurochem* 1994; **62**: 197–204.
36 Rangroo Thrane V, Thrane AS, Chang J, Alleluia V, Nagelhus EA, Nedergaard M. Real-time analysis of microglial activation and motility in hepatic and hyperammonemic encephalopathy. *Neuroscience* 2012; **220**: 247–255.
37 Miyashita T, Enosawa S, Suzuki S, Tamura A, Tanaka H, Amemiya H *et al.* Development of a bioartificial liver with glutamine synthetase-transduced recombinant human hepatoblastoma cell line, HepG2. *Transplant Proc* 2000; **32**: 2355–2358.
38 Tang NH, Wang XQ, Li XJ, Chen YL. Ammonia metabolism capacity of HepG2 cells with high expression of human glutamine synthetase. *Hepatobiliary Pancreat Dis Int* 2008; **7**: 621–627.
39 Labow BI, Souba WW, Abcouwer SF. Mechanisms governing the expression of the enzymes of glutamine metabolism--glutaminase and glutamine synthetase. *J Nutr* 2001; **131**: 2467S–2474S; discussion 2486S–7S.
40 Huang YF, Wang Y, Watford M. Glutamine directly downregulates glutamine synthetase protein levels in mouse C2C12 skeletal muscle myotubes. *J Nutr* 2007; **137**: 1357–1362.
41 Wang Y, Watford M. Glutamine, insulin and glucocorticoids regulate glutamine synthetase expression in C2C12 myotubes, Hep G2 hepatoma cells and 3T3 L1 adipocytes. *Biochim Biophys Acta* 2007; **1770**: 594–600.
42 He Y, Hakvoort TB, Köhler SE, Vermeulen JL, de Waart DR, de Theije C *et al.* Glutamine synthetase in muscle is required for glutamine production during fasting and extrahepatic ammonia detoxification. *J Biol Chem* 2010; **285**: 9516–9524.
43 Ganda OP, Ruderman NB. Muscle nitrogen metabolism in chronic hepatic insufficiency. *Metabolism* 1976; **25**: 427–435.
44 Girard G, Butterworth RF. Effect of portacaval anastomosis on glutamine synthetase activities in liver, brain, and skeletal muscle. *Dig Dis Sci* 1992; **37**: 1121–1126.
45 Desjardins P, Rao KV, Michalak A, Rose C, Butterworth RF. Effect of portacaval anastomosis on glutamine synthetase protein and gene expression in brain, liver and skeletal muscle. *Metab Brain Dis* 1999; **14**: 273–280.
46 Chatauret N, Desjardins P, Zwingmann C, Rose C, Rao KV, Butterworth RF. Direct molecular and spectroscopic evidence for increased ammonia removal capacity of skeletal muscle in acute liver failure. *J Hepatol* 2006; **44**: 1083–1088.
47 van Straaten HW, He Y, van Duist MM, Labruyère WT, Vermeulen JL, van Dijk PJ *et al.* Cellular concentrations of glutamine synthetase in murine organs. *Biochem Cell Biol* 2006; **84**: 215–231.
48 Rose C, Michalak A, Rao KV, Quack G, Kircheis G, Butterworth RF. L-ornithine-L-aspartate lowers plasma and cerebrospinal fluid ammonia and prevents brain edema in rats with acute liver failure. *Hepatology* 1999; **30**: 636–640.
49 Bosoi CR, Parent-Robitaille C, Anderson K, Tremblay M, Rose CF. AST-120 (spherical carbon adsorbent) lowers ammonia levels and attenuates brain edema in bile duct-ligated rats. *Hepatology* 2011; **53**: 1995–2002.
50 Oria M, Romero-Giménez J, Arranz JA, Riudor E, Raguer N, Córdoba J. Ornithine phenylacetate prevents disturbances of motor-evoked potentials induced by intestinal blood in rats with portacaval anastomosis. *J Hepatol* 2012; **56**: 109–114.
51 Odena G, Miquel M, Serafín A, Galan A, Morillas R, Planas R *et al.* Rifaximin, but not growth factor 1, reduces brain edema in cirrhotic rats. *World J Gastroenterol* 2012; **18**: 2084–2091.
52 Cunningham SC, Kok CY, Spinoulas A, Carpenter K H, Alexander IE. AAV-encoded OTC activity persisting to adulthood following delivery to newborn spf(ash) mice is insufficient to prevent shRNA-induced hyperammonaemia. *Gene Therapy* 2013; **20**: 1184–1187.
53 Lee EK, Hu C, Bhargava R, Ponnusamy R, Park H, Novicoff S *et al.* AAV-based gene therapy prevents neuropathology and results in normal cognitive development in the hyperargininemic mouse. *Gene Therapy* 2013; **20**: 785–796.
54 McGivan JD, Bungard CI. The transport of glutamine into mammalian cells. *Front Biosci* 2007; **12**: 874–882.
55 Urabe M, Ding C, Kotin RM. Insect cells as a factory to produce adeno-associated virus type 2 vectors. *Hum Gene Ther* 2002; **13**: 1935–1943.
56 Mena JA, Ramírez OT, Palomares LA. Titration of non-occluded baculovirus using a cell viability assay. *Biotechniques* 2003; **34**: 260–264.
57 Keil GM, Klopfleisch C, Giesow K, Blohm U. Novel vectors for simultaneous high-level dual protein expression in vertebrate and insect cells by recombinant baculoviruses. *J Virol Methods* 2009; **160**: 132–137.