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A novel biochemically salvageable animal model of hyperammonemia devoid of N-acetylglutamate synthase

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Abstract

All knockout mouse models of urea cycle disorders die in the neonatal period or shortly thereafter. Since N-acetylglutamate synthase (NAGS) deficiency in humans can be effectively treated with N-carbamyl-L-glutamate (NCG), we sought to develop a mouse model of this disorder that could be rescued by biochemical intervention, reared to adulthood, reproduce, and become a novel animal model for hyperammonemia. Founder NAGS knockout heterozygous mice were obtained from the trans-NIH Knock-Out Mouse Project. Genotyping of the mice was performed by PCR and confirmed by Western blotting of liver and intestine. NCG and L-citrulline (Cit) were used to rescue the NAGS knockout homozygous (*Nags*$^{-/-}$) pups and the rescued animals were characterized. We observed an 85% survival rate of *Nags*$^{-/-}$ mice when they were given intraperitoneal injections with NCG and Cit during the newborn period until weaning and supplemented subsequently with both compounds in their drinking water. This regimen has allowed for normal development, apparent health, and reproduction. Interruption of this rescue intervention resulted in the development of severe hyperammonemia and death within 48 hours. In addition to hyperammonemia, interruption of rescue supplementation was associated with elevated plasma glutamine, glutamate, and lysine, and reduced citrulline, arginine, ornithine and proline levels. We conclude that NAGS deprived mouse model has been developed which can be rescued by NCG and Cit and reared to reproduction and beyond. This biochemically salvageable mouse model recapitulates the clinical phenotype of proximal urea cycle disorders and can be used as a reliable model of induced hyperammonemia by manipulating the administration of the rescue compounds.

Keywords

hyperammonemia; urea cycle; N-acetylglutamate; N-acetylglutamate synthase; N-carbamylglutamate; mouse model

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The authors declared that they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Introduction

In mammals, nitrogen waste in the form of neurotoxic ammonia is converted in the liver to urea, which is then excreted in the urine[1]. Ureagenesis requires at least eight proteins and is initiated in the mitochondria via the activation of carbamyl phosphate synthetase 1 (CPS1) by N-acetyl-L-glutamate (NAG)[2]. NAG is produced by NAG synthase (NAGS) from L-glutamate and acetyl-CoA[3]. Inherited or acquired deficiency of NAGS catalytic activity causes a functional block in ureagenesis, due to the corresponding reduction or abolishment of CPS1 activity, leading to the accumulation of ammonia in the blood [4,5]. The clinical presentation and biochemical features of NAGS deficiency are indistinguishable from those of CPS1 deficiency and include elevated plasma glutamine, reduced citrulline, and normal levels of urinary orotic acid and orotidine [6].

Mouse models of complete enzyme deficiency and hyperammonemia have been created for each of the urea cycle enzymes with the exception of NAGS[6–13]. However, their usefulness for research has been rather limited due to neonatal lethality and difficulties in rescue by biochemical interventions for any significant length of time. Among these models, the one produced by Schofield et al. [7] for complete CPS1 deficiency (homozygous knockout) proved to be lethal within one day after birth. The availability of a mouse with a severe urea cycle defect that could be salvaged by a biochemical intervention would enhance research into various aspects of hyperammonemia and ureagenesis. We reasoned that if such a murine model could be reared to reproductive age, a large number of homozygous mice could be produced and studied. Furthermore, hyperammonemia could be induced and suppressed at will by manipulating the biochemical rescue allowing studies during, and off acute disease.

Unlike all other urea cycle disorders, NAGS deficiency in humans is amenable to effective treatment with N-carbamyl-L-glutamate (NCG) [14], a functional analog of NAG that has been shown to be resistant to *in vivo* degradation. There is good evidence that this molecule reaches the liver, enters the mitochondria and activates CPS1 *in vitro* and *in vivo* [2,15]. We therefore surmised, that if a NAGS knockout mouse could be created, the homozygous pups ($Nags^{-/-}$) could potentially be rescued by treatment with NCG allowing them to survive beyond the neonatal period and perhaps even to adulthood.

We document herein, the successful creation of the first salvageable mouse model of a urea cycle enzyme deficiency. We demonstrate that the $Nags^{-/-}$ mouse, which shows neonatal lethality similar to $Cps1^{-/-}$ mice, can be rescued by combinational therapy of NCG and L-citrulline (Cit), and consequently reach adulthood and reproduce to create litters of exclusive $Nags^{-/-}$ animals. We describe the procedure for rescue of $Nags^{-/-}$ mice, characterization of this new hyperammonemia mouse model, and show that the animal wellbeing depends on continued supplementation of NCG with the addition of Cit.

Materials and Methods

Generation of the NAGS knockout mouse

The trans-NIH Knock-Out Mouse Project (KOMP, www.komp.org), an NIH supported strain repository at the University of California Davis, was successful in transferring null alleles for *Nags* in two C57BL/6 embryonic stem cell lines and cryo-achieving germ line transfer embryos (C57BL/6N-$Nags^{tm1(KOMP)Vlcg}$). The strategy used for generating the ES cell clones with deletion of the entire gene is illustrated in Fig. 1A. Homologous recombination allows the entire *Nags* gene to be replaced with the ZEN-UB1 cassette within the bacterial vector, containing the coding sequences for lacZ and neomycin genes. NIH grants to Velocigene at Regeneron Inc. (U01HG004085) and the CSD Consortium

(U01HG004080) funded the generation of gene-targeted ES cells for 8500 genes in the KOMP Program which are archived and distributed by the KOMP Repository at UC Davis and CHORI (U42RR024244)[16]. The KOMP Repository provided us four C57BL/6N-Nags$^{tm1(KOMP)Vlcg}$ heterozygous mice from the cryo-archive.

These mice (*Nags*$^{+/-}$) were crossed with wild type Swiss Webster (Charles River Laboratories). The resulting first generation of heterozygous was crossed to obtain homozygous mice (*Nags*$^{-/-}$). Of these homozygous animals, those that were reared to reproductive age were crossed to obtain the homozygous mice used for the described experiments.

Animal care and husbandry

Mice were cared for in accordance with the guidelines set forth by the American Association for Accreditation of Laboratory Animal Care and all the studies were approved and supervised by the Children's National Medical Center Institutional Animal Care and Use Committee (protocol number 247-09-11). Mice were cared and bred in the Research Animal Facility (barrier facility) under controlled temperature (68°F) and a 12 hours light/dark period. They were fed Harlan Tecklad irradiated chow (2018) and sterilized water.

Genotyping and identification

Newborn pups were identified using tattoo patterns (Ketchum Manufacturing Inc.). Genomic DNA was isolated from tail biopsies with the Puregene Core Kit A (Qiagen). Fig. 1B illustrates the strategy used to genotype the pups using the PromegaGoTaq system. The strategy involves a three-primer PCR assay. Primer A (5'-ACTGTCAGAGAAAAGCGCTCAGGA-3') corresponds to a region in the 5' end of the *Nags* gene. Primer B (5'-ACATACACTTCATTCTCAGTATTGTTTTGCC-3') corresponds to a region within the neomycin selection cassette in the knockout allele. Primer C (5'-CTGTTTTTCAGACACATCAGATCCCG-3') is a "common" primer corresponding to a region downstream of the *Nags* gene. The product of Primer A and Primer C is a 1707 bp amplicon, which corresponds to the wild type allele. The combination of Primer B and Primer C produces a 1082 bp amplicon and corresponds to the knockout allele. Following one cycle of denaturation at 95 °C for 5 min, a total of 33 cycles were performed each consisting of 95 °C for 15 sec, 60 °C for 20 sec, and 72 °C for 1 minute. Both alleles were separated by electrophoresis in a 1% agarose gel, and a depiction of this gel with patterns for wild type, heterozygous, and knockout alleles is show in Fig. 1C, with a 1 Kb+ ladder as a reference (Invitrogen). Wild type and heterozygote control DNA were derived from tissue collected from original stock mice with known genotype.

Western blots

Frozen mouse liver was pulverized in liquid nitrogen. Small intestine epithelial cells were isolated according to Evans et al.[17]. Cells were lysed in RIPA buffer (Sigma) containing PhosSTOP protease inhibitor tablets (Roche). Protein concentrations were quantified using Protein Assay dye reagent (Bio-Rad) according to manufacturer's instructions. Proteins in cell lysate were resolved on 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and blocked in Starting Block (Thermo Scientific) with 0.5% Surfact-Amps 20 (Thermo Scientific). Ten micrograms of total liver protein and 60 μg of small intestinal epithelial protein were used to probe with NAGS antibody (1:5000 dilution of primary rabbit anti-NAGS antibody raised against recombinant mouse NAGS) and HRPT-conjugated donkey anti-rabbit secondary antibody (1:50,000) (Pierce). CPS1 was probed in 1 μg of total liver protein and 10 μg small intestinal epithelial protein using rabbit anti-CPS1 primary antibody (AbCam 1:5000) and HPRT-conjugated donkey anti-rabbit secondary antibody (Pierce 1:10,000). OTC was probed in 1 μg of total liver protein and 10 μg small intestinal

epithelial protein using rabbit primary antibody raised against recombinant ornithine transcarbamylase (OTC) (1:5000) and HPRT-conjugated donkey anti-rabbit secondary antibody (Pierce 1:10,000). Protein bands were visualized using Super Signal West Pico kit (Pierce). Tubulin, probed with the anti-tubulin primary antibody (Abcam) at 1:200 dilution followed by goat-anti-rabbit (1:5,000) (Abcam), was used as a loading control. The density of NAGS, CPS1, OTC, and tubulin bands were quantified using a GS-800 calibrated densitometer (Bio-rad) and analyzed with Quantity One software, version 4.5.2 (Bio-rad). NAGS, CPS1, and OTC bands were normalized against tubulin density and then expressed as a percentage of Wild type control.

Chemical Rescue of *Nags*$^{-/-}$ mice

Heterozygote breeding pairs were given water supplemented with NCG (1.0 g/L) (Orphan Europe, Paris) and L-citrulline (1.0 g/L) (VWR). *Nags*$^{-/-}$ pups were given daily intraperitoneal injections of NCG and citrulline dissolved in Lactated Ringer's solution starting at birth and continuing until weaning. The initial dose was 250 mg/kg/day for NCG and 1000 mg/kg/day for Cit, and as the animals grew, the dose was tapered to approximately 130 mg/kg/day NCG and 525 mg/kg/day Cit. After weaning (typically 21 days), *Nags*$^{-/-}$ mice were maintained on water supplemented with NCG and Cit (1.0 g/l each).

Plasma ammonia and amino acid measurements

Plasma was collected into a pre-chilled heparinized syringe from anesthetized mice via heart puncture. The sample was transferred immediately to a pre-chilled heparinized tube and centrifuged at 4°C for 10 min to separate the plasma. The plasma was transferred to cryogenic tubes and immediately frozen in dry ice and stored at −80°C until analysis within 48 hr. Plasma ammonia measurements were performed on a Dade Behring Dimension RXL clinical chemistry system (Dade Behring, Newark, DE), using an adaptation of the glutamate dehydrogenase enzymatic method of van Anken and Shiphorst [18] which substitutes NADPH for NADH, eliminating interference from other NADH-consuming reactions. The reaction was carried out in a Dimension® Ammonia (AMON) Flex reagent cartridge (K863840; Dade Behring, Newark, DE). The disappearance of NADPH was measured by spectrophotometry.

Amino acid concentrations in plasma were measured by ion-exchange chromatography on a Biochrom amino acid analyzer. Plasma proteins were first precipitated with an equal volume of 7% sulfosalicylic acid and centrifuged for 10 minutes at 13200 rpm. 5 μL of 2.5 N LiOH and 10 μL stock internal standard (S-2-aminoethyl-L-cysteine hydrochloride) were then added to 100 μL of plasma supernatant before injection into the analyzer.

Statistical Analysis

All statistical analyses were performed using Prism software, version 5 (GraphPad, San Diego, CA). The minimal level of confidence at which experimental results were considered significant was $p<0.05$. Statistical significance of differences in weight gain among the strains and between their amino acid levels was determined by a two-way ANOVA with Bonferroni posttest analysis. Statistical significance of differences in ammonia concentrations was determined by one-way ANOVA with Bonferroni posttest analysis. Analysis of genotype segregation from heterozygous matings was performed using a Chi-squared test.

Results

Breeding of mice

C57Bl/6N mice, heterozygous for the disrupted *Nags* allele (*Nags*$^{+/-}$) were crossed to obtain homozygote animals. After several litters, we observed that the overall survival rate of the pups was only 45% in these crossings, as mothers were not taking care of their pups. We also observed an increased incidence of birth defects due, possibly, to the inbreeding of the strain used to develop these mice. Therefore, *Nags*$^{+/-}$C57BL/6N mice were subsequently crossed with wild-type mice of the Swiss-Webster strain to increase the genetic heterogeneity. SW;B6-Nags$^{tm1(KOMP)Vlcg}$ heterozygous females were able to produce larger litters and maintain them, with an overall survival rate of 87%, thus markedly reducing confounding factors with respect to survival and health of the pups. No birth defects were observed using SW;B6-Nags$^{tm1(KOMP)Vlcg}$ heterozygous mice for crossing. Pups were genotyped within 48 hours of birth by PCR (Fig. 1B and 1C). Analysis of 11 liters (127 pups) from heterozygote crosses revealed that the genotypes of offspring were consistent with the expected Mendelian inheritance (1:2:1) (X^2= 0.890, df=2, p=0.6409) indicating improbable *in utero* lethality of *Nags*$^{-/-}$ mice when the mothers are maintained on NCG and Cit supplementation in the drinking water (Table 1).

Analysis of the NAGS protein in the liver and small intestine was performed using Western blotting. The analysis confirmed the genotypes, showing absence of NAGS in these organs in *Nags*$^{-/-}$ mice. The abundance of NAGS protein in the liver and small intestine of *Nags*$^{+/-}$ mice ranged between 50–70% and 15–25% respectively, of *Nags*$^{-/-}$ littermates (Fig. 2). This confirms that *Nags* disruption abolished its expression in both the liver and small intestine. Expression of remaining mitochondrial urea cycle enzymes, CPS1 and OTC, in both the liver and small intestine were similar in *Nags*$^{-/-}$, *Nags*$^{+/-}$ and *Nags*$^{+/+}$ littermates (Fig. 3).

Biochemical rescue of *Nags*$^{-/-}$ mice

Heterozygote breeding pairs were supplemented with NCG (1.0 g/l) and Cit (1.0 g/l) in the drinking water. This likely ensured that *Nags*$^{-/-}$ fetuses received NCG and Cit during *in utero* development. However, supplementation of heterozygous pregnant females with NCG and cit was proven to be insufficient to rescue the newborn *Nags*$^{-/-}$ pups and they died within 24–48 hours of birth if no other intervention was enacted (Fig. 4A). This suggested that insufficient amount of NCG and/or Cit was transferred to the pups via the mother's milk to mitigate the enzyme deficiency and resulting hyperammonemia. To improve delivery of the rescue chemicals, homozygous pups were administered intra-peritoneal injections of NCG and Cit dissolved in Lactated Ringer's solution within a few hours following birth and continuing once every day until weaning. The initial dose was 250 mg/kg/day NCG and 1000 mg/kg/day Cit, and as the animals grew, the dose was tapered to approximately 130 mg/kg/day NCG and 525 mg/kg/day Cit. The dosage for mice was determined based on dose per weight recommendation for NAGS deficient patients[19]. The addition of Cit to the treatment regimen enhanced survival of the mice. Without Cit, approximately 50% of the *Nags*$^{-/-}$ pups die before weaning, while the NCG plus Cit supplement increases survival to 85% (Fig. 4A). After weaning (typically 21 days), homozygous pups were switched to supplementation with NCG plus Cit in the drinking water, which has proved sufficient for survival to adulthood and reproduction.

In order to investigate the dependency on NCG plus Cit in the adult period, 6-week-old male and female mice were given water with various combinations of supplements. Mice on plain water died within 24 hours, and mice drinking water with only Cit supplementation died within 24–48 hours (Fig. 4B). Mice given water with NCG only or NCG/Cit were monitored

for survival over a 30-day period. At the end of 14 days, 50% of the mice on NCG alone were alive, whereas 89% of the mice on NCG/Cit supplementation were alive (Fig. 4B). These results indicates that while Cit is not absolutely required in the adult period, it does play a beneficial role in overall health and survival of the *Nags*$^{-/-}$ mice. Based on the concentration of NCG and Cit that was provided, a 25 g mouse would consume approximately 150 mg/kg/day of NCG and Cit. This dosage is within the range of the standard dosage recommended for patients.

Appearance of Nags$^{-/-}$ mice

At birth, *Nags*$^{-/-}$ mice are indistinguishable from their heterozygous and wild type littermates. As they grow older, a subtle phenotype arises. *Nags*$^{-/-}$ mice appear to have less fur compared to their *Nags*$^{+/-}$ littermates (Fig. 5A and 5B). However, this phenotype is not present in every homozygote, and is more obvious in the mice with white fur. Some homozygotes may develop a full coat of fur as they get older. As a result, distinguishing homozygotes by fur pattern is not reliable. Fur abnormalities are also seen in the two partial OTC deficiency mouse models, OTC^{spf} and OTC$^{spf\text{-}ash}$ [8,20] suggesting perhaps that this phenotype is related to Cit or arginine deficiency as proposed previously [21]. In order to determine if *Nags*$^{-/-}$ mice have growth defects, litters were weighed daily until weaning. Overall, the weight of rescued *Nags*$^{-/-}$ mice is lower during the pre-weaning period compared to their *Nags*$^{+/-}$ and *Nags*$^{+/+}$ littermates and this difference is statistically significant (Fig. 5C) ($p<0.01$).

Metabolic phenotype of *Nags*$^{-/-}$ mice

NCG and Cit were withdrawn from 2-month-old *Nags*$^{-/-}$ mice in an evening prior to the dark period. Behavior related to hyperammonemia was assessed according to the scoring system outlined in Ye, X et al [22]. Mice were sacrificed when they showed symptoms of severe hyperammonemia (lethargy, seizures, lying on side, decerebrate posture). All *Nags*$^{-/-}$ mice deprived of NCG and Cit supplements developed signs of severe hyperammonemia, however time of onset of symptoms was variable ranging from 10 to 28 hours. There was no significant difference in plasma ammonia levels between *Nags*$^{+/+}$, *Nags*$^{+/-}$, and *Nags*$^{-/-}$ littermates while on the NCG and NCG/cit treatment. However, *Nags*$^{-/-}$ mice deprived of NCG/Cit supplements displayed plasma ammonia levels in the range of 1000–3000 μmol/L (approximately 10-fold higher than their littermates) ($p<0.0001$), confirming that the development of severe hyperammonemia following treatment withdrawal is a characteristic of these mice and can be measured using methods similar to human subjects (Fig. 6A).

Plasma was also analyzed to determine the effect of NCG plus Cit supplementation on amino acid levels. *Nags*$^{+/+}$, *Nags*$^{+/-}$, and *Nags*$^{-/-}$ on NCG/Cit supplementation have similar plasma amino acid levels except for an increased glutamate level in the *Nags*$^{-/-}$ ($p<0.0001$) (Fig. 6B). Hyperammonemic (NCG/Cit deprived) *Nags*$^{-/-}$ mice exhibit decreased levels of plasma arginine, citrulline, ornithine, proline (Fig. 6B) and alanine ($p<0.0001$) (Fig. 6D), and increased levels of glutamine ($p <0.0001$), glutamate (Fig. 6B), and lysine ($p <0.0001$) (Fig. 6D). All other amino acids (Fig. 6D), including branched chain amino acids (Fig. 6C), remained unchanged.

Discussion

Using gene replacement techniques available through KOMP, we have produced a knockout mouse model of hyperammonemia due to complete NAGS deficiency. Genotypic analysis of the progeny from matings between *Nags*$^{+/-}$ animals kept on NCG/Cit supplementation shows Mendelian segregation of the *Nags* null allele thus excluding increased embryonic lethality in this model. Therefore, a potential role of NAGS in fetal development [19] seems

unlikely to explain the extreme rarity of the NAGS deficiency in humans. *Nags*$^{-/-}$ mice lack NAGS protein in both the liver and small intestine (Fig. 2). Thus, when this gene is absent, ureagenesis and *de novo* citrulline and arginine production do not occur resulting in neonatal death of *Nags*$^{-/-}$ pups if they are not supplemented with NCG and Cit (Fig. 4A). Fortunately, *Nags*$^{-/-}$ mice can be rescued with a combination of NCG and Cit, and we have obtained an 85% success rate in rearing these pups to weaning and subsequently to reproductive age. Since we did not lose *Nags*$^{+/+}$ and *Nags*$^{+/-}$ pups that were injected like the *Nags*$^{-/-}$ pups, we believe that the 15% loss in the neonatal period has probably been caused by NAGS deficiency in spite of rescue therapy. It is possible, that the stress of daily intra-peritoneal injections could have contributed to this loss. A portion of this 15% loss includes smaller newborn mice that died within 10–15 days after birth. We have not tested whether larger dosage of NCG and citrulline could mitigate this early lethality in all or a portion of these mice.

The requirement for NCG/Cit continues into adulthood, since *Nags*$^{-/-}$ mice become hyperammonemic within hours following withdrawal of the rescue chemicals from water. If not treated with NCG/Cit, the *Nags*$^{-/-}$ mice experience a 10 fold increase in ammonia levels within a day or two (Fig. 6A), similar to the phenotype reported in other mouse models with complete deficiency of urea cycle genes which cannot be salvaged [7,11,12,23,24], as well as the ornithine aminotransferase (OAT) knockout mouse when not supplemented with arginine in the neonatal period [25]. Although the addition of Cit does not seem to be absolutely essential for survival of the *Nags*$^{-/-}$ mice, it appears to play a beneficial role as 50% of adult *Nags*$^{-/-}$ mice treated only with NCG in drinking water die within 14 days of being on this supplement alone (Fig. 4B), compared to only 11% if treated with NCG plus Cit. Among the tests that were performed, the only significant difference found between *Nags*$^{-/-}$ mice on NCG vs. NCG/Cit is an elevated plasma glutamine level in mice supplemented with NCG only (Fig. 6B), suggesting increased nitrogen load in these animals [26,27]However, these mice did not display reduced plasma arginine or Cit levels, nor did they manifest chronic hyperammonemia (Fig. 6). Although arginine is only a semi-essential amino acid, its catabolism results in biosynthesis of creatine, ornithine, glutamine, proline, and polyamines [28]. Arginine also plays a role in signaling function, being a substrate for nitric oxide production [28] and in wound healing [29,30] and thus supplementation is recommended after physical stress such as post-surgery or trauma [31,32].

In humans, NCG is the standard treatment for NAGS deficient patients [33–35]. We chose a regimen of NCG and Cit based on a study by Kim et al. [15] that showed that an intraperitoneal injection of NCG and arginine protected rats against lethal doses of ammonia. We chose the dosage plan based on information on patient dosage [19]. Additionally, in a mouse model of OAT deficiency, arginine injections every 12 hours over the newborn period were necessary to compensate for a neonatal arginine deficiency [25]. We believed that Cit would be more efficient in this regard since arginine is rapidly degraded in the liver as a result of a high arginase activity [28] while citrulline would bypass the liver and be converted to arginine by the kidney [36].

Additionally, we have evidence suggesting that adult mice exhibiting early signs of hyperammonemia can be rescued with an intra-peritoneal injection of NCG/Cit (data not shown). This observation suggests that their hyperammonemia could be reversed upon early intervention, which should allow studying recovery of the brain from hyperammonemia under controlled conditions. The *Nags*$^{-/-}$ mouse represents the first mouse model in which hyperammonemia can be prevented with a chemical intervention and induced with the removal of said chemical. The creation of this mouse will allow the study of global effects of hyperammonemia, especially on the central nervous system. It should also prove useful as

a model for the study of liver gene therapy in complete urea cycle disorders, which would be challenging in animals with complete enzyme deficiency that die in the neonatal period.

Highlights

A mouse model of a urea cycle disorder (NAGS deficiency) that can be rescued by biochemical intervention, reared to adulthood and reproduce has been developed and characterized.

This mouse recapitulates a severe proximal urea cycle disorder that can be induced at will by a simple manipulation.

This mouse is a “clean” and reproducible model of hyperammonemia and can be used for studies on the effect of hyperammonemia on the brain, gene therapy interventions and other metabolic and biological investigations of nitrogen metabolism.

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Abbreviations

CPS1	Carbamyl phosphate synthetase 1
NAG	N-acetyl-L-glutamate
NAGS	N-acetylglutamate synthase
NCG	N-carbamyl-L-glutamate
Cit	citrulline
KOMP	Knock-out Mouse Project
OTC	ornithine transcarbamylase

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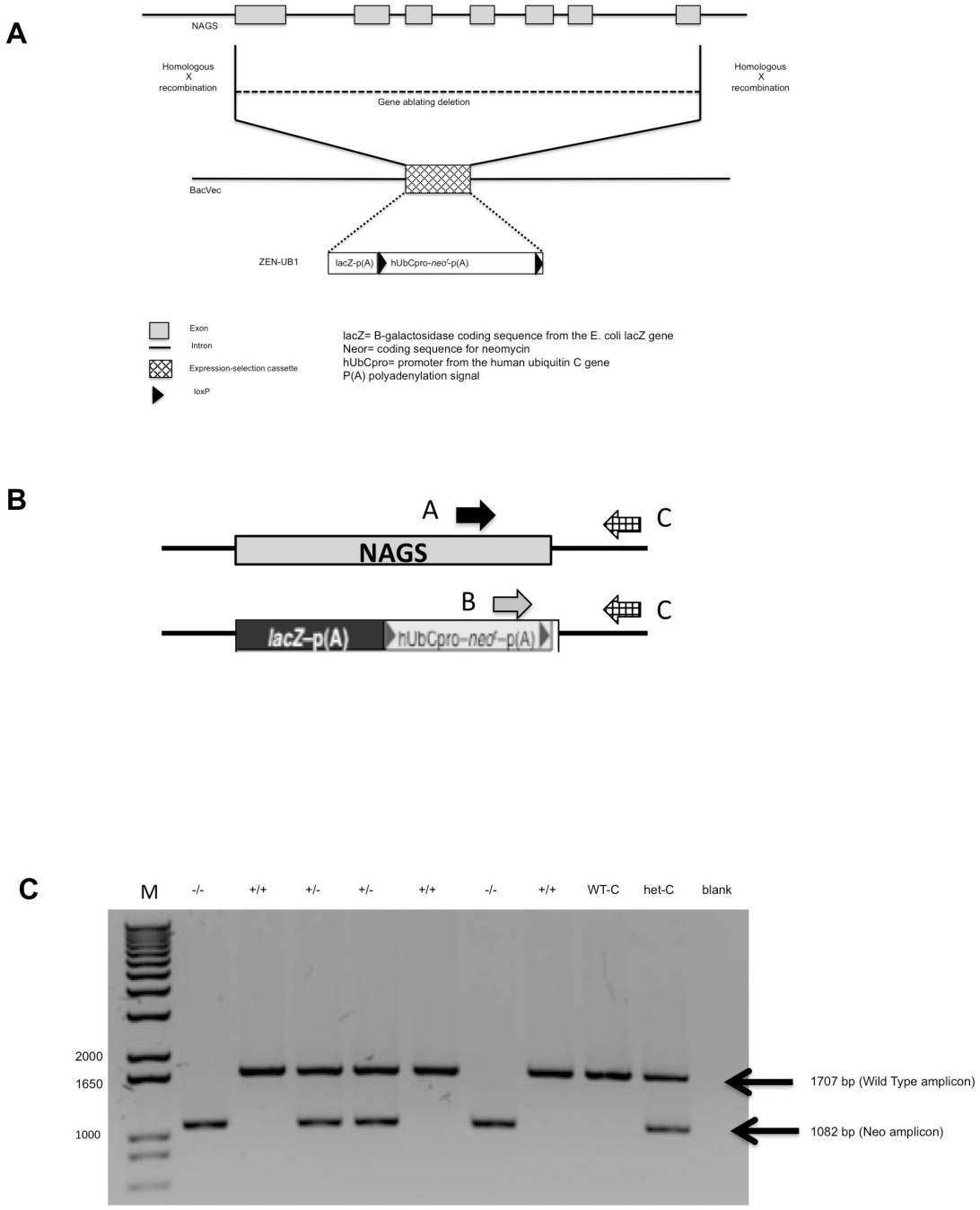

Figure 1. Strategy for deletion of the *Nags* gene and for genotyping

(A) Diagram of the *Nags* gene (top) and the large BAC-based targeting vector (bottom). (B) The combination of primers A and B and A and C amplifies a 1707 bp fragment from the normal *Nags* allele and a 1082 bp fragment from the targeted *Nags* allele respectively. (C) PCR products that indicate the animals' genotype. Genotype of each sample is shown at the top of each lane (WT-C: wild type control; het-C: heterozygote control sample; M: marker)

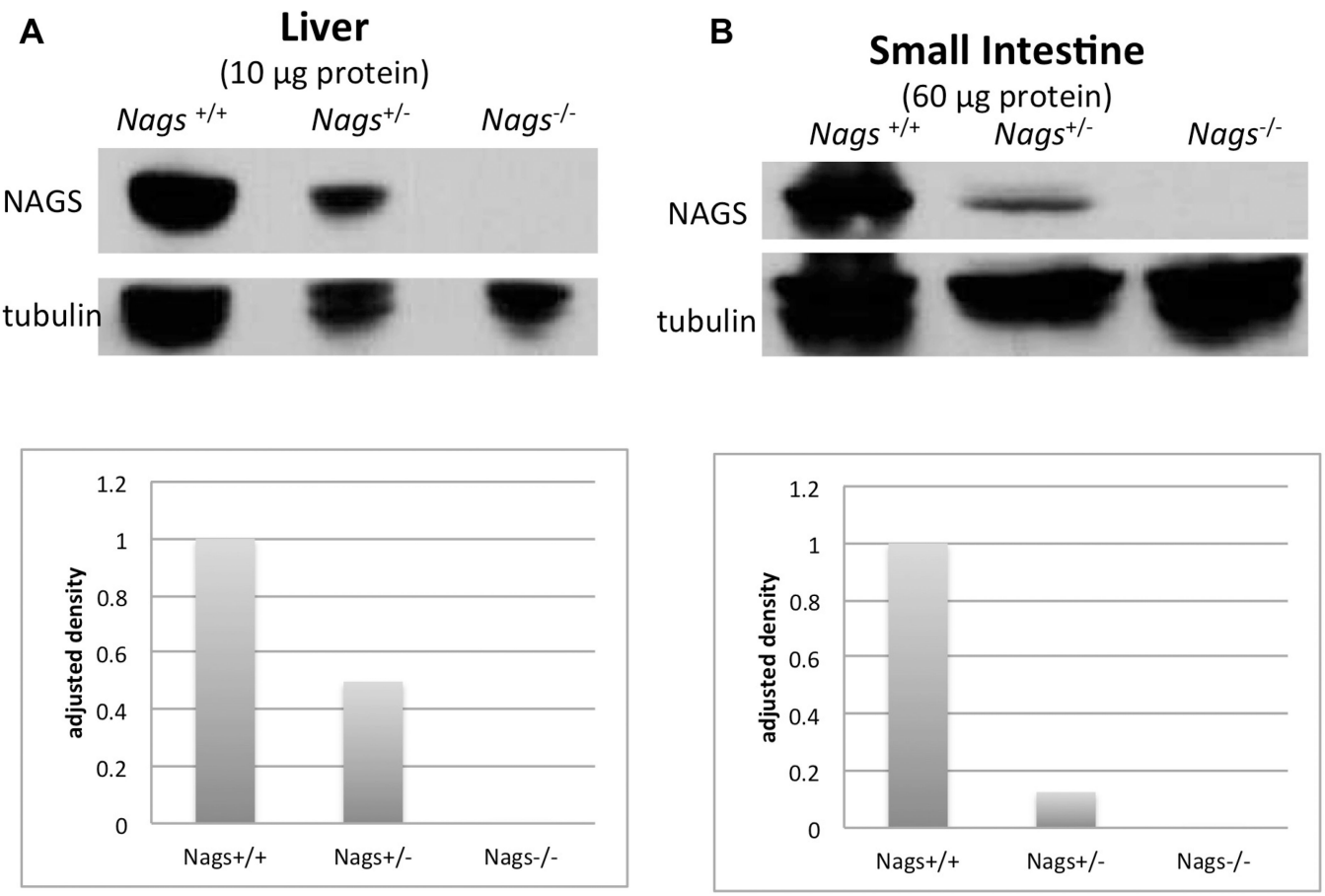

Figure 2. Western blots of NAGS from liver (A) and small intestine (B)

10 µg of liver protein and 60 µg of small intestine protein were loaded in each lane to detect expression of NAGS protein in ***Nags***$^{+/+}$ **,** ***Nags***$^{+/-}$**, and** ***Nags***$^{-/-}$ mice. Densitometry analyses of the blots are shown. Data were normalized to the tubulin protein and then expressed as a fraction of the respective protein in the wild type (*Nags*$^{+/+}$) mice.

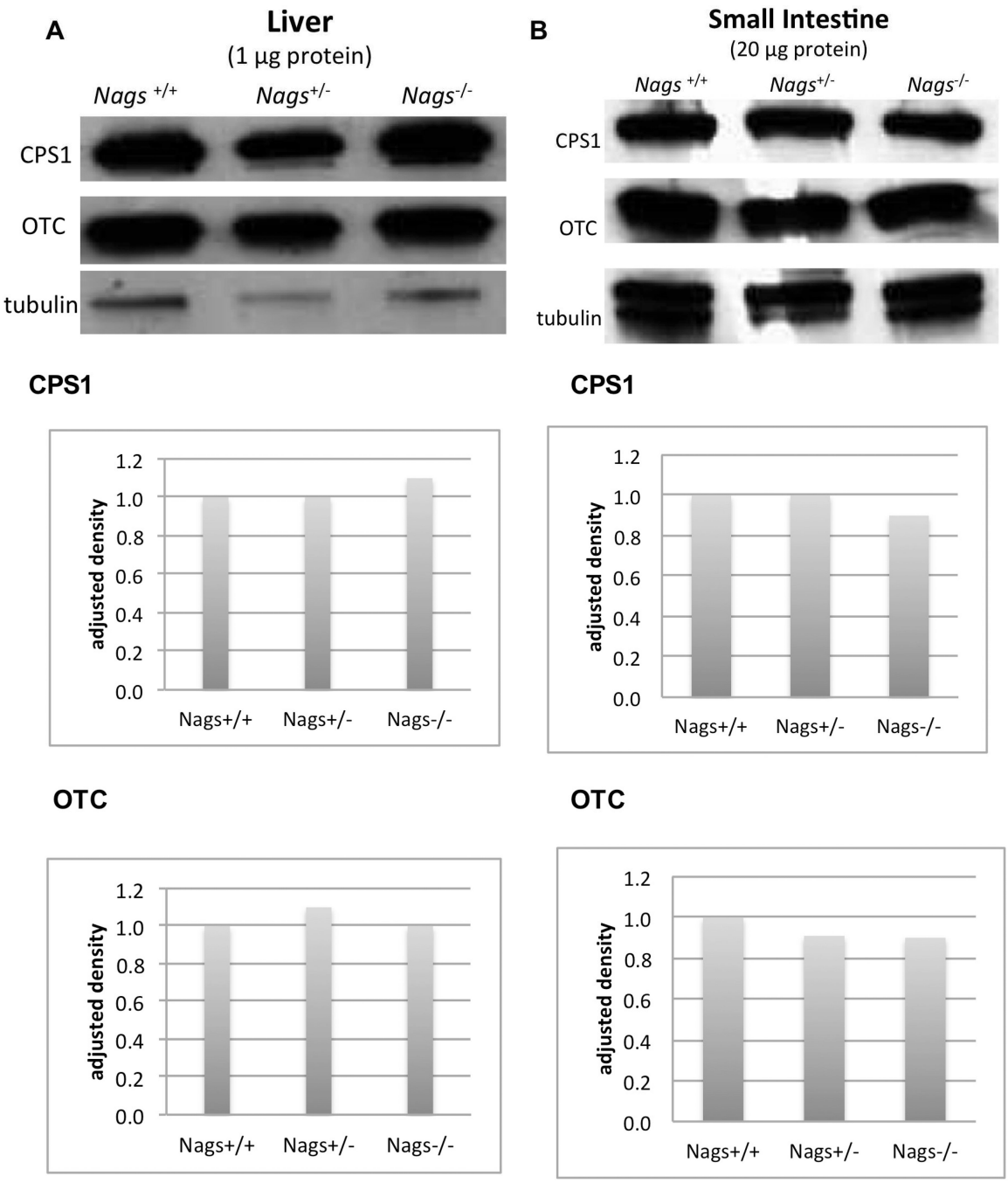

Figure 3. Western blots of CPS1 and OTC in liver (A) and small intestine (B)

1 µg of liver protein and (B) 20 µg of small intestine protein were loaded in each lane to detect expression of CPS1 and OTC proteins in *Nags* $^{+/+}$, *Nags*$^{+/-}$, and *Nags*$^{-/-}$ mice. Corresponding densitometry analyses of the blots are shown. Data were normalized to the tubulin protein and then expressed as a fraction of the respective protein in the wild type (*Nags*$^{+/+}$) mice.

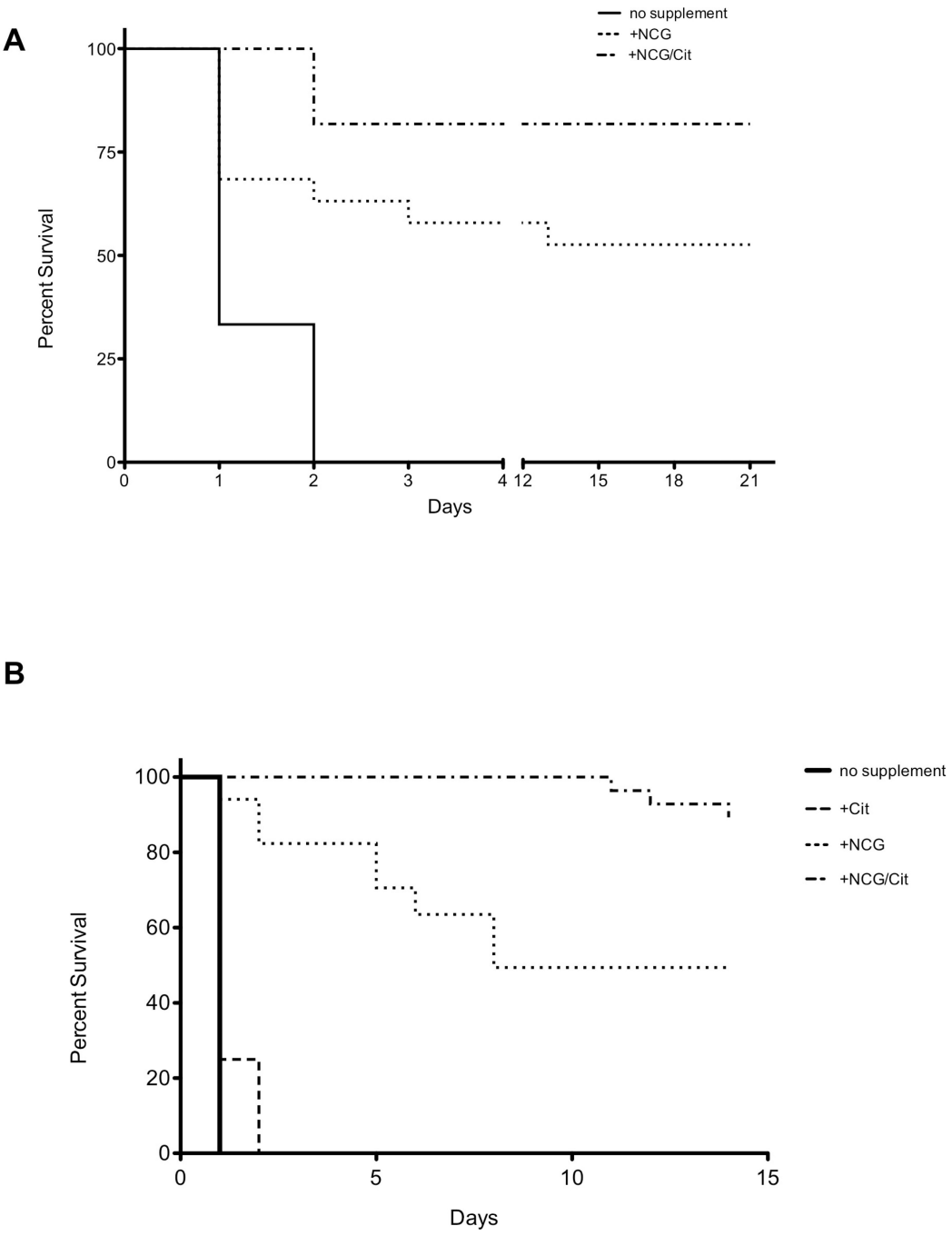

Figure 4. Biochemical rescue of *Nags*$^{-/-}$ mice

(A) Kaplan-Meier survival curves of pups on and off NCG ± Cit treatment. *Nags*$^{-/-}$ pups were given either no supplementation or intra-peritoneal injections of NCG or NCG/cit daily starting at birth and assessed for survival. (B) Kaplan-Meier survival curves of six-week-old mice were given different supplements in water (NCG, Cit, or NCG/Cit).

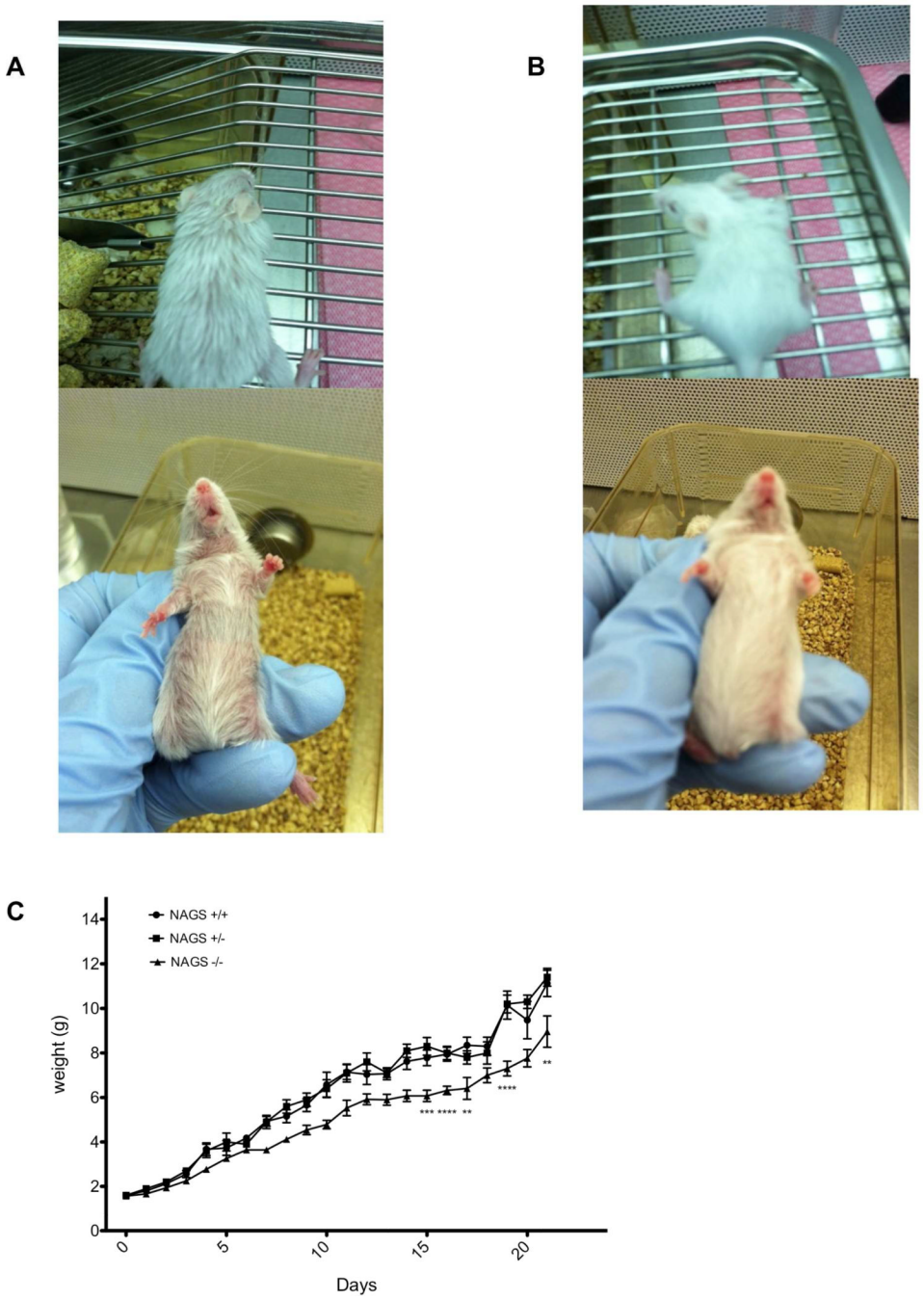

Figure 5. Phenotype and growth of *Nags*$^{-/-}$ mice

Outward phenotype of 6-week-old *Nags*$^{-/-}$ (A) and its *Nags*$^{+/-}$ (B) littermate. (C) *Nags*$^{+/+}$, *Nags*$^{+/-}$, and *Nags*$^{-/-}$ mice were weighed daily during the pre-weaning period. Weight differences were analyzed using a two-way ANOVA followed by a Bonferroni post-test.

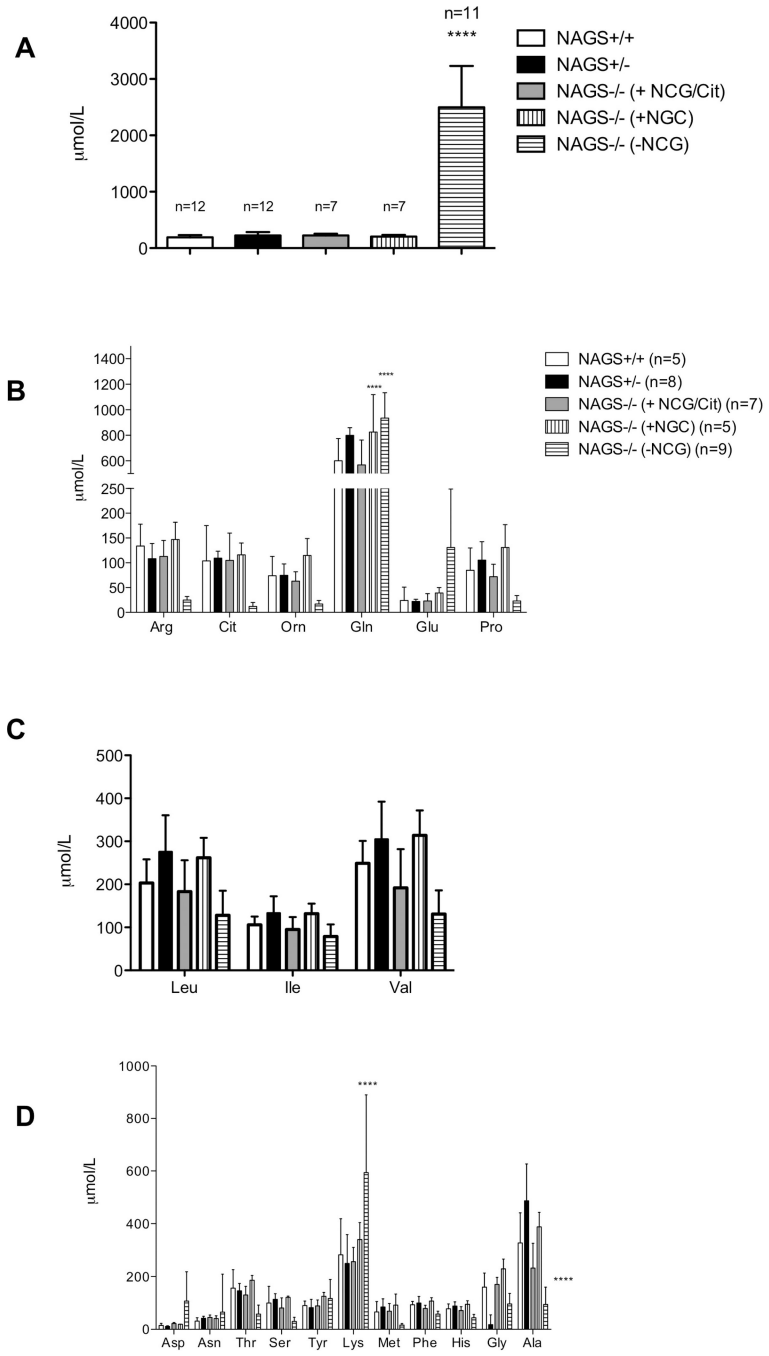

Figure 6. Metabolic phenotype of *Nags*$^{-/-}$ mice

(A) Plasma ammonia levels in *Nags* mice were measured following withdrawal of NCG and citrulline from *Nags*$^{-/-}$ mice. When mice showed signs of hyperammonemia they were sacrificed along with *Nags*$^{+/+}$, *Nags*$^{+/-}$, and *Nags*$^{-/-}$ controls. Data were analyzed using a one-way ANOVA with Bonferroni post-test, ($p<0.0001$). Plasma concentrations of (B) urea cycle related amino acids, (C) branched chain amino acids, (D) other amino acids. Graphs show mean and SD. Statistical significance was analyzed using a two-way ANOVA followed with Bonferroni post-test ($p<0.0001$), comparing to wild type mice.

Table 1

Distribution of genotypes among 127 progeny of *Nags*+/− matings

Progeny from 11 heterozygous matings were analyzed using a Chi-squared test to determine if *Nags* alleles show Mendelian segregation.

Genotype	Observed	Expected	P Value
$NAGS^{+/+}$	32	31.75	0.6409
$NAGS^{+/-}$	59	63.50	
$NAGS^{-/-}$	36	31.75	
Total	127	127	