AAV-based gene therapy prevents neuropathology and results in normal cognitive development in the hyperargininemic mouse

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INTRODUCTION
Arginase deficiency is a rare autosomal recessive metabolic disorder, resulting from a loss of arginase 1 (ARG1), the final enzyme in the urea cycle, which is the major pathway for the detoxification of ammonia in mammals. ARG1 is expressed most prevalently in hepatocytes, where in coordination with the other enzymes of the urea cycle, sequestration of nitrogen as urea occurs. ARG1 hydrolyzes arginine into ornithine, which can then re-enter the urea cycle; urea is then excreted as waste. Arginase deficiency is the least severe of the urea cycle disorders and results in hyperargininemia.

Neonatal and early infantile presentation of ARG1 deficiency with severe hyperammonemia does occur but is rare; ARG1 deficiency usually presents later in life, beginning in late infancy to the second year of life with microcephaly, spasticity, seizures, clonus, loss of ambulation, progressive mental impairment, growth retardation, periodic episodes of hyperammonemia and failure to thrive associated with hyperargininemia. Patients often manifest spastic diplegia; while the exact mechanism is not completely defined, it appears that the origin of this abnormality is in upper motor neuron function impacting the motor cortex, the basal ganglia and the corticospinal tract. Affected nerves (here, the ones controlling the lower extremities) perpetually activate their corresponding muscles, resulting in rigidity and hypertonicity that may be indistinguishable from cerebral palsy. Although the exact cause is not known, the neurological manifestations seen in arginase deficiency may arise from the accumulation of arginase or its metabolites, or an increase in guanidino compounds (GCs; putative neurotoxins) present from hyperargininemia. ARG1-deficient patients usually avoid the catastrophic hyperammonemic crises characteristic of the other urea cycle disorders and tend to survive much longer. Currently long-term therapy rests on provision of a low-protein diet and administration of sodium benzoate and sodium phenylbutyrate. Although these onerous dietary and pharmaceutical interventions can partially alleviate ARG1 deficiency, there is no completely effective therapy available today.

Arg1-deficient mice were previously generated in our laboratory by replacing exon 4, the active site of the Arg1 gene with the neomycin resistance gene. No Arg1 RNA was found on northern blot nor was any cross-reacting material detected. These mice completely lack hepatic ARG1 activity. In contrast to the human disease in which patients can survive into adulthood, National Institutes of Health (NIH)-Swiss mice with Arg1 deficiency typically die between postnatal days 14 and 21 (average day 17) with severe hyperammonemia. We have previously described the

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neurodevelopmental phenotype of the untreated Arg1<sup>+/−</sup> mice. These mice demonstrate microcephaly, spasticity, loss of ambulation, seizure-like activity and failure to thrive followed by death. Plasma ammonia levels of Arg1<sup>+/−</sup> mice in metabolic crisis are increased greater than 10-fold and their livers are abnormal with histopathological features similar to those seen in human arginase-deficient patients who died with hyperammonemia later in life. We have developed a serotype rh10 adeno-associated virus (AAV)-mediated gene transfer approach, where 3 × 10<sup>13</sup> viral particles per kg are administered intravenously on the second day of life to correct the biochemical defect in a neonatal murine model of ARG1 deficiency. The vector contains the ubiquitously-expressing chicken β-actin promoter/cytomegalovirus (CMV) enhancer; however, it is unclear if functional protein can be produced in extrahepatic tissues. We have previously described using this vector for reporter gene studies and long-term factor VIII expression using a similar neonatal approach in the murine model of hemophilia A. The treated Arg1-deficient mice have survival comparable to littermate controls, and while being slightly smaller and demonstrating less visceral and subcutaneous fat than littermates, it otherwise appear grossly phenotypically indistinguishable. Enzymatic activity is sustained as demonstrated by an examination of plasma ammonia levels and amino-acid profiles: plasma ammonia levels are ~2.5 times that of littermate controls and arginine levels are normal in these mice.

A detailed examination of the neurological system and cognitive abilities of these mice has not been previously performed. The goal of the studies described herein was to (1) systematically examine the brain and cognitive development of AAV-mArg1<sup>+/−</sup>-treated animals, and determine if subtle findings suggestive of neurological abnormalities may be present; (2) examine brain amino acids and other compounds (that is, GCs) that have been hypothesized to be important in the cause of the neurological dysfunction in hyperargininemia, and examine for level of correction with AAV-based gene therapy and (3) assess the durability of the gene-based therapy of these animals to an exogenous ammonium challenge.

RESULTS
Gene therapy results in normal brain development
Although Arg1<sup>+/−</sup> mice are indistinguishable in phenotype from littermate controls at birth, the onset of central nervous system (CNS) dysfunction is heralded initially by gait instability that leads to ataxia, lethargy, seizure-like activity and death over the subsequent 24 h. With an effective therapy, the treated animals survive; however, it is unclear if the anatomic and functional brain developments are normal, if there are histopathological abnormalities, or if there is overt or subtle behavioral dysfunction. To address these questions, treated Arg1<sup>+/−</sup> mice and littermate controls were observed and weighed for 1 year (n = 13 AAV-mArg1-treated Arg1<sup>+/−</sup> mice, n = 20 littermate controls) (Figure 1). At 4 months (n = 3), groups were euthanized and brains were examined grossly and histologically and computed axial tomography was performed of the brain and skull (Figure 2). And at 1 year of age (n = 8), brains were examined grossly and histologically (Figure 3). As the animals were followed over 1 year, they did demonstrate overall similar length, AAV-treated Arg1<sup>+/−</sup> mice remain lean, whereas littermate controls develop obesity (Figure 1). The olfactory bulbs, cerebral cortex, basal ganglia, hippocampus, thalamus, midbrain, cerebellum, pons and medulla were examined with comparable regions from littermate control section. The brains of littermate control (Figure 2a–d) and treated (Figure 2e–h) Arg1<sup>+/−</sup> mice at 4 months and littermate control (Figure 3a–c) and treated mice (Figure 3d–f) at 1 year were found to be similar and there were no lesions in any of these main regions. Computed axial tomography (CT) imaging of the cranial vault at 4 months (Figure 2i–l) demonstrated similar size and shape compared with littermate controls with no evidence of microcephaly. Average skull width (± s.d.) of heterozygote littermates was 10.0 ± 0.3 vs 9.6 ± 0.5 mm for treated Arg1<sup>+/−</sup> mice (n = 3 per group, P = 0.33). Average skull length (± s.d.) of heterozygote littermates was 21.7 ± 0.5 vs 21.3 ± 0.4 mm for treated Arg1<sup>+/−</sup> mice (n = 3 per group, P = 0.26).

Neurobehavioral testing suggests treated animals are similar to littermate controls
To determine whether treated Arg1<sup>+/−</sup> mice had any cognitive or motor deficits, AAV-mArg1-treated Arg1<sup>+/−</sup> mice (n = 8) and littermate controls (n = 8) at 11–12 weeks of age were subjected to a battery of behavioral tests by a blinded examiner to determine the CNS function of these animals. These studies included (1) the SmithKline Beecham Pharmaceuticals (Middlesex, UK); Harwell, MRC Mouse Genome Center and Mammalian Genetics Unit; Imperial College School of Medicine at St Mary’s Royal London Hospital, St Bartholomew and the Royal London School of Medicine; Phenotype Assessment (SHIRPA) to score defects in gait, posture, motor control and coordination, changes in excitability and aggression, autonomic function such as lacrimation, piloerection, defecation and muscle tone; (2) open field (OF) to examine exploratory activity; (3) elevated plus maze (EPM) to measure anxiety; (4) Morris water maze (MWM) to test spatial learning; (5) hot plate pain assay for pain detection and (6) Rotarod to test cerebellar function. No significant differences were found by any of these tests comparing treated Arg1<sup>+/−</sup> mice with littermate controls (Table 1 and Figures 4–8) and mice did demonstrate evidence of learning with the MWM.

SHIRPA primary screen. Quantitative measures of SHIRPA primary screen are presented (Table 1). No differences were observed between littermate controls (n = 8 mice and treated Arg1<sup>+/−</sup> mice n = 8 mice).

The open field test. The OF test is a commonly used qualitative and quantitative measure of general locomotor activity and willingness to explore in mice. Time spent in the center and periphery of the field and total distance traveled in first 20 min of activity were examined. We found no significant differences (± s.e.m.) from littermate controls and the treated Arg1<sup>+/−</sup> mice on the distance traveled in the OF (35 291 ± 2765 vs
25 571 ± 2768 mm, respectively \((P = 0.89)\) (Figure 4a). We also calculated the average velocity \((\pm \text{s.e.m.})\) for each group and found no significant differences in the treated Arg1\(^{−/−}\) compared with littermate controls \((22.12 ± 2.69\) vs \(29.80 ± 2.445\) \text{mm s}^{-1}\) \((P = 0.81)\)). Finally, we calculated the amount of time mice spent in the central zone away from the wall of the OF, and once again found no significant differences of treated Arg1\(^{−/−}\) compared with littermate controls \((P = 0.80)\) (Figure 4b). Thus, there were no deficits detected on a task requiring simple locomotion in an OF comparing the treated Arg1\(^{−/−}\) mice with the littermate controls.

The elevated plus maze. EPM tests the innate fear response (anxiety). The anxiety is induced by the height off of the ground of 100 cm, and animals can choose to go to a safe place where they...
will not fall. The open-arm is considered anxiety-provoking and the closed arm is considered safe. The data does not add up to 100% owing to the time animals spend in the center, while the number of entries refers to leaving the center to the open arms or closed arms and further assesses anxiety level (Figure 5a). There was no statistically significant difference detected between the littermate controls and the AAV-treated Arg1\(^{-/-}\) mice of time spent leaving the center to enter the open arm or closed arm (\(P = 0.85\)) (Figure 5a) or in the open (Figure 5b) (\(P = 0.85\)) or closed arms (Figure 5c) (\(P = 0.43\)).

### Table 1. SHIRPA primary screening score in littermate controls (n = 8) and Arg1\(^{-/-}\)–treated mice (n = 8) at 4 months of age

|                | Controls | AAV-mARG1–treated ARG \(^{-/-}\) | P-value |
|----------------|----------|---------------------------------|---------|
| **Viewing jar** |           |                                 |         |
| Body position  | 3.00 ± 0.00 | 3.00 ± 0.00 | 1.00    |
| Spontaneous activity | 1.63 ± 0.18 | 1.88 ± 0.13 | 0.28    |
| Tremor         | 0.00 ± 0.00 | 0.25 ± 0.16 | 0.17    |
| Urination      | 0.00 ± 0.00 | 0.00 ± 0.00 | 1.00    |
| Defecation     | 2.13 ± 0.48 | 1.25 ± 0.31 | 0.15    |
| **Arena**      |           |                                 |         |
| Transfer arousal | 3.13 ± 0.13 | 3.25 ± 0.16 | 0.55    |
| Locomotor activity | 20.00 ± 2.20 | 19.00 ± 3.57 | 0.82    |
| Palpebral closure | 0.00 ± 0.00 | 0.00 ± 0.00 | 1.00    |
| Piloerection   | 0.00 ± 0.00 | 0.00 ± 0.00 | 1.00    |
| Startle response | 0.75 ± 0.16 | 1.00 ± 0.00 | 0.17    |
| Gait           | 0.00 ± 0.00 | 0.00 ± 0.00 | 1.00    |
| Pelvic elevation | 2.00 ± 0.00 | 2.00 ± 0.00 | 1.00    |
| Tail elevation | 1.00 ± 0.00 | 1.25 ± 0.16 | 0.17    |
| Touch escape   | 3.00 ± 0.00 | 2.88 ± 0.13 | 0.35    |
| **Above arena** |           |                                 |         |
| Positional passivity | 4.00 ± 0.00 | 4.00 ± 0.00 | 1.00    |
| Visual placing | 3.00 ± 0.00 | 3.00 ± 0.00 | 1.00    |
| **Reflex**     |           |                                 |         |
| Grip strength  | 3.00 ± 0.00 | 3.00 ± 0.00 | 1.00    |
| Body tone      | 1.00 ± 0.00 | 1.00 ± 0.00 | 1.00    |
| Toe pinch      | 3.00 ± 0.00 | 3.00 ± 0.00 | 1.00    |
| Wire maneuver  | 0.38 ± 0.38 | 0.00 ± 0.00 | 0.35    |
| **Other**      |           |                                 |         |
| Righting reflex | 0.00 ± 0.00 | 0.00 ± 0.00 | 1.00    |
| Contact righting reflex | 1.00 ± 0.00 | 1.00 ± 0.00 | 1.00    |
| Negative geotaxis | 0.00 ± 0.00 | 0.50 ± 0.33 | 0.17    |

Abbreviations: AAV, adeno-associated virus; ARG1, arginase I. Values are given as mean ± s.e.m. Unpaired t-test, significance is achieved at \(P < 0.05\).

The Morris Water Maze. Mental retardation is an important comorbidity of hyperargininemia. To examine whether treated Arg1\(^{-/-}\) mice have adversely affected spatial cognition, we trained treated mice and littermate controls on the hidden version of the MWM. Over a training period of 5 days, time was measured from being placed in the chamber to finding the platform. In littermate controls and the AAV-treated Arg1\(^{-/-}\) mice, training in the visible version of the MWM revealed normal escape latencies (Figure 6a).

As escape latencies generally are a poor indicator of spatial learning,\(^{10}\) we tested the accuracy with which the platform location was learned by delivering a probe trial (during which the platform was removed from the pool) after completion of acquisition training. The entire procedure took 6 consecutive days. In brief, mice were trained to locate a hidden platform based on distal visual cues to escape from the pool. Mice received four training trials per day (with different start points) for 5 consecutive days. In addition to training trials, only on day 3, before trials, the platform was removed and probe test 1 was performed to measure the animal’s long-term memory; the assessment was made as to the time spent looking for the platform after a random location of entry. Although we do detect a trend for learning in the treated animals with the probe 1 test (if learning, more time is spent in target quadrant (Figure 6b, AAV-treated Arg1\(^{-/-}\) target bar)), these animals may not be completely suitable for the MWM due to potential, previously described (however unknown to us) visual impairment in the NIH Swiss mouse strain (rd1 mutation at the Pde6b locus conferring retinal degeneration\(^{11}\)). On day 6, the platform was removed and a probe test 2 was performed. This time there was no preference for the target quadrant (T) in littermate controls or AAV-treated Arg1\(^{-/-}\) mice (Figure 6c).

However, we do know that the mice have some visual acuity by the results of the SHIRPA evaluation (where the contextual cues are in part visual).

The hot plate pain assay. Hot plate pain assay testing examines time to lifting and licking hind paw (supraspinal response) when the animal steps on a surface at 52 °C. Between the treated Arg1\(^{-/-}\) mice and littermate controls, there was no difference in pain perception (± s.e.m.) as demonstrated by latency to paw withdrawal (14.0 ± 0.73 vs 14.63 ± 0.87 s, respectively (\(P = 0.67\)) (Figure 7)).

The Rotarod test. Mice were tested by being placed on a rotating rod that starts at 5 r.p.m. and then accelerates to 20 r.p.m. in 10 s and remains at that velocity for 180 s (constant velocity testing) with scoring based on latency to fall (Figure 8a). In a second test,
the rod turns at 5 r.p.m. and increases to 60 r.p.m. over 60 s, remaining at that velocity for 2 min (accelerating velocity testing) (Figure 8b). There was no cerebellar-dependent deficit detected in either test (±s.e.m.) between groups (constant velocity: littermate control 24.50 ± 14.57 s, treated Arg1/C0/C0 mice 33.25 ± 11.91 s (P = 0.75)); accelerating velocity: littermate control 17.50 ± 4.79 s, treated Arg1/C0/C0 mice 22.25 ± 7.53 s (P = 0.48).

Serum ammonia and brain amino acids are improved with AAV-mArg1 gene therapy
Untreated Arg1/C0/C0 mice die as juveniles before weaning. Although they die with evidence of cerebral dysfunction, it has been previously unclear as to the extent of the biochemical derangement in their CNS and whether this can be mitigated with gene therapy. By analyzing intracellular metabolites and amino acids in brain tissue of untreated animals, demonstrating signs of cerebral dysfunction at day 20 of life (n = 5 per group), we detected diffuse alterations in the levels of amino acids and other compounds in brain tissue (Table 2A). Elevations are detected in alanine, the branched chain amino acids, proline, histidine, threonine, the aromatic amino acids, arginine, citrulline, ornithine, asparagine and methionine, with a marked elevation in glutamine. Decreased levels of cystine, lysine, glutamic and aspartic acid and homocarnosine are also detected; g-aminobutyric acid (GABA) is not statistically different from littermate controls.

With AAV-mArg1 administration, 3-week-old AAV-mArg1-treated Arg1/C0/C0 mice demonstrate resolution of nearly all of these metabolic abnormalities at this early time point (Table 2A). In fact brain arginine and citrulline are at levels below that of control animals, as are tyrosine and threonine, whereas ornithine is twice that of littermate controls. Only the elevation in CNS glutamine suggests there is some underlying urea cycle dysfunction (34% above controls).

The level of Arg1 transgene expression declines with vector loss over time as was previously described, but it does persist to at least 1 year of age (see Supplementary Figure 1). In fact brain arginine and citrulline are at levels below that of control animals, as are tyrosine and threonine, whereas ornithine is twice that of littermate controls. Only the elevation in CNS glutamine suggests there is some underlying urea cycle dysfunction (34% above controls).

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Figure 5. EPM testing of littermate controls and Arg1/C0/C0 mice after AAV-mArg1 gene therapy demonstrate no anxiety abnormalities. Time spent leaving the center to enter the open arm or closed arm (a) or in the open (b) or closed arms (c) (5 min). (Data presented as mean ± s.e.m.).

Figure 6. MWM training demonstrates ability to learn in Arg1/C0/C0 mice after AAV-mArg1 gene therapy and littermate controls. (a) Learning curve as indicated by the latency to locate a hidden platform (up to 60 s) during a 5-day training period. The average of four trials per day is presented. (b) Probe test 1 on day 3 (the platform is removed), showing the percentage of time spent in each pool quadrant (in 60 s); note additional time spent in target (T) area by AAV-treated Arg1/C0/C0 mice indicating learning; however, with Probe test 2 on day 6 (c), time spent in the four quadrants is roughly equivalent between both types of mice. (T = target, R = right, O = opposite, L = left) (Data presented as mean ± s.e.m.).

Figure 7. Hot plate testing demonstrates no hyperreactivity to thermal sensory stimuli in Arg1/C0/C0 mice after AAV-mArg1 gene therapy compared with littermate controls. Latency to paw withdrawal from a plate at 52.5 °C (up to 45 s) demonstrated no difference between treated mice and littermate controls. (Data presented as mean ± s.e.m.).
addition, homocarnosine (which is present in excitable tissues but whose function is unknown) was normal at 3 weeks, but decreases while GABA is not statistically different from heterozygous controls.

Serum ammonia was followed long-term in littermate control and treated Arg1−/− mice. Untreated Arg1−/− mice before death (n = 7) at about 3 weeks of life (range 19–23 days of life) have ammonia levels of 182.9 ± 1073.4 μmol l⁻¹. Littermate controls (n = 11) and AAV-treated Arg1−/− (n = 4) mice have ammonia levels of 124.5 ± 65.7 and 101.6 ± 18.4 μmol l⁻¹, respectively. By 6 months of age, ammonia levels of littermate controls (n = 4) are 92.7 ± 23.4 μmol l⁻¹, whereas AAV-treated Arg1−/− (n = 4) mice have ammonia levels of 236.3 μmol l⁻¹. By 1 year of life, ammonia levels of littermates are 90.5 ± 19.6 μmol l⁻¹ and in AAV-treated Arg1−/− mice the ammonia level is 258.3 ± 15.1 μmol l⁻¹.

Serum arginine was also followed long-term in littermate control and treated Arg1−/− mice. Untreated Arg1−/− mice before death (n = 4) at about 3 weeks of life (range 19–23 days of life) have arginine levels of 1825.2 ± 52.5 μmol l⁻¹. Littermate controls (n = 5) and AAV-treated Arg1−/− (n = 4) mice have arginine levels of 198.6 ± 100.8 and 101.8 ± 24.3 μmol l⁻¹, respectively. By 6 months of age, arginine levels of littermate controls (n = 4) are 178.2 ± 35.2 μmol l⁻¹, whereas AAV-treated Arg1−/− (n = 4) mice have arginine levels of 155.8 ± 9.8 μmol l⁻¹. By 1 year of life, arginine levels of littermates are 132.7 ± 40.6 μmol l⁻¹ and in AAV-treated Arg1−/− mice the arginine level is 182.1 ± 60.9 μmol l⁻¹ (P = 0.22).

Elevated GCs are improved with AAV-mArg1 gene therapy

The serum GCs α-keto-δ-guanidinovaleric acid (α-K-δ-GVA), guanidinoacetic acid, α-N-acetylgarginine (α-N-AA), β-guanidinopropionic acid (data not shown), creatinase, creatine, γ-guanidinobutyric acid, homoarginine and arginic acid (ArgA) were all markedly elevated in the untreated Arg1−/− mice (n = 5 per group) (Table 3), whereas serum guanidinosuccinic acid, guanidine and methylguanidine were low. However, while not completely normalized, all elevated GCs demonstrated substantial reductions and all depressed GCs demonstrated substantial increases with AAV-based treatment when examined at 4 months of age (n = 3 in treated group, n = 5 littermate controls).

Effect of ammonium challenge on nitrogen metabolism reveals persistent urea cycle dysfunction

Amino acids and ammonia were measured in blood collected before and 20 min after challenge with 4 mmol kg⁻¹ NH₄Cl (Figure 9) in 8- to 10-month-old male AAV-treated Arg1−/− mice and male littermate controls (n = 5 per group); an additional ammonia level was also measured at 1 h after injection. Baseline serum ammonia and some amino acids were different in treated Arg1−/− mice compared with littermate controls. In particular, concentrations (± s.d.) of serum ammonia (263.2 ± 65.3 μmol l⁻¹ in treated Arg1−/− mice (n = 5) vs 100.7 ± 45.3 μmol l⁻¹ in littermate controls (n = 5) P = 0.01) and glutamine (1459.9 ± 302.5 μmol l⁻¹ in treated Arg1−/− mice (n = 5) vs 519.7 ± 89.6 μmol l⁻¹ in littermate controls (n = 5) P < 0.05) were higher in the treated Arg1−/− mice. In the littermate controls, the 4 mmol kg⁻¹ NH₄Cl challenge resulted in an increase in serum ammonia levels to 793.1 ± 269.5 μmol l⁻¹ 20 min after administration; this declined to 231.4 ± 93.3 μmol l⁻¹ 1 h after administration. In the treated Arg1−/− mice, serum ammonia increased to 2591.4 ± 725.2 μmol l⁻¹ (P = 0.004) after 20 min; at 1 h, the ammonia level was unchanged at 2591.4 ± 820.4 μmol l⁻¹ (P = 0.002).

Examining activity scores (± s.d.) 15 min after injection, littermate controls demonstrated an average activity score of 7.0 ± 0.0 (perfect = 7, moribund = 0), whereas AAV-treated Arg1−/− mice demonstrated scores of 4.8 ± 1.5 (P = 0.01), (for scoring method see Supplementary Table 1). At 1 h after NH₄Cl administration, heterozygous littermate controls demonstrated an unchanged average activity score of 7.0 ± 0.0 while AAV-treated Arg1−/− mice demonstrated a score of 4.0 ± 2.0 (P = 0.01); two of these mice were hypothermic at 1 h.

The most substantial changes in the serum amino acids noted from baseline to 20 min after injection were of citrulline and ornithine. Both groups demonstrated a decline in ornithine levels after NH₄Cl injection. AAV-treated Arg1−/− mice demonstrated baseline ornithine levels of only 58.3% of littermate controls pre-ammonium challenge (49.9 vs 85.6 μmol l⁻¹, respectively). Serum ornithine in heterozygous controls declined 46.1% after NH₄Cl administration to 46.2 μmol l⁻¹, whereas ornithine declined only 34.6% in treated Arg1−/− mice to 32.7 μmol l⁻¹. Citrulline, however, followed a different relationship. Owing to the administration of ammonium with normal ornithine transcarbamoylase enzymatic activity, an increase in citrulline (± s.d.) occurred in heterozygous control mice after NH₄Cl administration from 73.5 ± 12.2 to 97.7 ± 17.9 μmol l⁻¹ (a 33% increase). However, in the treated Arg1−/− mice, on average citrulline declined after the administration of NH₄Cl from 127.6 ± 17.1 to 125.5 ± 16.7 μmol l⁻¹ (a 1.7% decrease), suggesting that the baseline deficiency in ornithine may be insufficient to produce enough citrulline to compensate for the increased nitrogen loading.

**DISCUSSION**

Although the primary function of ARG1 in the liver is to catalyze the urea cycle, it is believed that the same reaction, in extrahepatic sites, may direct metabolites into other, quite different, pathways (Figure 10). In this setting, arginine and its hydrolytic products become substrates for the synthesis of a number of important neurotransmitters, secondary signaling molecules and DNA.
support molecules, including glutamate, GABA, proline, creatine, nitric oxide, citrulline and polyamines. 

Deficiency of the enzyme may lead to abnormalities in these other pathways and that likely these abnormalities are the cause of the characteristic phenotype detected in these patients. The purpose of the present series of investigations was to examine in gene therapy-corrected animals if (1) there are metabolic or structural abnormalities of the brain in AAV-mArg1-treated Arg1−/− mice; (2) determine if genetic correction could lead to normal behavioral and cognitive development in treated animals; (3) examine the details of the gene correction by analyzing the metabolites in the serum and tissue). 

**Table 2A.** Brain amino-acids levels in 3-week-old untreated Arg1−/−, AAV-mArg1-injected Arg1−/− and littermate control mice

| Brain amino-acid (nanomoles per ml per 100 mg tissue) | Monoamino, monocarboxylic | Branch chain | Mercapto | Heterocyclic |
|--------------------------------------------------------|---------------------------|-------------|----------|-------------|
| **3 Weeks**                                            |                           |             |          |             |
| Control littermates                                    |                           |             |          |             |
| treated                                              |                           |             |          |             |
| Treated ARG1−/−                                       |                           |             |          |             |
| Untreated ARG1−/−                                     |                           |             |          |             |
| P-value untreated*                                     |                           |             |          |             |
|                | Alane                      | Glycine      | Isoleucine| Leucine     | Valine       | Cystathionine| Cystine     | Taurine     | Proline     |
| Control      | 96.2 ± 19.3                | 122.2 ± 55.5 | 6.4 ± 2.0 | 13.6 ± 3.1 | 13.6 ± 3.0  | 6.8 ± 6.7   | 1.0 ± 0.7  | 1156.8 ± 77.8 | 14.8 ± 2.5 |
| Treated ARG1 | 105.2 ± 16.4               | 89.0 ± 18.3  | 6.8 ± 1.2 | 12.8 ± 1.9 | 14.0 ± 1.7  | 2.6 ± 0.8   | 0.4 ± 0.5  | 1183.2 ± 69.0 | 14.2 ± 1.9 |
| Untreated ARG1| 276.8 ± 135.2              | 133.0 ± 18.7 | 11.6 ± 2.7| 26.2 ± 7.7 | 23.6 ± 6.1  | 13.2 ± 4.6  | 0.0 ± 0.0  | 1086.2 ± 179.0 | 59.6 ± 18.1 |
| P-value treated*                                      | 0.50                      | 0.29        | 0.73      | 0.67        | 0.82        | 0.25        | 0.23        | 0.63          | 0.71        |
| P-value untreated*                                     | 0.03                      | 0.72        | 0.01      | 0.02        | 0.02        | 0.15        | 0.05        | 0.50          | 0.001       |

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| Brain amino-acid (nanomoles per ml per 100 mg tissue) | Monoamino, monocarboxylic | Branch chain | Mercapto | Heterocyclic |
|--------------------------------------------------------|---------------------------|-------------|----------|-------------|
| **3 Weeks**                                            |                           |             |          |             |
| Control littermates                                    |                           |             |          |             |
| treated                                              |                           |             |          |             |
| Treated ARG1−/−                                       |                           |             |          |             |
| Untreated ARG1−/−                                     |                           |             |          |             |
| P-value untreated*                                     |                           |             |          |             |
|                | Diamino                    | Lysine       | Glutamic acid| Aspartic acid| Serine      | Threonine    | Homocar sinus| Carnosine     | GABA        |
| Control      | 9.4 ± 1.0                  | 38.8 ± 7.4   | 860.8 ± 29.8| 365.2 ± 30.9| 84.6 ± 6.6 | 47.4 ± 13.4 | 2.4 ± 1.2   | 3.8 ± 1.3     | 3296.8 ± 74.8|
| Treated ARG1 | 11.8 ± 1.0                 | 32.0 ± 4.7   | 918.6 ± 70.4| 372.2 ± 18.4| 89.0 ± 13.8| 29.6 ± 3.5 | 2.4 ± 0.5   | 5.0 ± 1.1     | 2896.8 ± 26.9|
| Untreated ARG1| 30.0 ± 10.5                | 18.2 ± 5.5   | 661.4 ± 84.9| 221.8 ± 13.0| 73.2 ± 9.2 | 62.2 ± 41.1 | 0.6 ± 0.5   | 7.2 ± 4.7     | 2740.8 ± 23.8|
| P-value treated*                                      | 0.01                      | 0.16        | 0.17      | 0.71        | 0.58        | 0.03        | 0.01        | 0.20          | 0.34        |
| P-value untreated*                                     | 0.005                     | 0.002       | 0.002     | 0.27 – 05   | 0.08        | 0.03        | 0.02        | 0.20          | 0.19        |

**Table 2A.** Brain amino-acids levels in 3-week-old untreated Arg1−/−, AAV-mArg1-injected Arg1−/− and littermate control mice

| Brain amino-acid (nanomoles per ml per 100 mg tissue) | Monoamino, monocarboxylic | Branch chain | Mercapto | Heterocyclic |
|--------------------------------------------------------|---------------------------|-------------|----------|-------------|
| **3 Weeks**                                            |                           |             |          |             |
| Control littermates                                    |                           |             |          |             |
| treated                                              |                           |             |          |             |
| Treated ARG1−/−                                       |                           |             |          |             |
| Untreated ARG1−/−                                     |                           |             |          |             |
| P-value treated*                                      | 1.00                      | 0.04        | 0.24     | 0.01        | 0.07        | 0.03        | 0.27        | 0.02          | 0.08        |
| P-value untreated*                                     | 0.002                     | 0.001       | 0.001    | 0.001       | 0.001       | 0.001       | 0.05        | 1.1E – 07      | 0.04        |

Abbreviations: AAV, adenoviral-associated virus; ARG1, arginase I; GABA, γ-aminobutyric acid. Values are given as mean ± s.d. (as nanomoles per ml per 100 mg of tissue). *Compared to heterozygote. Bold values indicate P < 0.05.
human hyperargininemia,\textsuperscript{14,15} and in this mouse model, and it has been suggested that these GCs may be contributing to the unique CNS pathology detected in these patients (progressive dementia, epilepsy and spasticity, as well as cortical and pyramidal tract deterioration\textsuperscript{17}). This includes increased plasma $\alpha$-K-\textgreek{GVA}, $\alpha$-N-\textgreek{AA}, homoaarginine and ArgA.$\textsuperscript{14-16}$

Supporting this hypothesis was data that demonstrated intravenous loading of arginine had resulted in a pronounced increase in the formation of $\alpha$-K-\textgreek{GVA}, ArgA and $\alpha$-N-\textgreek{AA} in patients with ARG1 deficiency, while the corresponding increase in healthy children was slight.$\textsuperscript{17,18}$ $\alpha$-K-\textgreek{GVA}, $\alpha$-N-\textgreek{AA} and ArgA have been demonstrated as epileptogenic compounds in rabbits and rats, and it is hypothesized that this occurs through inhibition of neuronal responses to GABA and glycine by the blockage of chloride channels, which has been demonstrated in culture.$\textsuperscript{19-21}$ As compared with arginine, $\alpha$-K-\textgreek{GVA}, $\alpha$-N-\textgreek{AA} and ArgA were all previously found to be much more potent GABA receptor antagonists, with ArgA being the most potent.$\textsuperscript{20}$

In the studies performed herein, substantial improvement of GCs occurred in the group treated with AAV-based gene therapy.
However, while the levels are improved, they are not corrected, while CNS function in these animals is normal. A recent examination of human patients with ARG1 deficiency has raised doubt as to whether GCs are the culprit leading to CNS dysfunction. Although the exact cause of the unique neurological features of human patients with hyperargininemia has not been determined, the finding of abnormal levels of GCs and normal CNS function in the treated animals in this study further raises questions as to the effect of GCs in the CNS dysfunction of this disorder.

These studies demonstrate that the metabolic abnormalities present in hyperargininemia can be mitigated or improved in mice with AAV-based gene therapy, beginning in the neonatal period and that long-term survival (of at least 1 year) is possible. Furthermore, these studies demonstrate that the unique neurological features characteristic of this disorder can be prevented and result in normal brain development, cognition and activity. These studies support further investigation of using AAV-based gene therapy for this urea cycle disorder.

**Figure 9.** Ammonia challenging of ARG1 null mice after AAV-mArg1 gene therapy demonstrate reduced nitrogen clearance. AAV-mArg1-treated Arg1 KO mice and littermate controls (n = 5 per group) were injected with 0.4 M NH₄Cl and serum ammonia was measured 20 and 60 min after administration. AAV-mArg1-treated Arg1 KO mice demonstrated substantially reduced ammonia clearance (P < 0.05 between groups at both 20 and 60 min). (Data are presented as mean ± s.d.).

**Figure 10.** The arginine metabolic pathway. The arginine metabolic pathway showing the relationship of arginine with the urea cycle and side reactions to amino-acid biosynthesis, guanidino biosynthesis pathways and the polyamine biosynthesis pathways. (ADC, arginine decarboxylase; AGMase, agmatinase; ODC, ornithine decarboxylase; OAT, ornithine aminotransferase; HG, hydrogenation; AS, argininosuccinate; AI, arginase I; All, arginase II; AGAT, arginine glycine amidino transferase; Harg, homoarginine; GSA, guanidinosuccinic acid; GAA, guanidinoacetic acid; CT, creatine; CTN, creatinine; α-N-A, α-N-acetylgarginine; α-K-6-GVA, α-K-6-guanidinovaleric acid; GMT, guanidino glutamate; ODC, ornithine decarboxylase; Proline; PSC, 1-pyrroline-5-carboxylate).

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**MATERIALS AND METHODS**

**Mouse procedures**

The targeted Arg1 allele contains a deletion in exon 4 of the Arg1 gene and were generated and backcrossed to achieve a homogeneous NIH-Swiss strain background, genotyped and housed in 12 h light and 12 h dark cycle and maintained in approved mouse housing areas. All mice were housed under specific pathogen-free conditions; food and water were provided ad libitum. All mice were kept according to the NIH guidelines, and all experimental procedures were conducted in accordance with guidelines for the care and use of research animals at our institution. Newborn pups on the second day of life were injected with 3.0 × 10⁴ gc kg⁻¹ of AAVrh10 CBA-mArg1-WPRE diluted in pharmaceutical grade saline by the superficial facial vein. The injections were performed in a total volume of 50 μL. Males and females were equally represented throughout the behavioral studies. Because of sensitivity of female mice to ammonium challenge (also reported with ornithine transcarbamylase mice (personal communication Lily Wang PhD, University of Pennsylvania, 11 January 2012)), only male mice were used for ammonium challenge. Serum was frozen immediately and stored at −80°C until analysis. Mice were fed standard mouse chow with added carbohydrate (Picolab Rodent diet 20, catalog no. 5053; Labdiet/PMI Nutrition International, St Louis, MO, USA).

**PCR genotyping**

Genomic DNA was prepared from tail tip by standard methods. Anion-exchange column-purified genomic DNA was subjected to PCR for genotyping. Primer sets for wild-type (Wt) gene: knock out (KO) reverse primer: Exon5 reverse 5'-ACGATGTCTTTGGCAGATATGC-3' and Wt forward primer: mAI forward 5'-AAACCAGACCTCTAAGGTCTATGG-3'. Primer sets for KO: Wt/KO reverse primer: Exon5 reverse 5'-ACGATGTCTTTGGCAGATATGC-3' and KO forward primer: Neo forward 5'-GCCCATTCGGCCAGATATGC-3'. Cycle parameters: denaturation: 94°C for 30 s, annealing: 60°C for 30 s, elongation: 72°C for 3 min for 40 cycles using DNA polymerase (catalog no. RR006A; Takara, Mountain View, CA, USA).

**Biochemical analysis of serum**

Serum amino-acid analysis was performed on a Biochrom 30 HPLC amino-acid analyzer (Biochrom Ltd., Cambridge, UK). In brief, 30–50 μL of serum was mixed with equal volumes of Biochrom Seraprep and Lithium dilution buffer. Protein was precipitated by centrifugation and
Guanidinosuccinic acid, arginine (Arg), ArgA, homoarginine, presented as nanomoles per milliliter per 100-mg starting tissue.

Determination of GC levels was accomplished with a Biotronic LC 5001 (Biotronic, Mainz, Germany) amino-acid analyzer adapted for determination of GCs.\textsuperscript{23,24} The GCs were separated over a cation exchange column using sodium citrate buffers and detected with the fluorescence ninhydrin method as previously reported in detail.\textsuperscript{23} A Jagso Model FP-920 fluorescence detector (Jasco International, Tokyo, Japan) was used at 395 and 500 nm for excitation and emission, respectively. Detected compounds include creatine, creatinine, α-K-GVA, guanidinoacetic acid, guanidinosuccinic acid, arginine (Arg), ArgA, homoarginine, α-N-AA, β-guanidinopropionionic acid and γ-guanidinobutyric acid. Urea was determined in serum/plasma using the method of Ceriotti.\textsuperscript{25}

Biochemical analysis of brain tissue

Brain amino-acid levels were measured in Arg\textsuperscript{1-7/8}, AAV-mArg1-injected Arg\textsuperscript{1-7/8} and Arg1 \textsuperscript{7/8} mice injected and collected at 3 weeks and 1 year. For each sample, the tissue was weighed and homogenized in water to a concentration of 0.2 g/mL.\textsuperscript{25} The supernatant was then removed and sonicated. 1 mL was removed and 70 mg of sulfovaseline acid was added. The samples were then centrifuged. Supernatants were removed and sent on dry ice to the laboratory of Stephen Goodman (University of Colorado Health Sciences Center, Denver, CO, USA) for amino-acid analysis on a Beckman System 6300 Amino Acid Analyzer (Beckman Coulter, Brea, CA, USA) utilizing a 3-Lithium buffer method, as described previously\textsuperscript{26} and presented as nanomoles per millilitre per 100-mg starting tissue.

Ammonia analysis of serum

Ammonia was determined in serum samples, by reductive amination of 2-oxoglutarate and oxidation of NADPH, employing a commercial kit (Sigma-Aldrich) using 20 μL of serum for each sample tested. Results are presented as mean ± s.d.

Behavioral analysis

Eight mice per genotype were evaluated for each behavioral test (except Rotarod, where it was four mice per group). Mice were tagged with an ear tag number. Experimenters were blinded to the genotype during testing and analysis. Behavioral tests were performed in the UCLA behavioral test core and analyzed with TopScan (Clever Sys Inc., Reston, VA, USA) automated system.

**SHIRPA** (or SmithKline Beecham Pharmaceuticals; Harwell, MRC Mouse Genome Centre and Mammalian Genetics Unit; Imperial College School of Medicine) at St Mary’s Royal London Hospital, St Bartholomew and the Royal London School of Medicine; Phenotypic Assessment) Before testing any mouse, we used the SHIRPA as a quantitative and standardized method to provide phenotypic data about an individual animal’s general health and performance.\textsuperscript{27} This primary observational screen based on a modified Irwin profile\textsuperscript{29} was performed as described previously.\textsuperscript{29} Behavioral and functional profile screen included quantitative scoring to detect deficits in gait and posture, motor control and coordination, changes in excitability and aggression, autonomic function such as lacrimation, piloerection, defecation and muscle tone.

Open field. Exploratory activity was measured in a square Plexiglas enclosure (27.5 cm × 27.5 cm) for 20 min. Movement within the OF area was recorded by the computerized Topscan Package (Clever Sys Inc.) Measured parameters included distance traveled, time spent in peripheral and center zones of the box, average velocity, stereotypic behavior time, resting time. Zone analysis was performed to determine inner sector occupancy of mice; low scores in this measure can indicate high levels of anxiety. Results are presented as mean ± s.e.m.

Elevated plus maze. The EPM contained four arms (each arm 29 cm long and 8 cm wide), two of which were open, whereas the other two arms had walls on the sides (16.5 cm high). The maze was mounted 100 cm above the floor. For the behavioral session, mice were placed in the center part of the EPM and were allowed to explore the maze for 5 min. The behavior was recorded and analyzed using Topscan tracking system (Clever Sys Inc.). We used both the time spent exploring the open arms and closed arms. Animals were considered to be exploring the open arms when all four paws were on one of the open arms. This assay has good face, construct and predictive validity with respect to measuring anxiety.\textsuperscript{30,31} Low scores in the measure ‘time spent in open arms’ indicate high levels of anxiety. An unpaired t-test was used to statistically compare the time spent exploring the open arms across genotypes. Results are presented as mean ± s.e.m.

Morris water maze. The MWM is a test of spatial learning for rodents that relies on distal cues to navigate from start locations around the perimeter of an open swimming arena to locate a submerged escape platform. Spatial learning is assessed across repeated trials and reference memory is determined by preference for the platform area when the platform is absent.\textsuperscript{32} Results are presented as mean ± s.e.m.

For the hidden version of the MWM, we trained mice with four training trials per day for 5 consecutive days. The escape platform was hidden 1 cm under the water surface in a constant location of the pool. We released mice into the pool from one of seven starting locations. Training trials ended when the mouse was on the platform or 60 s had elapsed, whichever came first. Mice remained for 5 s on the platform before they were removed from the pool. We gave training trials in blocks of two spaced about 90 min apart. We assessed spatial learning with a probe trial (during which the platform was removed from the pool) on 3rd day and 6th day. We used two-way analysis of variance with repeated measures followed by Bonferroni-Dunn post hoc tests. Tracking information was processed by the Topscan Package (Clever Sys Inc.).

Hot plate pain assay. The Hot Plate assay involved placing the subject on an air bath (housing at 30°C). At 5°C, the animal will jump, lick or lift one of the hindpaws or with all four feet leaving the hotplate to the nearest 0.1 s.\textsuperscript{33} The subjects were first habituated to the hot plate before it is turned on by individually placing each mouse on the plate for 30 s. The habituation is then repeated while the plate is still cold. After all the mice have been habituated, the hot plate was turned on and heated to a constant temperature of 52.5°C. Results are presented as mean ± s.e.m.

Subjects were not placed on the plate during the 15-min heating period. Heating takes about 15 min and the temperature remained constant during the actual test period. Once the subject responded by licking or licking a hindpaw, or by jumping, they were immediately removed from the hot plate. The cutoff time was the maximum time a subject is allowed to stay on the hotplate and is chosen to prevent burn injury. For hot plate testing at 52.5°C the cutoff time was 45 s.

Accelerating Rotarod. To assess motor coordination and ability of the mice to handle a complex motor task that involves balance, coordination and motor skill learning, we used an accelerating Rotarod (RotoRod 3375-S; TSE Systems, Hamburg, Germany). Each trial started at a constant forward rotational speed of 5 r.p.m. For a constant velocity the rod rotates at 5 r.p.m. and then accelerates to 20 r.p.m. in 10 s and remains at 20 r.p.m. for 180 s. For accelerating, speed begins at 5 r.p.m. and increases to 60 r.p.m. over 60 s, remaining at that velocity for 2 min. Trials were completed after experimental subjects fell off the rod or 300 s at a maximum speed of 60 r.p.m. had elapsed, whichever came first. They received one trial on constant speed and 4 h later received another trial on accelerating speed. Primary measure of interest was latency to fall. Results are presented as mean ± s.e.m.

Histology

Mice were euthanized by inhalation of isoflurane. For light microscopy, brains were carefully dissected out of the skull and immersion-fixed in 4% formalin for 48 h followed by immersion in 70% alcohol solution. The brain was then coronally sectioned into four parts after routine processing and embedding in a single paraffin block using standard techniques. Further, 4-μm sections of the block were routinely cut, stained with hematoxylin-eosin and analyzed using an Olympus BX40 microscope. Microscope slides were digitized with a ScanScope scanner and viewed with Aperio Image Scope software V11.2.752 (both from Aperio, Vista, CA, USA).

Immunohistochemistry for arginase expression

Livers were removed from euthanized animals and placed in 4% paraformaldehyde (PFA) for 18–24 h. After rinsing with tap water for 15 min tissues were placed in 70% ethanol followed by routine processing and embedding in paraffin. Tissues on slides were deparaffinized and...
rehydrated with ethanol and xylene by routine procedures. Slides were removed from tap water and placed in a microwaveable vessel filled with sodium citrate buffer (10 mM sodium citrate, 0.05% Tween-20, pH 6.0) for antigen retrieval. Tissues were then permeabilized with 1 x 3% buffered saline (TBS) + 0.2% Triton X-100 for 5-10 min followed by 1 x TBS + 0.025% Triton X-100 for 5 min. Tissues were blocked with protein blocker + 0.1% Tween-20 for 20 min. Primary antibody was applied to the sections: Rabbit ARG1 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) diluted in protein blocker plus 0.1% Tween-20 at 1:50 ratio and incubated overnight at 4°C. After rinsing twice for 5 min with 1 x TBS 0.025% Triton the slides were incubated in 0.3% H2O2 in TBS for 15 min followed by application of the secondary antibody (Goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology Inc.) (1:100 in protein blocker + 0.1% Tween-20)) and incubated for 1 h at room temperature. After rinsing three times with 1 x TBS, slides were developed with ImmPACT DAB kit (Vector, Burlingame, CA, USA) for 3 min at room temperature. Slides were counterstained with hematoxylin and coverslipped.

Imaging protocol
All mice were imaged in a multimodality chamber designed to allow maintaining the mice on isoflurane anesthesia at 1–2% during imaging and provide heating to maintain the mices’ body temperature. The chamber also enables reproducible positioning to <1 mm, thus minimizing any attenuation variability due to animal positioning. Mice were imaged in a MicroCAT II small animal CT system (Siemens Preclinical Solutions, Knoxville, TN, USA). Exposure settings were 70 kVp, 500 mA, 500 ms exposure time and 360° rotation in 1° steps with 2.0 mm aluminum filtration. Images were reconstructed using a modified Feldkamp process to a cubic voxel size of 0.20 mm. Results are presented as mean ± s.d.

Ammonium challenge
To simulate a nitrogen challenge, mice (control and virus-treated) were injected with a 0.4 M solution of NH4Cl intraperitoneally. All ammonium challenges were performed at the same time of day, between 1400 and 1500h, to minimize the effect of food consumption on plasma amino acids. At 15 min after injection, the mice were evaluated behaviorally by scoring from two blinded observers using the scale outlined developed by Ye et al.[2] (Supplementary Table 1). Blood was collected from the retro-orbital plexus prior to beginning the studies, 20 min after injection, and at 1 h after the injection when they were euthanized. The serum was collected and immediately frozen until analyzed for ammonium and amino acids. Results are presented as mean ± s.d.

Statistical analysis
For analysis of behavioral data, GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA) was used. For SHIRPA, OF, hot plate and EPM analyses, unpaired t-test was used for the MWM, two-way repeated measures analysis of variance was used followed by Bonferroni–Dunn post hoc tests. Unpaired t-test was used to analyze the calvarial size and metabolite analyses.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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