Implications of arginine deficiency for growth and organ maturation. Studies on hair, muscle, brain and lymphoid organ maturation

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Chapter IV

Effect of arginine deficiency on circulating and tissue amino acids and guanidino compounds, and on behavioral development

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Abstract

Arginine is an intermediate of the ornithine cycle and serves as a precursor for the synthesis of nitric oxide, creatine, agmatine, and proteins. It is considered to be a conditionally essential amino acid, because endogenous synthesis only barely meets daily requirements. In rapidly growing suckling rats, endogenous arginine biosynthesis is crucial to compensate for the insufficient supply of arginine via the milk. Evidence is accumulating that the intestine rather than the kidney plays a major role in arginine metabolism in this period. Accordingly, ectopic expression of hepatic arginase in murine enterocytes by genetic modification induces a selective arginine deficiency. The ensuing phenotype, the severity of which correlates with the level of transgene expression in the enterocytes, could be reversed with arginine supplementation. We now report that these transgenic mice continue to suffer from an arginine deficiency after the arginine biosynthetic enzymes disappear from the enterocytes. Post-weaning catch-up growth in arginine-deficient mice is characterized by increased levels of all amino acids except arginine. Furthermore, amino acid concentrations, including arginine, are lower in adult male than in adult female transgenes. Decreases in the concentration of plasma and tissue arginine lead to corresponding decreases in most metabolites of arginine. However, the accumulation of the toxic guanidino compounds guanidinosuccinic acid and methylguanidine corresponds with arginine deficiency, possibly reflecting a higher oxidative stress under hypoargininemic conditions. Hypoargininemia is also associated with disturbed neuromotor behaviour, although brain levels of toxic guanidino compounds and ammonia are normal.
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Abbreviations

CPS [EC 6.3.4.16] : carbamoylphosphate synthetase
OTC [EC 2.1.3.3] : ornithine transcarbamoylase
ASS [EC 6.3.4.5] : argininosuccinate synthetase
ASL [EC 4.3.2.1] : argininosuccinate lyase
A-I [EC 3.5.3.1] : hepatic arginase
A-II [EC 3.5.3.1] : non-hepatic arginase
AGAT [EC 2.1.4.1] : L-arginine-glycine transamidinase
ND : neonatal day, days after birth
Harg : homoarginine
ArgA : argininic acid
GAA : guanidinoacetic acid
CT : creatine
CTN : creatinine
MG : methylguanidine
G : guanidine
GABA : γ-aminobutyric acid
γ-GBA : γ-guanidinobutyric acid
β-GPA : β-guanidinopropionic acid
δ-GVA : δ-guanidinovaleric acid
α-K-δ-GVA : α-keto-δ-guanidinovaleric acid
GSA : guanidinosuccinic acid
α-NAA : α-N-acetylgarginine
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Introduction

Arginine is an intermediate of the ornithine cycle and serves as a precursor for the synthesis of nitric oxide (NO), creatine, agmatine, and proteins (Wu and Morris, 1998). Arginine is not considered to be an essential amino acid. In adults, a major source of arginine biosynthesis from citrulline is the renal proximal convoluted tubule (Featherston et al., 1973; Levillain et al., 1992). Citrulline, in turn, is synthesized in the small intestine from glutamine and proline (Windmueller and Spaeth, 1981). Although arginine is not considered to be an essential amino acid in adult humans, its endogenous biosynthetic capacity only amounts to approx. 20% of daily expenditure (Visek, 1986). Hence, its bioavailability may become insufficient under conditions of increased demand, such as growth (Visek, 1986) and tissue repair (Thornton et al., 1997), or as a result of decreased dietary supply (de Lorgeril, 1998). For this reason, arginine is regarded as a conditionally essential amino acid.

In rapidly growing suckling rats, endogenous arginine biosynthesis is crucial to compensate for the insufficient supply of arginine via the milk (Davis et al., 1993). Evidence is accumulating that the intestine rather than the kidney plays a major role in arginine biosynthesis in the suckling period. Thus, the enterocytes of the small intestine express the enzymes required for arginine production from glutamine and proline (Wu, 1997) and do not express arginase (De Jonge et al., 1998). Furthermore, we have recently shown that overexpression of hepatic arginase in enterocytes induces an arginine deficiency (De Jonge et al, submitted). The intestine appears to play a similar role in human neonates as well, as destruction of the enterocytes in necrotizing enterocolitis also results in a selective decrease in circulating arginine (Zamora et al., 1997). In mice and rats, argininosuccinate synthetase and argininosuccinate lyase, the enzymes that synthesize arginine from citrulline, disappear from the enterocytes in the postweaning period, concurrent with the appearance of endogenous arginase (De Jonge et al., 1998), so that the role of the gut becomes confined to citrulline biosynthesis.
The reduction in circulating arginine concentration in transgenic mice that express different levels of arginase I in their enterocytes ("F/A" transgenes) depends on the expression level of transgenic arginase. The degree of arginine deficiency correlates, in turn, with the degree of retardation of hair- and muscle growth, and the development of the lymphoid tissue (De Jonge et al., submitted). Expression of arginase in all enterocytes is necessary to elicit arginine deficiency. Phenotypic abnormalities are reversed by daily injections of arginine, but not creatine. Because the phenotypic symptoms largely disappear after weaning, the question arose whether circulating levels of arginine in relation to the other important amino acids normalize in our transgenic mice when intestinal arginine biosynthesis ceases after weaning.

**Figure 1: Metabolic routes of arginine.** Enzymes that catalyze the indicated reactions are given in italics. Arginase accepts arginine as well as homoarginine as a substrate. Similarly, AGAT transaminates the guanidino group of arginine to glycine, but also to the other substrates indicated. The α-amino group of arginine can be transaminated to α-K-δ-GVA, and acetylated to α-NAA. GSA and MG are most probably formed from argininosuccinate (AS) and CTN, respectively, via a reaction with a reactive oxygen species.
We also investigated the accumulation of guanidino compounds, the metabolites of arginine that retain the guanidinium group. A schematic representation of the relation of arginine with the ornithine cycle and its guanidino metabolites is given in Fig. 1. Homoarginine, lysine, and homocitrulline (not shown) are homologues of arginine, ornithine, and citrulline, respectively, and as such are alternative intermediates of the ornithine cycle. A major metabolic route of the guanidino group of arginine is transamidination to glycine to yield guanidinoacetic acid (GAA), and subsequently creatine (CT) and creatinine (CTN). In addition to the physiological substrate glycine (α-aminoacetic acid), its homologues β-alanine (β-aminopropionic acid), γ-aminobutyric acid, and δ-aminovaleric acid can function as substrates of the enzyme AGAT, yielding β-guanidinopropionic acid (β-GPA), γ-guanidinobutyric acid (γ-GBA), and δ-guanidinovaleric acid (δ-GVA), respectively (Fritsch et al., 1999; Marescau et al., 1992b; Wyss and Kaddurah-Daouk, 2000). Furthermore, the α-amino group of arginine can be transaminated and acetylated, yielding α-keto-δ-guanidinovaleric acid (α-K-δ-GVA) and α-N-acetylarginine (α-NAA), respectively. Hydrogenation of α-K-δ-GVA produces argininc acid (ArgA) (Robin and Marescau, 1985). Methylguanidine (MG) forms after the reaction of an oxygen radical with CTN (Nakamura et al., 1991). Similarly, guanidinosuccinic acid (GSA) may be the product of the reaction of argininosuccinic acid and the action of an oxygen radical species (Aoyagi et al., 1999). Some guanidino compounds, in particular α-NAA, GSA and MG, are toxic and play a role in the pathology of renal (Tomida et al., 2000) and liver (Marescau et al., 1995) failure, in particular the metabolic (Horowitz et al., 1970) and neurological (da Silva et al., 1999; De Deyn et al., 2000; D'Hooge et al., 1996) consequences of these diseases. The neurotoxicity of guanidino compounds appears to be more pronounced in the growing than in the adult animal (D'Hooge et al., 1992; D'Hooge et al., 1994). The concentration of guanidino compounds was therefore determined in plasma and several tissues of 10-day old homozygous arginine-deficient transgenic mice, as well as in wild-type
controls. At this age, intestinal biosynthetic capacity for arginine is maximal (De Jonge et al., 1998) and the hypoargininemic phenotype of the transgenic mice most pronounced (De Jonge et al, submitted). The relation between circulating arginine concentration and the formation of guanidino compounds was investigated by arginine supplementation. Because of the neurotoxic potential of some guanidino compounds, like GSA and MG, and the possible long-term consequences of alterations of GABA and glycine levels on neurotransmission, we also determined the consequences of arginine deficiency on neuromotor development.
Methods

Animals. Mice were kept under environmentally controlled conditions (lights on at 8:00 a.m., off at 8:00 p.m.; water and rodent chow ad lib; 20-22 °C, 55% humidity). Animal experiments were done in accordance with the guidelines of the local Animal Research Committee. Litters discovered in the morning were assigned ND 0. The animals were weaned at three weeks of age. For arginine injections, nest size was adjusted to 7 pups. Pups received a subcutaneous injection of 5 mmol/kg of arginine-HCl (150 mM), twice daily (9:00 a.m. and 6:00 p.m.) from postnatal day 5 onward. Controls were injected with saline (0.9 % sodium chloride).

Generation of transgenics. Intestinal fatty-acid binding protein (FABPi)/arginase I transgenic mice (F/A) were generated in the FVB-strain. Generation of the transgenics is described in detail elsewhere (De Jonge et al, submitted). Briefly, arginase I (A-I) was specifically expressed in enterocytes by coupling the A-I structural gene to the FABPi promoter/enhancer element. Two lines, F/A-1 and F/A-2, which express A-I in all enterocytes, but which differ 2-fold in the level of A-I expression (F/A-2 = 2 * F/A-1), were analyzed.

Tissue and blood sampling. Pups were separated from their mother and kept at 37° C for one hour prior to sacrifice. After decapitation, blood was collected into heparin-containing tubes and centrifuged at 2,000 x g for 5 minutes at 4° C. Fifty μL of plasma was added to 4 mg of lyophilized sulphisalicylic-acid, mixed, frozen in liquid nitrogen and stored at -70° C. Tissue samples were collected, flushed in ice-cold phosphate-buffered saline (PBS), rapidly frozen in liquid nitrogen and stored at -70° C until analysis.

Determination of amino acid and guanidino compound concentrations. Jejunum and plasma amino acids were determined by fully automated HPLC as described
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(van Eijk et al., 1993). Norvaline was used as an internal standard. For analysis of guanidino compounds, plasma was deproteinized with an equal volume of 20% trichloroacetic acid, followed by centrifugation at 16,000 x g at 4 °C. The supernatant was diluted and injected into an LC5001 amino acid analyzer (Biotronic, Maital, Germany), adapted for guanidino compound determination. For guanidino compound determination in tissue, approximately 40 mg tissue was homogenized (Tissue Tearor®, model 985-370 type 2, Biospec Products, Bartesville, USA) in 350 μl of ice-cold water. Thirty μl was taken for urea determination. The tissue homogenizer was washed immediately with 350 μl 30% trichloroacetic acid, the wash fluid was added to the first pool and the total was vortexed. The acetic protein complexes were precipitated by centrifugation at 20800 x g at 4 °C. Diluted supernatant was injected. Guanidino compounds were separated on a cation-exchange column using sodium citrate buffers and detected with the ninhydrin fluorescence method (Marescau et al., 1986). Urea nitrogen was determined with diacetylmonoxime (Ceriotti, 1971). Blood ammonia concentration was determined using the using the ammonia test kit (Menarini Diagnostics, Florence, Italy), according to the manufacturer’s instructions.

**Behavioral tests.** Mice of 3 months of age were used. Each animal was put in a separate cage (16x22 cm²) between 3 infrared photobeams connected to a microprocessor counter. Cage-activity was recorded between 16:00 p.m. and 8:00 a.m., and expressed as total number of beam crossings during the recording period. Open field activity was recorded in animals on a reversed dark-light schedule during the dark phase of the cycle, and their movements in a brightly lit area were tracked for 10 minutes using a computerized video tracking system (San Diego Instruments, USA). Exploratory activity was additionally assessed in a dark/light transition box consisting of a large illuminated (45x75 cm²) and a smaller (10x75 cm²), dark compartment. A dividing wall allowed transition between compartments through four evenly spaced 4-cm holes. Animals on reversed dark-light cycle were
placed in the box for 10 min (starting from the dark compartment) during the dark phase of the light cycle, and exploration of the illuminated area was registered using two photobeams (at 1 and 7 cm distance to the dividing wall) and a microprocessor-based counter.

For the wire suspension test, animals were put with their front paws on a taut steel wire (0.6 mm diameter), 46 cm above tabletop, and were to remain suspended for 120 s using their front paws only. Latency of the first slip and number of slips within the 120-sec test period were recorded. In the rotarod test, animals received a 1-min training trial at 4 rpm followed by four testing trials at 10-min intervals on an accelerating rotarod (Ugo Basile, Varese, Italy). Each testing trial consisted of a 5-min session during which the rod accelerated linearly from 4 to 40 rpm. The time the animals were able to stay on the rod was recorded automatically. Finally, gait characteristics were recorded using a runway apparatus. With their hind paws wetted with ink, the animals walked on a strip of paper down a brightly lit corridor (40 cm long, 4.5 cm wide) towards a dark goal box. Recordings were made in duplicate, and maximal distances between prints of left and right paws were measured from the tracks.

Passive avoidance learning was tested in a two-compartment step-through box. Animals on reversed dark-light cycle were put in the small (5x9 cm²) brightly lit compartment of the box. After 5 sec, the sliding door was opened, leading to the larger (20x30 cm²), dark compartment. Upon entrance into the dark compartment, the door was closed and animals received a slight electric foot shock (0.3 mA, 1 sec). Twenty-four hrs later, the procedure was repeated, and step-through latency was recorded up to 300 sec.

Statistical analyses. Biochemical data on amino acids and guanidino compounds were tested with a repeated measure analysis of variance (ANOVA) per compound group. Because of significant interactions between compound, genotype and age, a two-way ANOVA (amino acids; factors age and genotype) or a one-way ANOVA (guanidino compounds; factor genotype) was carried out per compound. In case of
a significant effect of genotype or genotype-age interaction in these ANOVAs, Dunnett's multiple comparison test between genotypes was applied. Results were considered significantly different if \( p<0.01 \).

For the amino acids with a significant age effect, a mean amino acid level was calculated by removing the multiplicative variation between amino acids (J.M. Ruijter et al, submitted). To this end, the correction factors for removal of variation were deduced from a matrix of between amino acid ratios. The mean amino acid level of adult wild type mice was set at 100. Thus, in the resulting mean amino acid all included amino acids carry equal weight.
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Results

Levels of circulating amino acids in wild-type and transgenic mice during postnatal development.

F/A-1 and F/A-2 are transgenic mouse lines that were genetically modified to express hepatic arginase (A-I) in their enterocytes in order to annul their capacity to synthesize arginine (De Jonge et al, submitted). The level of hepatic arginase in the enterocytes of transgenic line F/A-1 is 50% of that of line F/A-2.

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To evaluate whether the apparent normalization of body and fur growth after weaning was associated with the normalization of circulating arginine levels, circulating amino acid concentrations, including arginine, were determined as a function of age. In suckling wild-type mice, the concentration of plasma arginine is 250 μM and gradually declines to 150-170 μM in adult male and female animals (Fig. 2A). In homozygous suckling and weanling F/A-1 mice, plasma arginine is reduced to 80-125 μM, whereas in homozygous F/A-2 suckling and weanling animals, plasma arginine averages only 70 μM.

Table 1: statistical analysis (two-way ANOVA) of changes in amino acid levels in WT, F/A-1 and F/A-2 postnatal and adult mice. Significant effects (p<0.01) of genotype (G), age (A), or genotype-age interaction (I) are given in each column. * Indicates genotype effects which depend solely on age group 5-10 weeks.
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Figure 2: Plasma amino acids as a function of age.
The plasma concentration (μM) of arginine (panel A), ornithine (panel B), glycine (panel C), tryptophan (panel D), citrulline (panel E), and the mean of all other amino acids that decline with age (panel F) in wild-type, homozygous F/A-1 and F/A-2 mice in the preweaning period (1-2 weeks of age), in the periweaning period (3-4 weeks), in the adolescent period (5-10 weeks), and in the adult period (>10 weeks). Arginine is significantly decreased, whereas glycine, tryptophan, and ornithine plasma concentrations are increased in the F/A transgenics. Note the higher amino acid levels in the adolescent period of F/A-2 mice and the
higher arginine levels in adult female (open symbols) compared to male (closed symbols) F/A transgenic mice. Panel F represents the means of plasma concentrations of the amino acids that significantly decline with age (asparagine, glutamate, histidine, lysine, ornithine, serine, isoleucine, methionine, valine, threonine and tyrosine). Since the concentrations were corrected for multiplicative variation (see materials and methods), all amino acids weigh equally in this mean. No difference between sexes was found in suckling and adolescent mice. Asterisks indicate significant differences between male and female (P < 0.01).

No sex differences were found in suckling and weanling animals. In adult female F/A-1 and -2 mice, arginine concentration increases to 130-150 μM, whereas in adult transgenic males, it remains depressed (P < 0.01) at 110 and 80 μM in F/A-1 and F/A-2 mice, respectively (Fig. 2A).

In the preweaning period, arginine deficiency is associated with an increased concentration of glycine and ornithine (De Jonge et al, submitted). Table 1 shows the effects of genotype and age, as well as the interaction between genotype and age on circulating amino acid concentrations in wild-type, F/A-1 and F/A-2 mice from birth to adulthood. As shown in Fig. 2A, the decline in circulating arginine concentration depends strongly on genetic background (p<0.0005). In addition to ornithine (Fig. 2B) and glycine (Fig. 2C), circulating tryptophan (Fig. 2D), serine, threonine and phenylalanine concentrations are increased in transgenics. The significance of the genotype-related difference in ornithine, serine, threonine and phenylalanine (Table 1) depends solely on the increase in plasma amino-acid concentration in 5-10 weeks-old F/A-2 mice, as exclusion of this age group from the ANOVA abolishes significant differences between wild-type, F/A-1, and F/A-2 mice (Table 1, genotype effects indicated by asterisks). Since the increased plasma concentration in 5-10 weeks-old F/A-2 mice is not limited to the amino acids mentioned (Fig. 2F and next paragraph), we felt justified to consider only tryptophan levels as being affected by the F/A genotype.

The Table further shows a significant age effect in the plasma levels of alanine, glutamate, methionine, ornithine, serine, taurine, threonine, histidine, lysine, tryptophan, asparagine, isoleucine, tyrosine, and valine, which is a decline with age in all mice (Fig. 2F), whereas the levels of citrulline (Fig. 2E), glutamine, and leucine do not change significantly in concentration during postnatal
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devolution (Table I). In wild-type mice, arginine, glycine and ornithine also exhibit an age-dependent decline, indicating that their circulating level is determined by both genotype and age. Fig. 2F shows the age-dependent decline of the mean concentration of the amino acids with a significant age effect. Two features are remarkable. First, the circulating concentration of these amino acids is significantly higher in fully-grown, adult transgenic females than in males, but not in wild-type control animals, showing that this gender difference is caused by the genotype, i.e. is the result of low circulating arginine concentrations earlier in life. Second, the circulating concentration of these amino acids is significantly higher in adolescent (5-10 weeks old) F/A-2 transgenic mice than in either wild-type or F/A-1 transgenic mice. The relatively high level of amino acids in adolescent F/A-2 mice coincides with the phase of catch-up growth from the growth retardation that they incur in the first 3 weeks of life (De Jonge et al, submitted).

**Guanidino compounds under arginine deficiency**

**Arginine, homoarginine and urea (Fig.3A)**

Arginine concentration of transgenic mice was significantly decreased in all organs analyzed. The effect was most pronounced in muscle, in which arginine levels decreased approx. 8-fold, and least in liver. No differences in urinary arginine concentration were observed. Despite the 2-fold difference in intestinal arginase expression and an associated aggravation of the phenotype (De Jonge et al, submitted), no striking differences in tissue and plasma arginine concentration between lines F/A-1 and F/A-2 were observed in this series of assays (cf. Fig. 2A). Arginine deficiency was accompanied by an even more pronounced decrease in the arginine homologue homoarginine. Unlike arginine, the decreased homoarginine levels resulted in a decline in urinary excretion. Furthermore, homoarginine levels in most organs were more affected in line F/A-2 than F/A-1. Significant increases in tissue urea concentration were only found in muscle and jejunum, that is, the
organ with the most pronounced drop in arginine concentration (muscle) and the organ with transgenic arginase activity (jejunum). Although urea levels tended to be higher in F/A-2 than F/A-1 and WT mice, plasma and urinary urea was not significantly increased in the transgenic animals.

**Figure 3A:** Tissue, plasma and urinary concentration of guanidino compounds. Concentration of guanidino compounds in wild-type (black bars), F/A-1 (gray bars), and F/A-2 (white bars) mice at ND 10. Panel A: arginine (Arg), homoarginine (Harg) and urea. Tissue concentrations are given in μmol/kg, plasma and urinary concentrations in μM, except for urea concentrations, which are in mmol/kg or mM. <DL is below detection limit. Values are means ± SEM, with n = 6-21. Asterisks indicate significant difference (p<0.05) from wild-type means using a one-way ANOVA.
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Figure 3B: Tissue, plasma and urinary concentration of guanidino compounds. panel B: the transamidination products, guanidinoacetic acid (GAA), β-guanidinopropionic acid (β-GPA), and α-guanidinobutyric acid (γ-GBA).

Other products of arginine metabolism (Fig. 3B and C).

Arginine deficiency causes significant decreases of the transamidination products GAA, β-GPA, and γ-GBA in most tissues (Fig. 3B). However, plasma GAA level was not affected. Tissue and urinary concentrations exceed plasma concentrations, indicating active transport.
Figure 3C: Tissue, plasma and urinary concentration of guanidino compounds.

Panel C: The GAA products creatine (CT) and creatinine (CTN), the transamination product α-amino,δ-guanidinovaleric acid (α-K-δ-GVA) and its hydrogenation product argininic acid (ArgA).
Figure 3D: Tissue, plasma and urinary concentration of guanidino compounds.
panel D: guanidinosuccinic acid (GSA), methylguanidine (MG) and guanidine (G), and the transacetylation product α-N-acetylarginine (α-NAA).
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The changes in transaminidation products are similar in F/A-1 and F/A-2 mice. Tissue creatine (CT) concentration in hypoargininemic animals follows the changes in GAA concentration, though in contrast to GAA, plasma and urinary CT levels are decreased in the transgenic animals (Fig. 3C). No effect of arginine deficiency was observed for the CT product CTN. The transamination product α-K-δ-GVA and its hydrogenation product ArgA are decreased in tissues and plasma, but not in urine (Fig. 3C). Generally, the decreases in α-K-δ-GVA, and argA were equal in F/A-2 and F/A-1 mice.

The formation of GSA, MG, G, and α-NAA (Fig. 3D)

GSA levels were increased in liver, kidney, jejunum and plasma of the transgenic mice. Furthermore, its excretion into urine was substantially increased. MG, which was only detectable in urine, was increased three-fold in F/A-2 mice. G, which was additionally detectable in jejunum and plasma, was increased only in the urine of the transgenic mice. Importantly, clear differences in the urinary excretion were observed between line F/A-1 and F/A-2, levels being the highest in the most affected line. In transgenic brain or muscle, GSA levels were unaltered, although brain arginine concentration is reduced to 40% and arginine in muscle to less that 15% of that of wild-type animals. In those tissues in which the α-amino acetylation product α-NAA was detectable, it was decreased in both transgenic lines, whereas its excretion into urine was increased.

Effect of arginine treatment

In order to demonstrate that the alterations in guanidino compounds in the transgenics are caused by arginine deficiency, we treated transgenic mice with twice daily subcutaneous injections of arginine, starting at ND5. Mice were sacrificed at ND10, 6 hours after the last arginine injection. At sacrifice, plasma arginine concentration was 560, 170 and 90 µM in wild-type, homozygous F/A-1 and F/A-2 mice, respectively, reflecting the influence of arginase activity in the
transgenic intestine. The difference in plasma urea, GAA, CTN, α-K-δ-GVA, ArgA, GSA and G between F/A-2 animals on the one hand and the wild-type and F/A-1 animals on the other, shows that urea production mirrored circulating arginine concentration. In a few cases (CTN, α-K-δ-GVA and G), the plasma guanidino compound concentration in F/A-1 animals was also significantly lower than that in wild-type animals. Similar differences were found for β-GPA, γ-GBA and CT, but these numbers did not reach significance. Urinary CT excretion paralleled the corresponding circulating compounds. The urinary data also reveal that the bulk of the injected arginine is metabolized by arginase and excreted as urea in both control and transgenic mice. Whereas the urinary excretion of most compounds increased or remained constant upon arginine supplementation, that of GSA decreased in the transgenic mice. In summary, the observed response of plasma and urinary guanidino compound concentration to arginine supplementation indicates that the altered concentrations of guanidino compounds that are found in F/A mice, reflect the decreased availability of arginine. Because of the rapid establishment of the equilibriums (< 6 hours), plasma guanidino compound concentrations can be interpreted as fluxes.

**Behavioral alterations in adult F/A transgenics**

Cerebral arginine concentrations are decreased to 50% and 40% in F/A-1 and F/A-2 transgenics, respectively. These decreased levels of arginine may limit cerebral NO synthesis, as has been shown for endothelial cells (Arnal et al., 1995) and activated macrophages (Norris et al., 1995), and decreased NO synthesis has been reported to impair long-term potentiation (Son et al., 1996). Furthermore, in both transgenic lines the cerebral content of creatine is decreased to 70%, while the concentration of the purported neurotoxins GSA and ammonia (WT: 39 ± 11, F/A-1 40 ± 11, F/A-2 44 ± 14 μM), are not altered. In view of this potential modification of metabolism in the brain of F/A transgenic mice, it was decided to evaluate its possible consequences for brain function.
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<tr>
<td>latency first slip (s)</td>
<td>104 ± 11</td>
<td>55 ± 14*</td>
<td>42 ± 12*</td>
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<tr>
<td>slips/120 s</td>
<td>0.2 ± 0.1</td>
<td>1.0 ± 0.2</td>
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<tr>
<td><strong>Rotarod (trial 4)</strong></td>
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<tr>
<td>time on the rod (s)</td>
<td>271 ± 14</td>
<td>232 ± 19</td>
<td>162 ± 20*</td>
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<tr>
<td><strong>Gait test</strong></td>
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<tr>
<td>maximal span (cm)</td>
<td>6.4 ± 0.2</td>
<td>6.2 ± 0.2</td>
<td>5.3 ± 0.3*</td>
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<tr>
<td><strong>Passive avoidance learning</strong></td>
<td></td>
<td></td>
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<tr>
<td>step-through latency (s)</td>
<td>300 ± 0.0</td>
<td>238 ± 32*</td>
<td>186 ± 24*</td>
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Table 2. Behavioral assessment in wild-type and transgenic groups.
Values are mean ± SEM, n=10-11. Asterisks indicate significant difference from wild-type using Dunnett’s multiple comparison test after significant between-genotype effect (ANOVA).

Table 2 shows that both F/A transgenic lines were impaired in several behavioral tests, and that the F/A-2 group generally deviated more from wild-type than the F/A-1 group.

Cage and open field activity were increased in the F/A transgenic mice compared to controls. In the open field tests, ambulatory activity measures like path length and corner entries, differed significantly between the groups, whereas exploratory activity measures, like entries and dwell in center did not. Accordingly, transitions between the compartments of the dark-light box were not significantly different between the groups. Together, these tests demonstrated general hyperactivity in the transgenic mice, with the F/A-2 line being most severely affected. This hyperactivity could not be reduced to a qualitative activity difference (e.g., increased exploration). Several additional, more specific neuromotor abilities were tested. Wire suspension (grip strength and endurance) and rotarod performance (equilibrium and coordination) showed significant decreases in F/A transgenics. Analysis of their gaits revealed that F/A mice walked with shorter paces than controls. Finally, passive avoidance learning was impaired since the
step-through latency was decreased in F/A-1 and F/A-2 transgenic mice compared to controls.
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Discussion

Arginine deficiency and amino acids

Endogenous arginine biosynthesis is a typical example of interorgan metabolic cooperation. The intestine is special in that it has the unique capacity to synthesize citrulline, whereas many other tissues express argininosuccinate synthetase and argininosuccinate lyase to metabolize the citrulline originating from the intestine or from local NO synthesis to arginine. In the adult mammal, 60% of arginine formation from citrulline occurs in the kidney (Yu et al., 1996). However, the intestine rather than the kidney appears to play a major role in arginine metabolism in the suckling period (Herzfeld and Raper, 1976; Hurwitz and Kretchmer, 1986). Accordingly, the F/A transgenic mice, which express different levels of arginase in their enterocytes, suffer from a graded reduction in circulating arginine concentration, in particular during the suckling period. The functional significance of the intestine in arginine metabolism is not yet fully understood, but the degree to which hair and muscle growth (De Jonge et al., submitted), and B-cell maturation (De Jonge et al., submitted) are disturbed in F/A transgenic mice, is directly related to the level of arginase that accumulates in the enterocytes. We now report that circulating arginine levels in F/A transgenes remain low after the capacity to synthesize arginine in the gut ceases to exist, probably due to the continued breakdown of circulating arginine by intestinal arginase. The accelerated disappearance of injected arginine in the transgenic mice shows that the capacity to catabolize circulating arginine already exists in suckling F/A mice. For this reason, we do not know at present to what extent overexpression of arginase in the enterocytes depresses circulating arginine by interfering with local biosynthesis or by increasing catabolism of circulating arginine. We also do not know why circulating arginine levels are lower in male than in female transgenic mice.

Arginine belongs to the group of amino acids, of which the circulating concentration declines concurrent with the declining growth rate in the course of postnatal development. The expression of arginase in enterocytes is sufficient to completely abolish the high circulating arginine levels in the suckling period,
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suggesting that the observed phenotype was a direct consequence of arginine deficiency. The mechanism by which low arginine concentrations prevent hair and muscle growth, remains to be solved. Because extremely arginine-rich proteins such as trichohyalin, are present in normal amounts in F/A-2 mice (De Jonge et al., submitted), the most obvious explanation, that is the precursor function of arginine for protein synthesis cannot account for the phenotype of hypoargininemia. Arginine is also known as a secretagogue of growth-promoting hormones, such as growth hormone (Barbul et al., 1983) and insulin (Palmer et al., 1975), via mechanisms dependent, as well as independent of the production of nitric oxide from arginine (Jun and Wennmalm, 1994). However, animals of the F/A-2 line show catch-up growth after weaning, even though the concentration of circulating arginine remains low. The association of this delayed growth spurt with the 50% increased concentration of virtually all amino acids, is probably not a coincidence. Together, these data suggest that the ratio between arginine and the other members of the age-dependent group of amino acids determines whether or not growth is inhibited. This hypothesis is supported by the difference in growth behavior of F/A-1, which grow normally, and F/A-2 mice, which are severely affected, even though the additional decrease of arginine levels is modest. We are presently following these and other leads.

Another intriguing observation is the rise in circulating arginine levels in adult F/A females, but not in males. The effect is more pronounced in the F/A-2 than in the F/A-1 line. Since the intestinal arginase expression is similar in both sexes and since arginine biosynthesis by e.g. the kidneys can be adaptively increased (Prins et al., 1999), this finding suggests that females are able to mount a more effective adaptive increase in arginine biosynthesis than males. Alternatively, the requirements for arginine may be higher in males than in females, e.g. for creatine synthesis, because creatine accumulates to very high levels in testis and muscle ((Moore, 2000); see also next paragraph). Since this gender difference is also observed for the other age-dependent amino acids in the
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F/A transgenics, the cause may also be more general, e.g. a lower rate of amino acid catabolism in female arginine-deficient animals. Irrespective, this gender difference may well be of clinical importance, since a common disease such as atherosclerosis, has been hypothesized to represent an arginine-deficiency disease (Cooke, 1998) and since males are more prone to develop atherosclerosis than females (Clarkson et al., 1985). In view of the atheroprotective effect of estrogens (Elhage et al., 2000), future studies should also address the interaction between estrogens and arginine metabolism.

The instant hydrolysis of newly synthesized and circulating arginine by transgenic arginase in the enterocytes of suckling F/A mice leads to a drop of circulating and tissue arginine levels. As described in Chapter III, arginine deficiency was associated with an increase in the levels of ornithine and glycine, when only the preweaning period is considered. The inclusion of the postweaning and adult mice in the current analyses reveals genotype-dependent increases in the levels of ornithine, glycine, serine, tryptophan, threonine and phenylalanine. The 40% increase in circulating glycine may well be due to the diminished synthesis of creatine. Creatine plays an essential role in the energy metabolism of muscle, nerve and testis (Walker, 1979), and accounts for a sizable portion of arginine catabolism (Visek, 1986). Moreover, GAA levels in brain, liver and kidney reach maximum levels in the suckling period (Watanabe et al., 1985), underscoring the importance of arginine metabolism for CT synthesis in this period. The diminished synthesis of creatine is due to the decreased concentration of available arginine and not to feedback inhibition of the transaminidase reaction by creatine or ornithine. Conceivably, ornithine concentration could be increased as a result of the increased flux through arginase, but increased ornithine levels were only observed in intestine, where creatine levels are not altered and transaminidase activity is virtually absent (McGuire et al., 1986). The genotype-related increases in serine, threonine and phenylalanine were found to be solely due to their increased level at 5-10 weeks concentrations in F/A-2 animals. Since all amino acids except arginine were elevated at this time (Fig 2F), we do not attach too
much importance to it. At present, we have no explanation for the increase in circulating tryptophan. Interestingly though, a similar increase in tryptophan was found in brain of OTC deficient spf mice (Bachmann and Colombo, 1984).

The absence of an increased urea concentration in tissue, plasma or urine is at first glance surprising in view of the overexpression of arginase in the enterocytes and the ensuing consumption of body arginine content. However, as also shown by the extremely low urinary urea content, amino acid catabolism in the suckling period is very low (Blommaart et al., 1993). Hence, dietary and endogenously synthesized arginine largely ends up in protein or creatine. Loss via the creatine/creatinine pathway virtually stops in the F/A mice. Arginine content in rodent protein is 3.5-4% (Davis et al., 1993), so that arginine degradation in the intestine only represents a tiny fraction of the total substrate for urea synthesis.

**Arginine deficiency and guanidino compounds**

The decreased arginine availability in F/A mice decreased the concentration of guanidino compounds that form via the transaminidation pathway, but caused an accumulation of GSA, MG and G. Strictly speaking, our data do not allow conclusions about fluxes. However, we observed that 6 hours after an arginine injection, the concentration of arginine and the arginine metabolites urea, GAA, β-GPA, γ-GBA, CTN, α-K-δ-GVA, ArgA, GSA, and G were still substantially increased in plasma of wild-type mice, but were similar to the corresponding control animals in F/A-2 mice, with animals from the F/A-1 line taking an intermediate position. The plasma levels of these compounds therefore correspond with the actual availability of arginine instead of with the administered arginine load, in other words, are a parameter for flux. The exception is homoarginine, which is not a metabolite of arginine. This conclusion implies that the decline in arginine concentration, and not the accumulation of transaminidation products (McGuire et al., 1984; Sipila, 1980), determines the decrease in flux through transamidinase.
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The increase in tissue, plasma and urinary GSA levels and urinary G levels corresponds with the increased severity of arginine deficiency. The higher concentration of GSA and G in the urine of transgenic mice upon arginine loading confirms that the formation of these compounds is enhanced under arginine-deficient conditions. Increased α-NAA, GSA, MG and G levels are also observed in renal failure (Cohen et al., 1968; De Deyn et al., 1986; Marescau et al., 1997), subtotal nephrectomy (Al Banchaabouchi et al., 1998; Levillain et al., 1995), endotoxin treatment (Deshmukh et al., 1997), and after consumption of an arginine-free diet by strict carnivores (Deshmukh and Rusk, 1989). As in the diseased states mentioned, the contribution of GSA and/or MG and G to the development of the arginine-deficient phenotype remains to be established.

It has been proposed that GSA is a transamidination product of arginine, when aspartate, instead of glycine, is the amide donor (Cohen et al., 1968). However, since the flux through transaminidase is slowed down due to arginine deficiency in F/A mice, this explanation is not favored. GSA concentrations positively correlate with plasma urea (Marescau et al., 1992a). Urea, in turn, inhibits argininosuccinate lyase (Menyhart and Grof, 1977), suggesting that argininosuccinate may accumulate locally. The hypothesis that GSA, as well as MG, form upon interaction of argininosuccinate and creatine with free radicals (Aoyagi et al., 1996) is therefore more attractive, the more since it has been shown that free radicals form in the neonatal intestine (Musemeche et al., 1993), and probably elsewhere under catabolic conditions.

Arginine deficiency and behavioral deficits

F/A transgenic mice displayed hyperactivity as well as several more specific deficits in coordinated neuromotor abilities. Since passive avoidance learning was also impaired in these animals, although the motor requirements of this task are minimal, these deficits are probably not caused by muscular problems. Behavioral deficits, comparable with the ones seen in the F/A mice, have been associated with increased levels of urea and CTN in nephrectomized mice (Al Banchaabouchi et
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al., 1999). Uremic toxins, in particular GSA, affect cerebral neurotransmitter systems and are thought to cause the psychomotor deficits seen in uremic encephalopathy (D'Hoooge et al., 1996). Behavioral alterations, similar to those seen in F/A mice, were found in spf-mutant mice (Batshaw et al., 1995). Unlike F/A mice, these mice suffer from increased ammonia levels. However, brain ammonia, urea, CTN and GSA concentrations are unchanged in F/A mice, virtually excluding these factors as causes of their neuromotor deficits. Similarly, GSA levels were not elevated in brain of spf mice (Batshaw et al., 1995). However, we cannot presently exclude that the slightly elevated circulating levels of tryptophan, a precursor for cerebral production of serotonin and quinolinate, a known excitotoxin (Batshaw et al., 1993), are responsible for the observed neuromotor deficits in F/A mice. Likewise, elevated brain tryptophan levels in spf mice (Bachmann and Colombo, 1984) have been associated with a two-fold increase in brain quinolinic acid, neuropathology (Robinson et al., 1995), and impaired passive avoidance (Batshaw et al., 1995).

As arginine is required for NO synthesis in neurons, the low level of circulating arginine in the F/A mice may be limiting for NO synthesis. Limitation of cerebral NO synthesis has been shown in spf mice, which also suffer from arginine deficiency (Ratnakumari et al., 1996). A hampered NO synthesis impairs synaptic plasticity, motor coordination and memory functions (O'Dell et al., 1994; Son et al., 1996). Neuronal NOS-deficient mice show behavioral alterations, but unlike F/A transgenics, wire suspension, open field activity, pole equilibrium and several other neuromotor tests were not altered in these animals (Nelson et al., 1995). Furthermore, mice lacking nNOS, especially males, were reported to be extremely aggressive, but we did not observe any signs of this aggressive behavior in F/A mice.
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Conclusion

F/A transgenic mice suffer from a life-long deficiency in circulating arginine and lasting behavioral deficits. Despite a low circulating arginine level, F/A-2 transgenics show a catch-up growth after weaning, which is temporally related to increased circulating levels of all other amino acids. Arginine levels in adult F/A females are higher than in adult F/A males, possible as a result of the extra requirement for CT synthesis in muscle and testis. Low levels of arginine lead to a decrease in the flux through transaminidase and accumulation of GSA and G, probably as a result of a higher oxidative stress under hypoargininemic conditions.

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