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ARTICLE

An AAV9 coding for frataxin clearly improved the symptoms and prolonged the life of Friedreich ataxia mouse models

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Friedreich ataxia (FRDA) is a genetic disease due to increased repeats of the GAA trinucleotide in intron 1 of the frataxin gene. This mutation leads to a reduced expression of frataxin. We have produced an adeno-associated virus (AAV)9 coding for human frataxin (AAV9-hFXN). This AAV was delivered by intraperitoneal (IP) injection to young conditionally knockout mice in which the frataxin gene had been knocked-out in some tissues during embryogenesis by breeding them with mice expressing the Cre recombinase gene under the muscle creatine kinase (MCK) or the neuron-specific enolase (NSE) promoter. In the first part of the study, different doses of virus were tested from 6×10^{11} v.p. to 6×10^{9} v.p. in NSE-cre mice and all leading to an increase in life spent of the mice. The higher and the lower dose were also tested in MCK-cre mice. A single administration of the AAV9-hFXN at 6×10^{11} v.p. more than doubled the life of these mice. In fact the MCK-cre mice treated with the AAV9-hFXN were sacrificed for further molecular investigations at the age of 29 weeks without apparent symptoms. Echography analysis of the heart function clearly indicated that the cardiac systolic function was better preserved in the mice that received 6×10^{11} v.p. of AAV9-hFXN. The human frataxin protein was detected by ELISA in the heart, brain, muscles, kidney, and liver with the higher dose of virus in both mouse models. Thus, gene therapy with an AAV9-hFXN is a potential treatment of FRDA.

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INTRODUCTION

Friedreich ataxia (FRDA) is an autosomal recessive neurodegenerative and cardiac disease, caused by a trinucleotide (GAA) repeat expansion in the first intron of the frataxin gene located in chromosome 9.[1] The mutation in the intron 1 of the frataxin gene leads to a reduced expression of the frataxin gene without changing the protein. The mechanism of this pathology has been reviewed by Pandolfo *et al.*[2,3] Neurons and cardiomyocytes are particularly sensitive to the reduction of frataxin.[4–6] Thus, neurological and cardiac symptoms appear in or before the second decade of life.[7–11] There are also some systemic involvements, such as diabetes mellitus and scoliosis. Cardiomyopathy and associated arrhythmias lead to early death.[9,12]

Mice homozygous for a conditional frataxin allele (Frda exon 4 located between 2 LoxP, named $Frda^{L3/L3}$) have been crossed by Puccio *et al*.[13] with mice heterozygous for the deletion of Frda exon 4 (Frda L-/L3) that also carried a tissue-specific Cre transgene under the control of the muscle creatine kinase (MCK) or the neuron-specific enolase (NSE) promoter. The MCK promoter induced a knockout of the exon 4 in the heart and the striated muscle and the resulting mice were called MCK-cre. The NSE promoter lead to a knockout of the Frda exon 4 not only in the brain but also in the heart, muscles, kidney, and liver and the resulting mice were designated as NSE-cre. These mice reproduced important progressive pathophysiological and biochemical features of the human disease: cardiac hypertrophy without skeletal muscle involvement. The NSE mice also express large sensory neuron dysfunctions without alteration of the small sensory and motor neurons.

In the present article, we tested different quantity of virus from 6×10^{9} virus particles (v.p.) to 6×10^{11} v.p. The injection of the virus clearly prolonged the life of the treated mice for all the quantity tested. Also, the administration of 6×10^{11} v.p. of an adeno-associated virus (AAV)9 coding for the human frataxin to the MCK-cre and to the NSE-cre not only increased the expression of the frataxin protein in these mouse models of FRDA but clearly reduced their heart hypertrophy and improved their heart function. We are thus proposing that an AAV coding for frataxin could be a treatment for FRDA.

RESULTS

Confirmation of the frataxin gene knockout in the experimental mice

PCR amplifications of the genomic DNA of various tissues were made to verify the KO of the frataxin gene (Supplementary Figure S1). The delta allele (L-) resulted in a 263 bp band due to exon 4 excision. Different organs were analyzed: the muscle (*Tibialis anterior*),

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the liver, the heart, the kidneys, and the brain. For the MCK-cre mice, a delta allele band was observed only in the muscle and in the heart corresponding with the specificity of the MCK promoter (Supplementary Figure S1b on the left). Sometimes, a very light delta band (L-) appeared in the brain of some animals. For the NSE-cre mice, a delta band was observed in all the investigated tissues (the brain, the liver, the heart, the skeletal muscles, and the kidney) (Supplementary Figure S1b on the right).

The expression of the frataxin gene was also investigated by RT-PCR in several tissues. The upper 332 bp band corresponded to the unfloxed frataxin mouse mRNA (Supplementary Figure S1c, left side). A second band was observed under 332 bp. As indicated by sequencing this lower band (designated as floxed band) corresponded to the mouse frataxin mRNA deleted of exon 4. For the MCK-cre mice, the unfloxed frataxin band was observed in liver, kidney, and brain and the floxed band was observed only in muscles and heart.

In the NSE-cre mice, the floxing of the conditional frataxin gene was different (Supplementary Figure S1c right side). For all these mice, we observed only the floxed mRNA in the heart. In the other organs of these NSE-cre mice, most of the time two bands were observed indicating that the frataxin gene was not floxed in all cells. The floxed frataxin allele was more abundant in the brain than in the muscle, liver, and kidneys. These results indicated that NSE-cre mice have a complete KO of the frataxin gene in the heart and a partial KO in the other organs investigated.

Table 1 Animal score

Score	*Characteristics*	*Action*
0	Lively and active animal	None.
	Smooth coat	
	No lack of comfort	Observe once per week.
1	Mouse moves normally but often on the tip of their toes.	Wet food into the cage.
	May have the back lightly curved.	Observe daily.
2	Mouse moves normally but on the tip of their toes.	Wet food into the cage.
	Mouse mostly lively.	Observe twice a day.
	Hair slightly spiky.	
	Back curved.	
	Could have a abnormal breathing but not difficult.	
3	Mouse has difficulties to walk and moves less.	Wet food into the cage.
	Back curved and hair very spiky.	Observe twice a day.
	Difficulties to eat.	
	Could have an abnormal and difficult breathing.	Should be sacrificed within 4 hours.
4	Mouse does not move or moves with pain.	Should be sacrificed immediately.
	Very spiky hair.	
	Could have lifeless, sunk or closed eyes.	
	Difficulties to eat.	
	Could have a breathing insufficiency.	

PART 1: study on the NSE-cre mice

Behavior of the experimental mice without the AAV9-hFXN treatment. The NSE-cre were initially normally active but had signs of kyphosis and they walked on the tip of their toes (score 1, Table 1). After weaning, they showed a rapid degeneration of their physical condition. They progressively became more curved, their fur appeared ruffled (bent back, bristly hairs) and finally their general activity decreased. At the end of their life, they lost progressively some body weight and had difficulties to move, to walk, to breathe and to feed themselves (score 3 and 4, Table 1). They had to be sacrificed due to ethical concerns around 35 ± 18 days (mean ± SEM) (Figure 1).

Effects of AAV9-FXN treatment on body weight. The growth curve of the NSE-cre was followed for the treated and not treated mice. The NSE-cre curve was significantly lower than that of the L3/L3 mice (Figure 1). When these mice received the AAV9-hFXN at 5–9 days, their growth curves were improved compared to the NSE-cre mice that did not receive the treatment. This was observed for all the viral doses tested. However, they did not reach the body weight of the L3/L3 mice for male (Figure 1a) and female mice (Figure 1b). Only the males receiving 6×10^{11} v.p. (d1/10) seem to follow the curve of the L3/L3 but there were only three mice in this group.

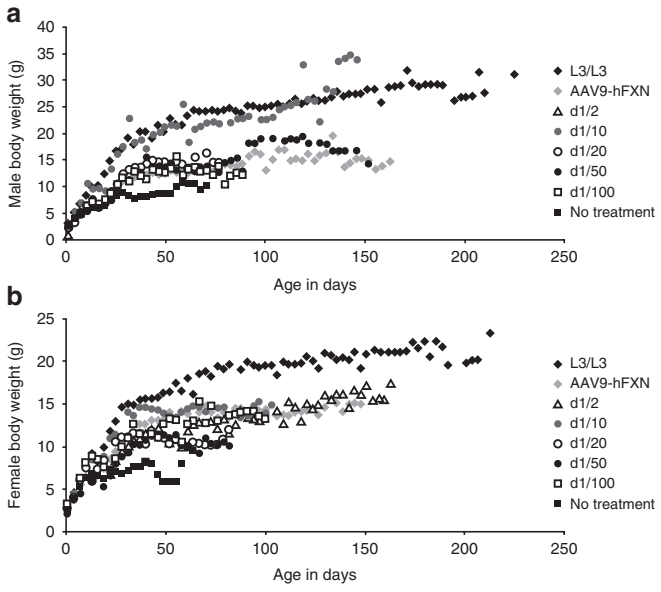

Figure 1 Evolution of neuron-specific enolase (NSE)-cre mouse weight. The body weight of the different groups of mice was followed until their sacrifice. In **a**, the male mice: L3/L3 ($n = 26$), NSE-cre mice treated with the AAV9-hFXN at 6×10^{11} v.p. (named AAV9-hFXN; $n = 12$), at 3×10^{11} v.p. (named d1/2; $n = 0$), at 6×10^{10} v.p. (named d1/10; $n = 3$), at 3×10^{10} v.p. (named d1/20; $n = 2$), at 1.2×10^{10} v.p. (named d1/50; $n = 5$), at 6×10^{9} v.p. (named d1/100; $n = 3$) and the untreated mice (named no treatment; $n = 33$). In **b**, the female mice: L3/L3 ($n = 20$), NSE-cre mice treated with the AAV9-hFXN at 6×10^{11} v.p. ($n = 17$), at 3×10^{11} v.p. ($n = 3$), at 6×10^{10} v.p. ($n = 2$), at 3×10^{10} v.p. ($n = 3$), at 1.2×10^{10} v.p. ($n = 3$), at 6×10^{9} v.p. ($n = 4$) and the untreated mice ($n = 33$). The untreated NSE-cre had the lowest body weight. The growth curve of the NSE-cre mice was clearly improved by the AAV9-hFXN treatment for both sex groups.

Detection of human frataxin after the AAV9-hFXN treatment. An AAV9 coding for the human frataxin (AAV9-hFXN) was injected IP in NSE-cre mice between 5 and 9 days of age. Their organs were collected at the time of the sacrifice. A PCR for the human frataxin was made on genomic DNA to detect the presence of the human frataxin transgene. This transgene was detected in all the investigated tissues (*i.e.*, brain, heart, muscle, kidney, and liver) for 6×10^{11} v.p. injected (Figure 2a on the left). Dilutions 1/10 and 1/20 were also tested in the same conditions. The human frataxin band was observed in muscle, liver, and heart for these dilutions. The intensity of the band decreased with the dilutions, but the strongest band was always detected in the heart. RT-PCR also produced similar results (Figure 2a on the right). For an injection of 6×10^{11} v.p., human frataxin expression was observed in all tissues tested, with the strongest band in the heart and the intensity of the bands were decreasing with the dilutions of the virus.

Finally, the presence of human frataxin protein was investigated using a specific dipstick test. No human frataxin was detected in L3/L3 mice and in model mice that were injected with saline rather than with the AAV9-hFXN (data not shown). However, the hFXN was detected in all tissues from both mouse models injected with 6×10^{11} v.p of AAV9-hFXN (Figure 2b). With the dilution, the intensity of the band diminished in all organs except for the heart where the intensity remained strong with the different quantities of virus. The human frataxin was not observed in the brain for the three lower doses of virus, probably because the limit of detection was reached.

Effects of AAV9-hFXN treatment on mouse survival. A score from 0 to 4 was created to estimate the NSE-cre health (0 corresponding to no symptoms and 4 a state of health requiring immediate sacrifice; cf Supplementary Information). The NSE-cre mice were evaluated every day after the weaning (21 days) because of the great variability of the progression of the disease and mortality. When the mice were treated with the AAV9-hFXN, the score was around 1.5 at 21 days, while the average score was 2.0 for the untreated NSE-cre. However, the score of the treated NSE-cre mice decreased (*i.e.*, they were less sick) over the following 3 days while the score of the untreated mice increased. The scores were significantly different between the two groups between 21 and 40 days. The time between the injection and the first score of 0 (*i.e.*, no symptom) was also estimated. In average, 20 ± 5.8 days after the viral injection for 6×10^{11} v.p. and 15.7 ± 2.6 days for 6×10^{9} v.p., all the symptoms of the disease had disappeared (Figure 3a). Some mice reached a 0 score but were later scored at 0.5 or 1. For the estimation of the time where no symptoms were detected, we considered the first time the mouse score reached 0. However, these NSE mice have later developed

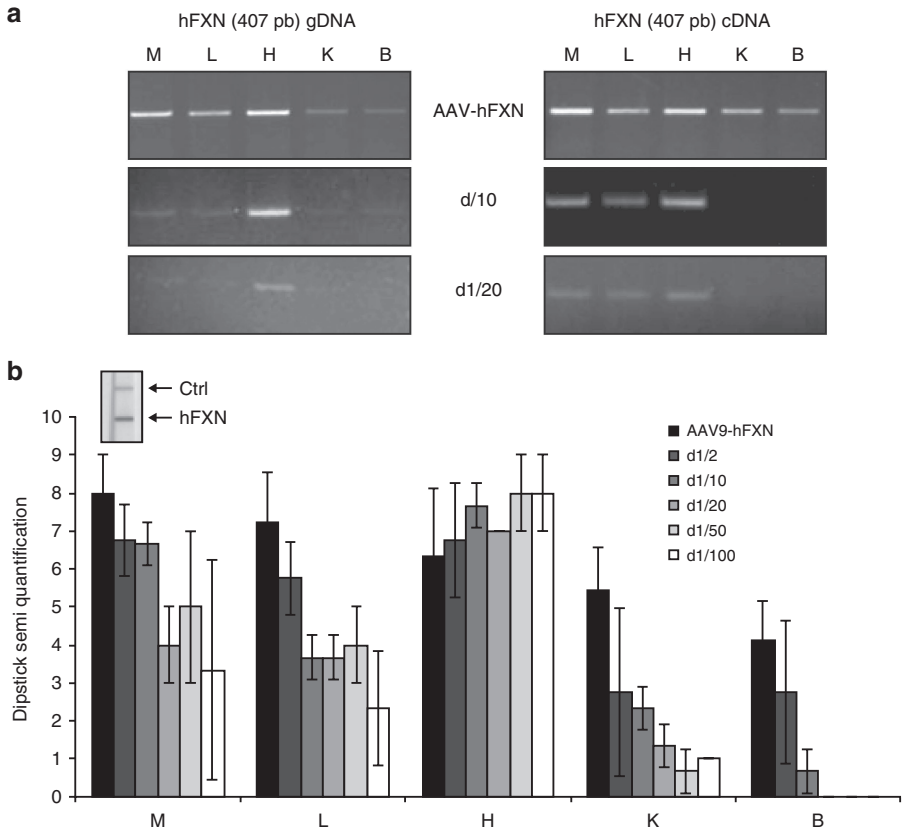

Figure 2 Detection of the hFXN transgene in various tissues of neuron-specific enolase (NSE)-cre. (**a**) on the left: The human frataxin (hFXN) transgene was detected by polymerase chain reaction (PCR) in the DNA of muscles (M), L (liver), heart (H), kidney (K), and brain (B) of mice injected with AAV-hFXN at 6×10^{11} v.p. (named AAV-hFXN) and in 6×10^{10} v.p. (named d1/10) and in 3×10^{10} v.p. (named d1/20). **a**, on the right: The expression of the human frataxin transgene (hFXN) was then detected by RT-PCR in all these tissues. (**b**) Finally, an ELISA test (Dipstick) was made to detect the human FXN protein in skeletal muscle (M), liver (L), heart (H), kidney (K), and brain (B). The human frataxin protein was estimated in these tissues in NSE-cre mice treated with AAV9-hFXN at 6×10^{11} v.p. ($n=9$), at 3×10^{11} v.p. ($n=4$), at 6×10^{10} v.p. ($n=3$), at 3×10^{10} v.p. ($n=3$), at 1.2×10^{10} v.p. ($n=3$), at 6×10^{9} v.p. ($n=3$). The presence of human frataxin was strong with the highest dose of virus and decreased in the tissues with the dilutions except for the heart where the frataxin was still strong at the lowest viral dose.

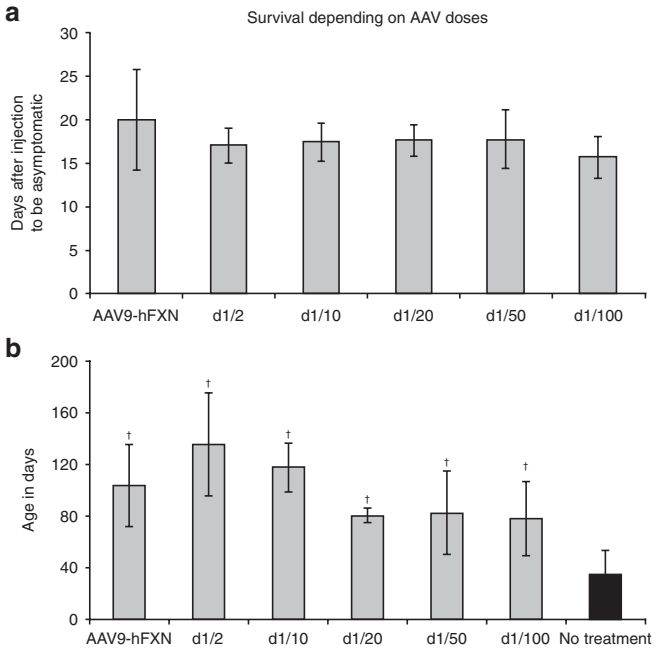

Figure 3 Improved life survival of the neuron-specific enolase (NSE)-cre mice treated with AAV9-frataxin. (**a**) A score was created to evaluate daily the health of the NSE-cre mice. The score goes from 0, which corresponds to a normal phenotype, to 4, where the signs of discomfort (loss of weight, difficulties to walk or to feed) led to the sacrifice of the mice. The score started after weaning, at 21 days. The delay between the injection and a score of zero (no symptoms) was determined for all doses of AAV9-hFXN at 6×10^{11} v.p. (named AAV9-hFXN; $n = 14$), at 3×10^{11} v.p. (named d1/2; $n = 7$), at 6×10^{10} v.p. (named d1/10; $n = 5$), at 3×10^{10} v.p. (named d1/20; $n = 5$), at 1.2×10^{10} v.p. (named d1/50; $n = 8$), at 6×10^{9} v.p. (named d1/100; $n = 6$). (**b**) The survival was also estimated for all these groups. The injection of AAV9-hFXN clearly improved the general health of the NSE-cre mice for all viral doses ($^{\dagger}P \leq 0.0001$): AAV9-hFXN at 6×10^{11} v.p. ($n = 18$), at 3×10^{11} v.p. ($n = 5$), at 6×10^{10} v.p. ($n = 4$), at 3×10^{10} v.p. ($n = 5$), at 1.2×10^{10} v.p. ($n = 8$), at 6×10^{9} v.p. ($n = 6$) when compared to the untreated mice ($n = 58$).

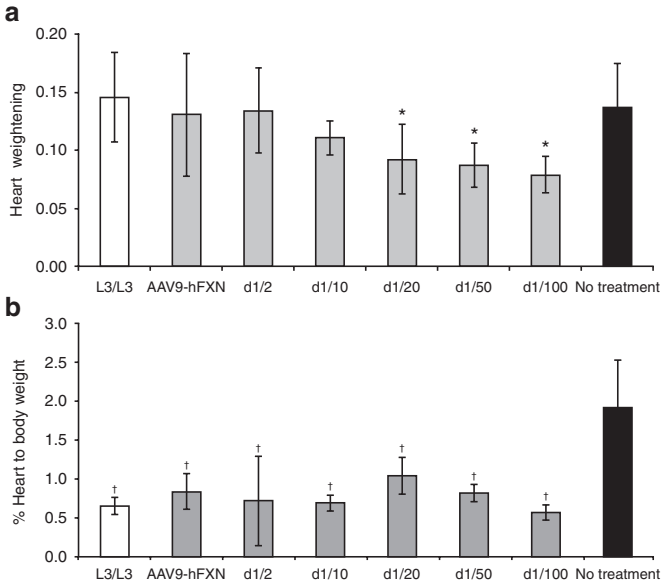

Figure 4 AAV-hFXN treatment reduced heart hypertrophy in neuron-specific enolase (NSE)-cre mice. The weight of the mouse heart treated or not with the AAV9-hFXN was measured (**a**) and expressed at sacrifice as a percentage of the whole body weight (**b**). The groups were composed of L3/L3 ($n = 20$), NSE-cre treated with AAV9-hFXN at 6×10^{11} v.p. (named AAV9-hFXN; $n = 11$), at 3×10^{11} v.p. (named d1/2; $n = 5$), at 6×10^{10} v.p. (named d1/10; $n = 4$), at 3×10^{10} v.p. (named d1/20; $n = 5$), at 1.2×10^{10} v.p. (named d1/50; $n = 8$), at 6×10^{9} v.p. (named d1/100; $n = 6$) and untreated mice ($n = 27$). The weight of the heart was significantly different from the control L3/L3 and from the untreated mice for the three lowest quantity of virus ($^{*}P < 0.05$; d1/20, d1/50, and d1/100) (**a**). The normalized weight was similar to that of L3/L3 for all the treated mice and significantly different ($^{\dagger}P \leq 0.0001$) from that of the nontreated NSE-cre mice (**b**).

nervous system symptoms, their score rapidly increased and they had to be sacrificed for ethical concerns.

The survival of the mice was also evaluated after the AAV9-hFXN injection. It is important to note that some mice were sacrificed voluntary to investigate cardiac parameters while other had to be sacrificed for ethical concerns. For treated NSE-cre mice injected with 6×10^{11} v.p., 6 mice from the 18 mice in that group were voluntary sacrificed at 96 and 122 days. Despite the voluntary sacrifice of some mice for the two highest quantities of virus injected, the average survival of the NSE-cre mice was significantly increased from 35 ± 18 days (untreated) to 103 ± 32 and 135 ± 40 with the injection of 6×10^{11} v.p. and 3×10^{11} v.p. of AAV9-hFXN respectively (Figure 3b). All the quantities of virus led to a significant increase of the survival compared to the untreated mice. The survival was doubled with v.p. dilutions from 1/100 to 1/20. The 6×10^{11} v.p dose of virus increased the survival by roughly threefolds.

Effects of AAV9-hFXN treatment on cardiac function. At the time of sacrifice, the weight of the heart of the mice treated or not with the different doses of AAV9-hFXN was measured (Figure 4a). There was no significant heart weight difference between the L3/L3, the NSE-cre, and the treated NSE-cre mice with 6×1011 v.p. to 6×10^{10} v.p. (d1/10). However, because of the difference in body weight, we also expressed the heart weight as a percentage of the total body weight (Figure 4b). This percentage was 0.57 ± 0.11 for L3/L3 mice. The effect of the treatment was strong for all the NSE-cre treated mice, the percentage passing from 1.91 ± 0.61 (not treated) to 0.81 ± 0.23 with the AAV9-hFXN treatment 6×10^{11} v.p. All the treated mice were significantly different from the nontreated mice and not significantly different from the L3/L3 mice.

PART 2: study on the MCK-cre mice

The results obtained in the first part with the NSE-cre mice showed a strong presence of human frataxin protein in the heart of the treated mice with a single injection of 6×10^{9} v.p. For the study of the MCK-cre mice, we thus decided to focus only on the highest and lowest doses of virus because the major health problem of the MCK-cre is due to the deletion of frataxin in heart.

Effects of AAV9-FXN treatment on body weight. The MCK-cre mice followed the same body weight growth curve as the L3/L3 mice. The growth curve of these MCK-cre mice was not modified by the administration of AAV9-hFXN at 6×10^{11} v.p. and 6×10^{9} v.p. (d1/100) (Figure 5a,b).

Detection of human frataxin after the AAV9-hFXN treatment. The presence of the virus was detected by PCR in genomic DNA (Figure 6a on the left) and by RT-PCR (Figure 6a on the right) following a treatment with 6×10^{11} v.p. As for NSE-cre mice, the strongest band was observed for the heart tissue. The presence of

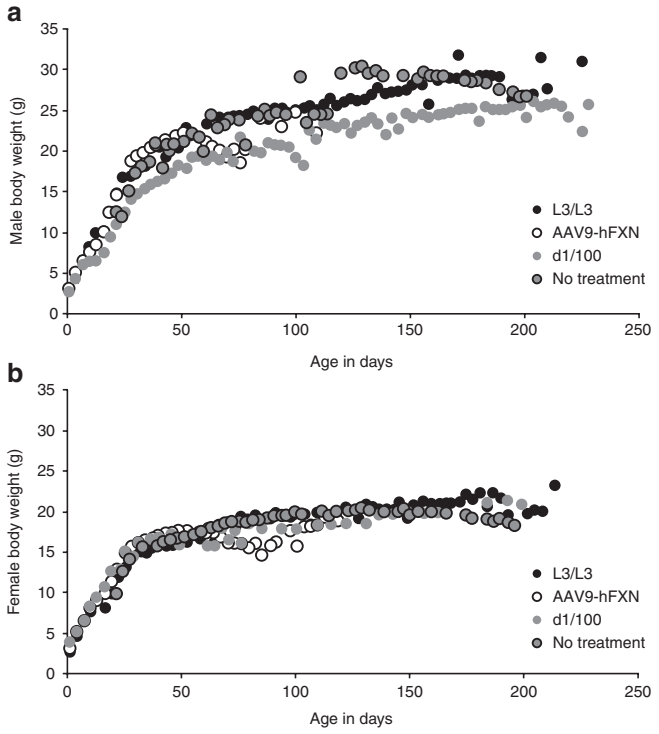

Figure 5 Evolution of muscle creatine kinase (MCK)-cre mouse weight. In **a**, the growth curves of the male mice: L3/L3 mice ($n = 26$), MCK-cre untreated (no treatment; $n = 67$) and MCK-cre mice treated with the AAV9-hFXN at 6×10^{11} v.p. (AAV9-hFXN; $n = 8$) as well as at 6×10^{9} v.p. (d1/100; $n = 2$). In **b**, the growth curves of the female mice: L3/L3 mice ($n = 20$), MCK-cre untreated ($n = 47$) and MCK-cre mice treated with the AAV9-hFXN at 6×10^{11} v.p. ($n = 7$) as well as at 6×10^{9} v.p. ($n = 3$). There was no difference between the L3/L3 and the different MCK-cre mice groups.

human frataxin protein was also detected with the dipstick method (Figure 6b). Following the injection of 6×10^{11} v.p. of AAV9-hFXN, the presence of frataxin was observed in all tissues tested, the stronger bands being in muscle, liver, and heart. Following the administration of 6×10^{9} v.p., the human frataxin protein was still observed in all the organs except in the brain where no protein was detected. However, the frataxin concentrations were lower than following the administration of the higher quantity of virus.

Effects of AAV9-hFXN treatment on survival and cardiac function. The MCK-cre mice had no behavioral difference with the L3/L3 mice when they were young. Indeed, their behavior and body weight gain were similar to those of L3/L3 mice. However, the mouse health regressed suddenly after 50 days. They had periods of difficulty to breathe, to walk and within 24–48 hours they had to be sacrificed due to ethical concerns. The age of sacrifice varied between 71 ± 20 days (mean ± SEM) (Figure 7a). 10 MCK-Cre mice treated with 6×10^{11} v.p. of AAV9-hFXN were voluntary sacrificed at 203 or 251 days. For MCK-cre mice, the survival went from 71 ± 20 days without treatment to 194 ± 67 days following a 6×10^{11} v.p. AAV treatment (Figure 7a). Both doses of AAV9-hFXN (*i.e.*, 6×10^{11} v.p. and 6×10^{9} v.p.) significantly improved the survival of these mice. The weight of the heart was also measured as well as the body weight. The heart weight of MCK-cre was significantly higher than that of control L3/L3 mouse heart, due to a clear cardiac hypertrophy (Figure 7b). Both doses of AAV9-hFXN reduced heart weight. The heart weight was expressed as a percentage of the total body weight. This percentage was higher for the untreated mice than for the control L3/L3 mice (Figure 7c). It was however significantly decreased with both doses of virus.

PART 3: behavior study and echocardiography on NSE-cre and MCK-cre injected with 6×10^{11} v.p. of AAV9-hFXN

Behavior and echocardiography tests were also made on untreated mice and mice treated with the highest dose of 6×10^{11} v.p. of AAV9-hFXN because human frataxin was detected in the mouse brain only at that dose.

Effects of AAV9-hFXN treatment on behavior. The following behavior parameters were analyzed: distance traveled, immobile time, rear duration, and episode immobile (Figure 8a–d). The behavior of the MCK-cre mice was not different from that of L3/L3 mice (Figure 8). On the contrary, the NSE-cre mice were clearly different from the L3/L3 for all these parameters. These mice were less active (Figure 8a,c). However, after the injection with the AAV9-hFXN, the NSE-cre mice (NSE-cre-AAV) got back to normal activities (Figure 8a–d). The NSE-cre treated mice appeared quite normal and were clearly different from the NSE mutants that did not receive the AAV9-FXN treatment.

Other more specific tests were also made on these mice but no significant effects were observed between the treated and untreated mice (data not shown).

Effects of AAV9-hFXN treatment on cardiac function. Echocardiography examination was also made on these mice (Figure 9). The injection of AAV9-hFXN ameliorated significantly the stroke volume (a), the cardiac output (b) and the systolic (c) and diastolic (d) diameters of the MCK-cre mice (Figure 9, on the left). The relative wall thickness, the fractional shortening and the fractional ejection were also ameliorated in the MCK-cre treated mice (Supplementary Figures S2 and S3). The injection of AAV9-hFXN had no effect on the stroke volume and the cardiac output of NSE-cre mice. However, the treatment restored the diastolic and systolic diameters of the heart of NSE-cre mice (Figure 9c,d, on the right).

DISCUSSION

FRDA is due to a reduced expression of frataxin following an expansion of a trinucleotide (GAA) repeat in intron 1.[1] Frataxin is a mitochondrial protein and its reduction leads in turn to a mitochondrial miss-function (for a review of the pathological mechanism, see Pandolfo *et al.*[3]). There is increased oxidative stress and accumulation of iron in the mitochondria. These changes, particularly the increased oxidative stress, lead to cell death, including neurons and cardiomyocytes. This progressive cell death results in several neurological and cardiac symptoms.

The transgenic NSE-cre and MCK-cre mouse models were developed by Dr Puccio.[13] The MCK-cre model of FRDA has floxed frataxin gene in the heart and the muscles in which the Cre recombinase is expressed. We have detected no expression of mouse frataxin in these tissues. However, for the NSE-cre mice, there was a complete knockout of the mouse frataxin gene only in the heart and a partial KO in the muscle, liver, kidney, and brain. Our results in the MCK-cre and the NSE-cre confirmed the previously published results.[13]

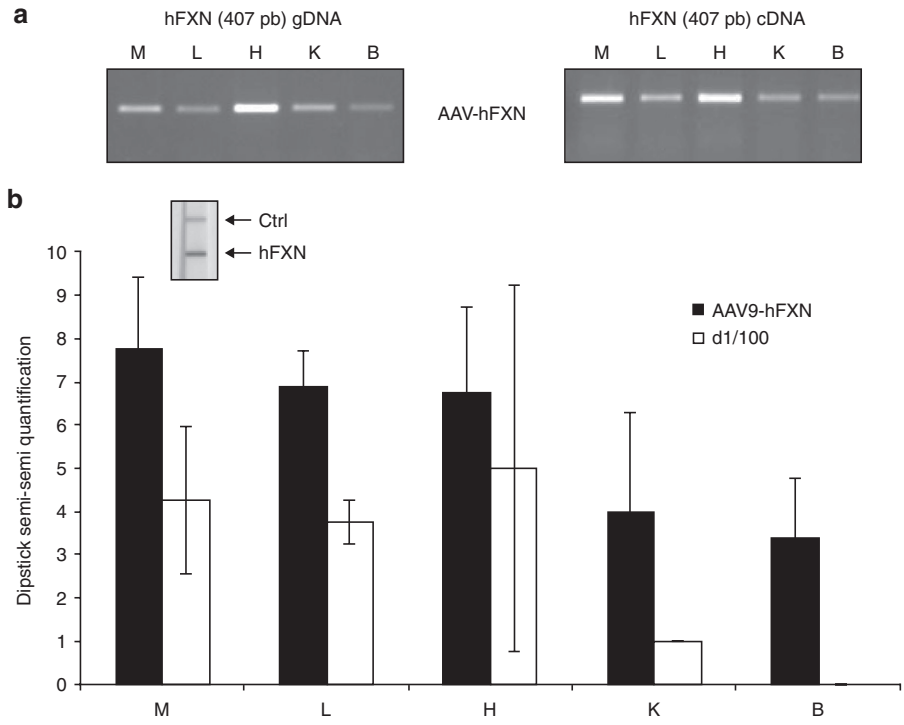

Figure 6 Detection of the hFXN transgene in various tissues of muscle creatine kinase (MCK)-cre. (**a**) The human frataxin (hFXN) transgene was detected by polymerase chain reaction (PCR) not only in the DNA of muscles (M), L (liver), heart (H), kidney (K), and brain (B) of the mice injected with AAV9-hFXN at 6×10^{11} v.p. (on the left) but also the expression of human frataxin (on the right). (**b**) Finally, an ELISA test (Dipstick) was made to detect the human FXN protein in the tissues of MCK-cre mice treated with AAV9-hFXN at 6×10^{11} v.p. (named AAV9-hFXN; $n = 8$) and at 6×10^{9} v.p. (named d1/100; $n = 4$). The presence of human frataxin was strongest with the highest viral doses and decreased in all tissues with the dilution.

The MCK-cre mice were used for this study because they have more specific cardiac symptoms of FRDA compared to the NSE-cre mice, which lack of frataxin in almost every organ. The NSE-cre mice were however useful because of its severe early onset symptoms, which permitted to detect rapidly clear differences following an effective treatment with the AAV9-hFXN.

This absence of frataxin expression starts during the embryological development of the conditional KO mice bred with a mouse expressing the Cre gene. Our results confirmed the previous report that the NSE-cre mice have a lower than normal weight and develop progressive neurological symptoms and hunched stance.[13]

The age of death of the untreated MCK-cre mice was similar in all research groups (76 ± 10 days)[13] and 65 ± 10 days[14] versus 71 ± 20 days for us. In the case of the NSE-cre mice, the age of death was a little higher in our results 35 ± 18 days than in those of Dr Puccio (24 ± 9 days)[13] and Dr Payne groups (28 ± 2 days).[15] The difference in mouse survival could be due to the difference in the mouse housing (the accessibility to the food and after the weaning, a wild type female remained in the cage with the sick mice to help them to make a nest and to take care of themselves).

Both mutants are thus very severe models of the disease and have a short life expectancy without treatment. Our results clearly demonstrated that the IP administration of an AAV9 vector coding for human frataxin significantly increased up to two times the life expectancy of both NSE-cre and MCK-cre mice with high and low quantity of virus. Vyas *et al.*[15] also increased by around 50% the survival of the NSE-cre mice by intrainjection of Tat-frataxin protein twice a week with a maximum of 88 days of survival. In our study, among the 18 NSE-cre mice treated with 6×10^{11} v.p. AAV9-hFXN, only one died at 46 days, the other ones lived more than 74 days and five were sacrificed for the cardiac analysis at 96 and 121 days. The lower AAV dose (6×10^{9} v.p.) increased significantly the survival of NSE-cre mice. The longer survival was obtained for the three highest viral doses even if some mice were voluntarily sacrificed for experiments. For the MCK-cre mice, among the 14 mice treated with 6×10^{11} v.p. AAV9-hFXN, only 4 died between 80 and 116 days, 10 were sacrificed for cardiac analysis (4 mice at 203 days, 4 mice at 251 days, and 2 mice at 253 days). Perdomini *et al.*[14] also increased significantly the survival of the MCK-cre mice through an intravenous injection of 5.4×10^{13} vg/kg of AAVrh10 (this corresponds to about 5×10^{11} v.p. per young mouse) coding for for the human frataxin. The viral dose injected per mouse or rat was around 10^{11} g.c. ou v.p. per animal in many publications.[14,16,17] Bish *et al.*[17] also tested various dilutions of the AAV vector (2.5×10^{11}, 2.5×10^{10}, and 2.5×10^{9} viral particules. They found that the expression in heart was diminished with the dilution contrarily to our results with the NSE-cre where the frataxin protein stayed at high quantity for all the dilution of virus tested in heart (Figure 2b). Our study clearly showed that with a quantity of 6×10^{11} v.p., the frataxin protein was detected in the brain of mice but with only 6×10^{9} v.p., the heart function was still improved as well as the survival of the mice. This suggests that the quantity of virus injected could be adjusted to the targeting organ, with a lower quantity for the heart and a higher quantity to reach the brain.

In Friedreich's patient, a hypertrophic cardiomyopathy was observed due to a thickening of the ventricular walls.[18] In general, the global systolic function is preserved but at the end of their life, the ejection fraction is reduced. The MCK-cre and NSE-cre model reproduce the cardiomyopathy as well as the biochemical features observed in FRDA patients.[13–15] In the MCK model, after 6 weeks, this cardiomyopathy is characterized by an increased in left ventricular end-distolic chamber dimensions and left ventricular end-systolic

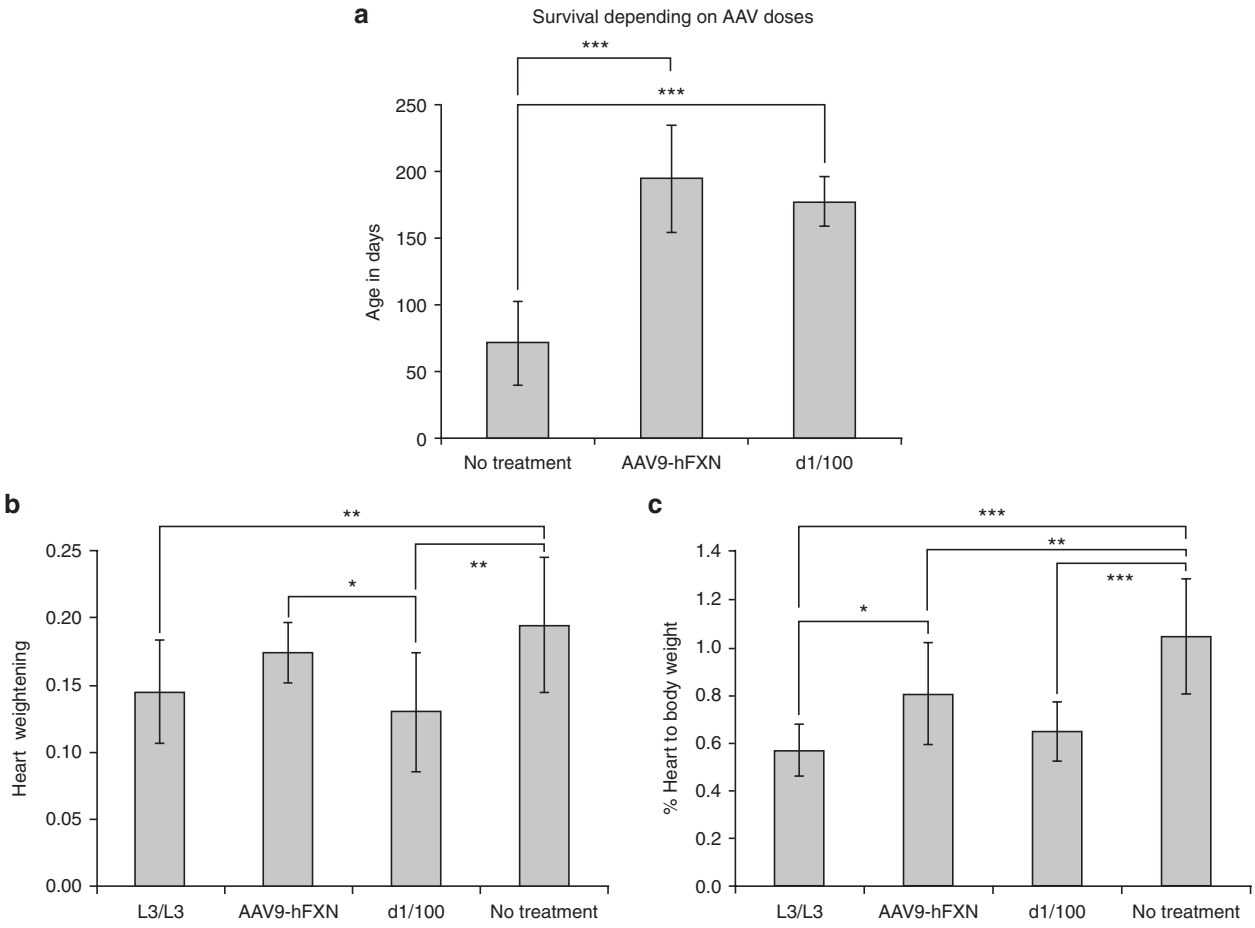

Figure 7 Improved life survival of the muscle creatine kinase (MCK)-cre mice treated with AAV9-frataxin. (**a**) Without treatment, the MCK-cre had to be sacrificed at an average age of 71 ± 20 days (no treatment; $n = 37$). However, the MCK-cre injected intraperitoneally (i.p.) with 6×10^{11} v.p. or 6×10^{9} v.p. of AAV9-hFXN were voluntary sacrificed after 180 days for further investigation. Thus, the average age of sacrifice of the treated mice was significantly higher than that of the untreated mice for both MCK-cre groups treated at the higher ($n = 14$) and the lower ($n = 5$) viral doses. The weight of the mouse heart treated or not with the AAV9-hFXN was measured at the time of sacrifice (**b**) and expressed as a percentage of the whole body weight (**c**). The heart weight of the MCK-cre was higher than that of the L3/L3; this hypertrophy was prevented by the AAV9-hFXN treatment. The normalized heart weights of the untreated MCK-cre mice were higher than that of the wt mice. The treatment with AAV9-hFXN ameliorated the percentage compared to the control and the lowest quantity led to the same value than the control L3/L3. The *** indicate significant difference at P less than 0.0001. $P \leq 0.05$*; $P \leq 0.001$**; $P \leq 0.0001$***.

chamber dimension and a reduction in left ventricular fractional shortening,[19] which were also observed in our study. In our studies, the average age of the untreated MCK-cre mice tested for echocardiography was 70 days (59–71 days for the 11 mice). However, one mouse was evaluated at 131 days corresponding to a late stage since the untreated mice lived an average of 71 days.

The percentage of fractional ejection was significantly reduced from 51% for the wt mice to 32% for the MCK-cre mice, a symptom similar to that of the FRDA patients. The AAV9-hFXN treatment permitted to increase the fractional ejection to 43% without completely restoring the function (Supplementary Figure S3). The same observation was made for the stroke volume, which was reduced in FRDA patient. The stroke volume of wt mice was 0.042 ml, this decreased to 0.02 ml in MCK-cre mice. The treatment of these mice when they were only 5–9 days old with AAV9-hFXN prevented the reduction of the stroke volume. Thus, these treated MCK-cre mice had an average stroke volume of 0.034 ml. The fractional heart shortening is reduced in the MCK-cre mice. This means that the left ventricle in diastole and in systole varies less during the cardiac cycle. The reason for this reduced variation is that the ventricle is contracting less during the systole. The stroke volume is the blood volume ejected by the left ventricle during the systole while the cardiac output corresponds to this volume multiplied by the heart frequency to obtain the ejected volume per minute. The stroke volume of the MCK-cre mice is about only haft of that of normal mice. Thus the MCK-cre mice have cardiac insufficiency. The fractional shortening, the stroke volume and the cardiac output were all significantly improved in the MCK-cre mice treated with AAV9-hFXN.

For the NSE-cre mice, less difference were observed compared to the wt mice. In our study, the stroke volume as well as the cardiac output was significantly lower for the NSE-cre mice while the ejection fraction and the fractional shortening was similar between the wt and the NSE mice (Figure 8 and Supplementary Figures S2 and S3). The same conclusion was made by Vyas *et al.*[15] study by comparing wt and NSE-cre mice. They used Tat-frataxin protein to treat the disease, and even though the survival of the treated mice was increased, these cardiac parameters were not significantly ameliorated, and this was also the case in our study for the stroke volume and the cardiac output.

However, it should be noted that although the AAV9-hFXN was detected as well as the protein in the brain of our mice injected with 6×10^{11} v.p., the mice did develop nervous symptoms (abnormal leg

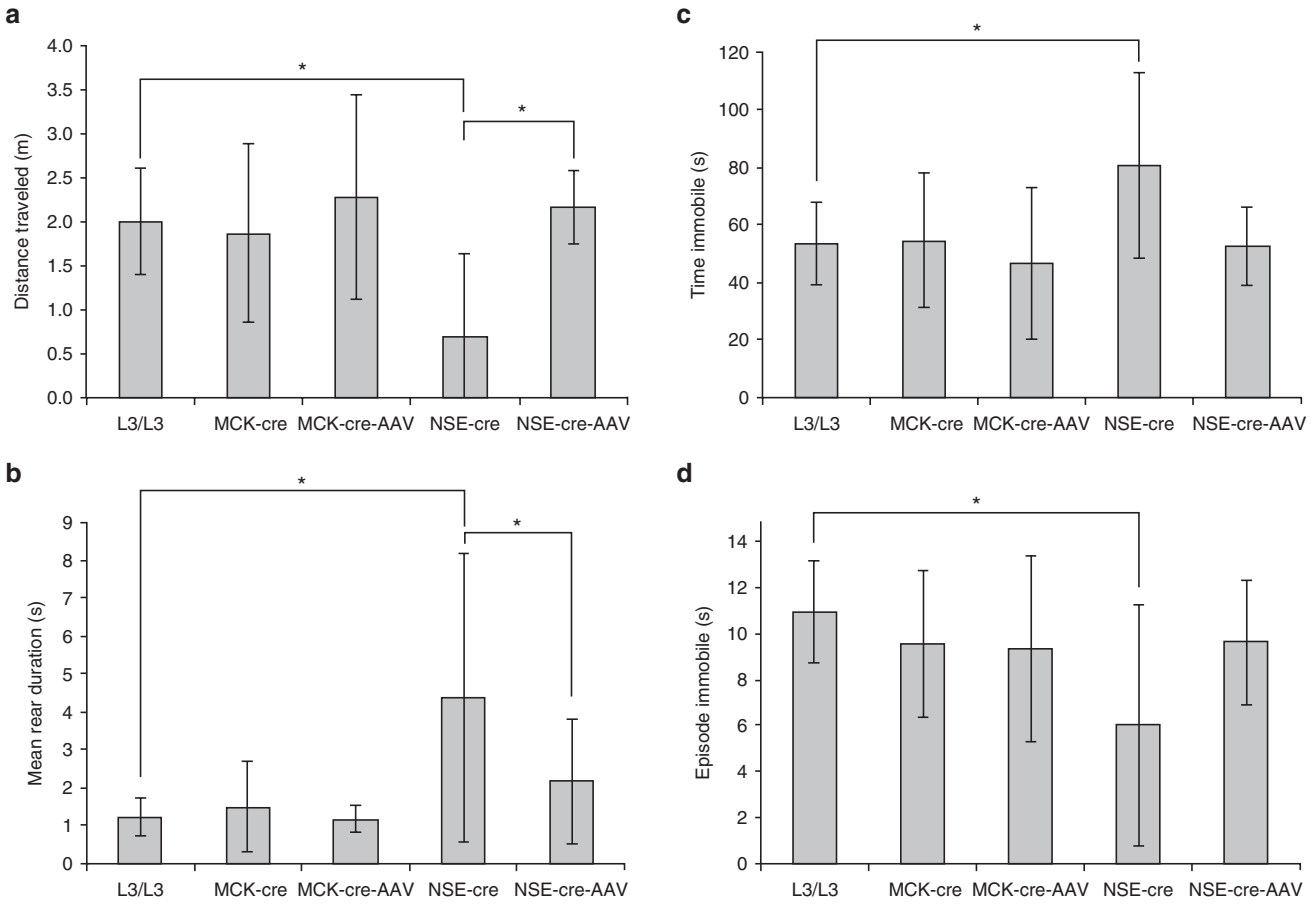

Figure 8 Cage activity. The activity was estimated for the different groups of mice (L3/L3, $n = 11$; muscle creatine kinase (MCK)-cre, $n = 15$; MCK-cre treated with 6×10^{11} v.p. of AAV9-hFXN, $n = 7$; neuron-specific enolase (NSE)-cre, $n = 4$ and NSE-cre treated with 6×10^{11} v.p. of AAV9-hFXN, $n = 5$) as the distance traveled in meters (**a**), the mean rear duration in seconds (**b**), the duration of immobility in seconds (**c**), and the number of immobile episodes (**d**). The injection of AAV9-hFXN significantly improved the distance traveled (**a**) and the mean rear duration (**b**) of the NSE-cre treated mice. The *** indicate significant difference at P less than 0.0001. $P \leq 0.05$*; $P \leq 0.001$**; $P \leq 0.0001$***.

movement when they were hold up by the tail (see Supplementary Video S1). These symptoms are potentially the consequence of insufficient expression of frataxin in the brain of these mice starting during early embryogenesis. It is reported that the NSE mice have spongiform degeneration as in brain cortex and the deletion of frataxin in other organs like the liver should explain the short lifetime.[13,20]

The recent article from Dr. Puccio's group showed that the intravenous injection of an AAVrh10-hFXN in 3 weeks old MCK mice also increased the survival and preserved of the heart's activity. The virus was particularly present in the heart, the liver, and the dorsal root ganglia but the expression was lower in skeletal muscles.[14] Piras *et al.*[16] indicated that AAV9 can deliver a shRNA with good efficacy to the heart while Aschauer *et al.*[21] indicated that it can infect neurons very effectively. Our genomic analysis indicated that the AAV9-hFXN injection resulted in strong presence of the human frataxin transgene in muscle, heart, and brain. The expression of human frataxin in the different organs parallels the intensity of bands in the genomic result. Nevertheless, the presence of human frataxin protein was also strong in liver in our mice. This strong expression of frataxin in the liver was also observed in a recent article from Puccio's group[14] by using a AAVrh10 to deliver the human frataxin to mice. Compared to this study, we demonstrated that an IP injection of an AAV9 led not only to a high detection of frataxin in heart, in liver (which was observed in Puccio's group) but also in skeletal muscle.

The mode of administration as well as the serotype of the AAV used are both very important for the diffusion and the expression in the organism. To treat specifically the heart, some experiments where made with a direct injection of AAV in the thoracic chamber or directly into the pericardia tissue. Piras *et al.*[16] showed that the AAV9 were detected in heart and in lung but the expression was only observed in the heart. Another article from Bish *et al.*[17] compared different AAV serotypes 1–9 injected intrapericardially. They found that AAV9 led to the higher expression in heart. They also shown that even through following intrapericardiac injection, the presence of the virus was detected in all other tissues it was a strongly expressed only in the heart.

In view to all the results obtained and with the different modes of injection described previously, we conclude that a simple IP injection is as effective as a venous or a direct cardiac injection to obtain a significant effect in the heart and in the brain, at least with the AAV_9 serotype. Therefore, our results suggest that the administration of an AAV9 coding for human frataxin may be able to increase the expression of frataxin in the brain and the heart of FRDA patients. The increased expression of the frataxin may reduce or prevent the development of nervous system and cardiac symptoms.

The exposition to an AAV has been shown to induce an immune response.[22–24] Thus, many humans have preformed antibodies to AAV and this has led to the failure of the early clinical trials conducted with AAV_2.[25] However, a recent clinical trial for hemophilia B using an

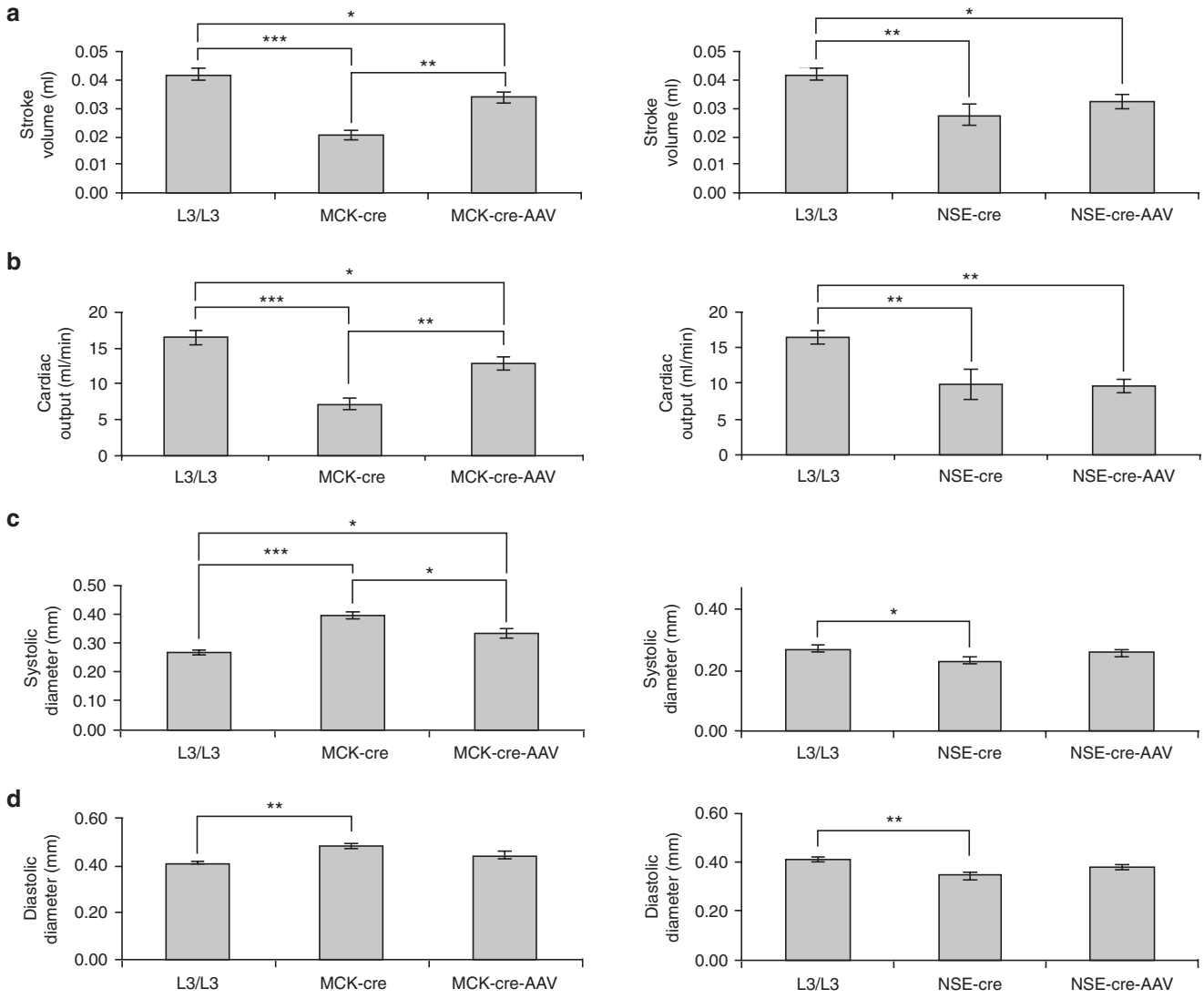

Figure 9 AAV9-frataxin treatment improved heart function of muscle creatine kinase (MCK)-cre mice. Stroke volume (**a**), cardiac output (**b**), systolic (**c**), and diastolic (**d**) diameters were evaluated by echocardiography under isoflurane anesthesia. All these parameters were improved in the MCK-cre that received 6×10^{11} v.p. of AAV9-hFXN compared to untreated MCK-cre mice. In neuron-specific enolase (NSE)-cre mice, only the systolic and diastolic diameters were enhanced. The *** indicate significant difference at *P* less than 0.0001. $P \leq 0.05$*; $P \leq 0.001$**; $P \leq 0.0001$***.

AAV_8 has been very successful because most humans do not have pre-formed antibodies to this serotype of the virus.[26] Puccio *et al.*[14] detected no immune reaction in mice after one intra-venous injection of AAVrh10-hFXN. Although an immune response will probably develop in the FRDA patients following the systemic delivery of an AAV8, AAV9, or AAVrh10 coding for frataxin, this will not be a problem since no readministration of the virus will be required. The main problem facing this potential therapy is that the large-scale production of AAV vector under GMP conditions remains difficult.

MATERIALS AND METHODS

AAV vector construction and viral vector production

The AAV vector plasmids were made and packaged in AAV9 capsids as previously described.[27–29] Specifically, the AAV vector plasmid dsAAV-CB-hFrataxin (AAV9-hFXN) was derived from plasmid dsAAV-CB-GFP[30] by replacing the GFP gene with the human frataxin coding sequence, which was polymerase chain reaction (PCR) amplified with the following primers: Forward: 5′ TAGTGCTAGCCACCATGTGGACTCTCGGGCGCCG 3′ and reverse: 5′ ACTGAAGCTTATCAAGCATCTTTTCCGGAATAG 3′. The forward primer also contained the Kozak sequence GCCACC before the start codon ATG and the reverse primer had two stop codons at the end of the coding sequence. The vectors were then packaged in AAV9 capsid using the adenovirus-free, triple plasmid DNA transfection method and purified twice with CsCl gradient (1). AAV vector genome (v.g.) copies/ml were titrated by a standard dot-blot assay.

Reproduction of the mutant mice

Supplementary Figure S1a summarizes the different mouse genotypes (Puccio 2001). Mice homozygous for a conditional allele of Frda ($Frda^{L3/L3}$) and heterozygous $Frda^{L3/L}$:NSE-cre (NSE-cre), which carried a tissue-specific Cre transgene under the control of the neuron-specific enolase (NSE), were gifts from Dr M Payne laboratory (Indiana University School of Medicine) following the permission of Dr Puccio (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France) who generated these mice.[13] These mice were bred in our animal facility to produce $Frda^{L-/L-}$:NSE-Cre (NSE-cre). In these mice, both conditional knockout frataxin genes are

Table 2 Polymerase chain reaction parameters

Gene name	*Primer sequences*	*Tm*	*Nb of cycles*	*Enzyme*
Delta FXN allele (Fw) (Rev)	5′-CTG TTT ACC ATG GCT GAG ATC TC-3′	62,5	35	Terra
	5′-GTA GCT GGC TGG GAA ATG AA-3′			
mFXN (Fw) (Rev)	5′-CTG CGT GGT GCA TTT GAG GAA C-3′	62	35	Taq 360
	5′-CAT GCA GAG ACA CGC CGT CAT GA-3′			
hFXN (Fw) (Rev)	5′-AAC GTG GCC TCA ACC AGA TTT G-3′	62	30	Taq 360
	5′-TAA GGC TTT AGT GAG CTC TGC G-3′			
m β-actin (Fw) (Rev)	5′-ATA CGC TGC GCT GGT CGT C-3′	58	30	Taq 360
	5′-AGG ATG GCG TGA GGG AGA GC-3′			

knocked out in the neurons and in other tissues expressing the Cre gene. Two hemizygous males B6.FVB(129S4)-Tg(Ckmm-cre)5Khn/J were also purchased from the Jackson laboratory (stock # 006475) and used to generate $Frda^{L-/L-}$:MCK-cre (MCK-cre). In these mice, both conditional KO frataxin genes are knocked in skeletal muscle fibers and in cardiac cells. For the experiments, $Frda^{L3/L3}$ mice were used as control mice in many experiments. All the experiences were approved by the animal care committee of the Centre Hospitalier de l'Université Laval.

The $Frda^{L3/L3}$ mice have the completed mouse frataxin gene but there is LoxP site on each side of the exon 4. When these mice were bred with mice expressing the Cre gene, mice without exon 4 were obtained because the Cre enzyme had cut both LoxP sites and deleted exon 4. This frataxin allele deleted of exon 4 is called the delta allele (L-). The deletion of exon 4 led to a nonfunctional frataxin gene.

Two mouse models were used in this study: the MCK-cre and NSE-cre mice. Homozygous conditional knockout mice $Frda^{L3/L3}$ were bred with heterozygote mice $Frda^{L3/+}$ also expressing the Cre gene under the MCK promoter. Some of the offsprings were homozygous for the conditional KO frataxin gene and also had the MCK-cre gene. Therefore these mice had both frataxin genes KO in their skeletal muscles and heart starting during embryogenesis. These mice are designated as "MCK-cre" mutants. MCK-cre mice (injected or not with saline) were used as untreated controls. Similarly, homozygous conditional knockout mice $Frda^{L3/L3}$ were bred with heterozygote mice $Frda^{L3/+}$ also expressing the Cre gene under the NSE promoter. Some of the offsprings were homozygous for the conditional KO frataxin gene and also had the NSE-cre gene. The mice were named "NSE-cre".

Administration of AAV9-hFXN

A genotype analysis was made in 4 days old mice using the tail tip. The MCK-cre or NSE-cre mice were then injected with a single intraperitoneal (IP) injection of viral particles in 60 µl of saline between 5 and 9 days. Control mice were injected with saline. A point of surgical glue was applied to the skin just after removing the needle to keep the liquid inside the mice. In the first part of the study, different quantities of viral particles were injected in NSE-cre mice: 6×10^{11}, 3×10^{11} (d1/2), 6×10^{10} (d1/10), 3×10^{10} (d1/20), 1.2×10^{10} (d1/50), and 6×10^{9} (d1/100) v.p. For the second part of the study, only the highest and lowest doses were tested (6×10^{11} and 6×10^{9} viral particles) on MCK-cre mice. In the third part, only the higher quantity of virus was used in NSE-cre and MCK-cre mice for more specific evaluations, such as echocardiography.

Mouse evaluation

The weight of the mice was evaluated after weaning and until their death. An estimation of the mouse behavior was done each week for the MCK-cre and twice a day for the NSE-cre mice because of the severity of their disease. Their health was scored from 0 (normal behavior) to 4 (severe cardiac or respiratory insufficiency (Table 1) requiring immediate sacrifice due to ethical concern. At the time of euthanasia, the body and heart weight was measured. Different tissues (*Tibialis anterior* muscle, the liver, the kidneys, the heart, and the brain) were recovered and directly frozen in liquid nitrogen for analyzes.

DNA extraction

DNA was extracted from different tissues (muscle, liver, heart, kidney, and brain). Briefly, a part of the recovered tissue was incubated with 50 µl of proteinase K (10 mg/ml) in a lysis buffer at 56 °C until the solution became clear. Digested tissues were then mixed with 500 µl of a solution of phenol/chloroform/isoamyl alcohol (25:24:1) and centrifuged 3 minutes at 13,000 rpm. The upper solution was recovered and mixed with the same volume of chloroform and centrifuged again. The upper solution was recovered and 50 µl of 5 mol/l sodium chloride was added before the addition of 1 ml of 100% ethanol. After a 8-minute centrifugation at 13,000 rpm, the pellets were washed in 70% alcohol before another centrifugation. The pellets were then dried before the suspension of the DNA in sterile water. PCR was performed with 50 ng of the genomic DNA solution using primers specific for delta and human frataxin (hFXN). The annealing temperatures and the PCR parameters are presented in the Table 2.

RT-PCR

The total RNA was extracted using the Trizol reagent from a part of the different tissues. First-strand cDNAs were synthesized using 1 µg total RNA with the oligo (dt) primer and Superscript III reverse transcriptase. PCR was performed with 2 µl of the cDNA solution using the primers specific for β-actin (reference gene), human frataxin (hFXN), and mouse frataxin (mFXN). The PCR parameters are presented in Table 2. PCR products were then separated by electrophoresis in 1% agarose gels and were stained with RedSafe (Chembio, St Albans, Herts AL2 3UG). A picture of the gel was obtained with the GelDoc program (BioRad, Mississauga, Ontario, Canada) under UV light.

Open-field activity

Mice were placed in a rectangular cage made of clear Plexiglas identical in size to their home-cage (width: 18 cm, length: 28 cm, height: 12 cm) and equipped with a video-tracking system (Side

view, Any maze, Stoelting, Wood Dale, IL). Each mouse was placed in this cage without its usual bedding for a single 2-minute session with the experimenter out of its view. Distance traveled, time spent immobile and rearing frequency and duration were recorded. After each trial, fecal boli were removed and the floor was wiped clean with a damp cloth and dried.

Quantification of the frataxin protein by ELISA

The human frataxin protein in various mouse tissues was quantified using the Dipstick Array of Abcam (cat # ab109881, Cambridge, MA). This is an immunologic sandwich assay using two monoclonal antibodies specific for two antigens present in the mature form of frataxin. One antibody is immobilized on the nitrocellulose membrane of the dipstick in a thin line perpendicular to the length of the dipstick while the other is gold-conjugated with gives the red signal proportional to the level of frataxin. To compare and to pool the different organs and mice, a range from 1 to 10 was established to "semi-quantify" the density of the frataxin band.

Evaluation of heart function by echocardiography

A complete M-Mode, 2D, and Doppler echocardiogram was performed on the mice under 1.5% inhaled isoflurane anesthesia using L17-7 and S12 probes with a HD11XE echograph (Philips Medical Imaging, Andover, MA). Different parameters (left ventricular dimensions, fractional shortening, stroke volume, and cardiac output and frequency) were evaluated as previously described.[31,32] At the end of the protocol and under anesthesia, the heart was quickly removed, freed from connective tissue, and weighed.

Statistical analyses

The experimental values were presented as means ± SD. Statistical analysis was performed using the StatView statistical package (StatView 5; SAS Institute, Cary, NC). Comparisons of different variables between groups were performed using analysis of variance techniques. Differences were considered statistically significant at $P \leq 0.05$*; $P \leq 0.001$**; $P \leq 0.0001$***.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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