RESEARCH ARTICLE

Neuroprotective Effects of Creatine in the CMVMJD135 Mouse Model of Spinocerebellar Ataxia Type 3

Sara Duarte-Silva, PhD,[1,2] Andreia Neves-Carvalho, PhD,[1,2] Carina Soares-Cunha, PhD,[1,2] Joana M. Silva, PhD,[1,2] Andreia Teixeira-Castro, PhD,[1,2] Rita Vieira, BSc,[1,2] Anabela Silva-Fernandes, PhD,[1,2] and Patrícia Maciel, PhD[1,2]*

[1]*Life and Health Sciences Research Institute, School of Medicine, University of Minho, Braga, Portugal*
[2]*Life and Health Sciences Research Institute/3B's–PT Government Associate Laboratory, Braga/Guimarães, Portugal*

ABSTRACT: Background and Objective: Mitochondrial dysfunction has been implicated in several neurodegenerative diseases. Creatine administration increases concentration of the energy buffer phosphocreatine, exerting protective effects in the brain. We evaluate whether a creatine-enriched diet would be beneficial for a mouse model of spinocerebellar ataxia type 3, a genetically defined neurodegenerative disease for which no treatment is available.

Methods: We performed 2 independent preclinical trials using the CMVMJD135 mouse model (treating 2 groups of animals with different disease severity) and wild-type mice, to which 2% creatine was provided for 19 (preclinical trial 1) or 29 (preclinical trial 2) weeks, starting at a presymptomatic age. Motor behavior was evaluated at several time points from 5 to 34 weeks of age, and neuropathological studies were performed at the end of each trial.

Results: Creatine supplementation led to an overall improvement in the motor phenotype of CMVMJD135 mice in both trials, rescuing motor balance and coordination and also restored brain weight, mitigated astrogliosis, and preserved Calbindin-positive cells in the cerebellum. Moreover, a reduction of mutant ataxin-3 aggregates occurred despite maintained steady-state levels of the protein and the absence of autophagy activation. Creatine treatment also restored the expression of the mitochondrial mass marker Porin and reduced the expression of antioxidant enzymes Heme oxygenase 1 (HO1) and NAD(P)H Quinone Dehydrogenase 1 (NQO1), suggesting a beneficial effect at the level of mitochondria and oxidative stress.

Conclusions: Creatine slows disease progression and improves motor dysfunction as well as ameliorates neuropathology of the CMVMJD135 animals, supporting this as a useful strategy to slow the progression of spinocerebellar ataxia type 3. © 2018 International Parkinson and Movement Disorder Society

Key Words: creatine; preclinical trial; polyglutamine diseases; Machado-Joseph disease; therapy

*Corresponding author: Dr. Patrícia Maciel, Life and Health Sciences Research Institute (ICVS), School of Medicine, ICVS/3B's – PT Government Associate Laboratory, University of Minho, Campus Gualtar, 4710-057 Braga, Portugal; pmaciel@med.uminho.pt

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Spinocerebellar ataxia type 3 (SCA3) is the most common autosomal dominant ataxia worldwide and is caused by a mutation leading to the introduction of an expanded polyglutamine stretch within the protein ataxin-3 (ATXN3).[1] The symptoms of SCA3 are variable, reflecting a multisystemic involvement, and include ataxia, ophthalmoplegia, diplopia, dysphagia, and dysarthria as well as amotrophy, fasciculations, dystonia, and/or spasticity.[2,3]

The direct neurodegeneration causative pathways remain unclear for most neurodegenerative diseases. Nevertheless, these disorders are thought to share pathogenic mechanisms and molecular events that ultimately lead to neuronal death. One such mechanism is impaired energy metabolism associated with mitochondrial dysfunction. In polyglutamine diseases, particularly in Huntington's disease (HD), mitochondrial

and energetic deficits are well described.[4,5] Recently, a study using a transgenic mouse model of SCA3 revealed a decreased mitochondrial DNA (mtDNA) copy number and the accumulation of mtDNA deletions with age in affected brain regions.[6]

Creatine is a natural substance, exogenously consumed in the diet and endogenously synthesized by humans and rodents[7,8] and that functions as a cellular energy buffer. Exogenous creatine administration has been tested as a therapeutic approach in several experimental models of neurodegenerative diseases, with promising outcomes.[9-14] In the context of polyglutamine diseases, creatine supplementation was shown to improve motor function, to reduce mutant protein aggregates, and to increase the survival rate or lifespan of HD transgenic mouse models.[11,12,15] Not surprisingly, because creatine is a natural compound and is present in the daily diet, the side effects of creatine supplementation were shown in phase I trials to be very few, both for healthy persons and patients.[16-18] Creatine supplementation to HD patients was shown to be safe in dosages up to 30 mg per day and to reduce the levels of the DNA damage marker 8-hydroxy-2'-deoxyguanosine(8OH2'dG).[19] A phase III clinical trial of creatine supplementation in HD was completed this year (with 650 participants enrolled, ClinicalTrials.gov identifier NCT00712426), demonstrating no beneficial effects for early manifesting HD patients.[20] Nevertheless, and considering the lack of knowledge of the effect of this compound in SCA3, we decided to test the efficacy of creatine in a transgenic mouse model of this disease—CMVMJD135—previously described by us and others[21,22] and shown to be a valuable model to perform preclinical trials.[21,23-26]

Methods

Ethics Statement

All procedures were conducted in accordance with European regulations (European Union Directive 86/609/EEC). Animal facilities and the people directly involved in animal experiments (S.D.S., A.N.C.) as well as the principal investigator (P.M.) were certified by the Portuguese regulatory entity Direção Geral de Alimentação e Veterinária.

All of the protocols performed were approved by the Animal Ethics Committee of the Life and Health Sciences Research Institute, University of Minho (Braga, Portugal).

Animals

Female CMVMJD135 mice (background C57BL/6) were used in the study. A detailed description of the animals used as well as housing conditions and welfare is found in the Supplementary Methods.

Preclinical Trial 1 (PCT1)

Creatine was supplemented in the standard diet at 2% (4RF21, Mucedola SRL, purchased at Ultragene, Portugal), as previously described.[15] At 5 weeks of age, the animals were sequentially assigned by cage into 4 groups of 10 to 12 animals each: CMVMJD135 and wild-type (wt) under normal diet and CMVMJD135 and wt under diet supplemented with creatine 2%. The treatment had the duration of 19 weeks until the animals reached 24 weeks of age. Creatine 2% treatment was started at 5 weeks of age because of the previous knowledge that the phenotype in this model starts at 6 weeks of age.[21]

Preclinical Trial 2 (PCT2)

The methods used were as described for PCT1, but used 15 animals per group and a treatment duration of 29 weeks until the animals reached 34 weeks of age.

Creatine Treatment

In both trials the amount of food intake per cage per week was determined. In the beginning of the week, a fixed amount of food (300 g) was placed in each cage, and 7 days later the remaining food was weighed and divided by the number of animals in that specific cage.

Phenotypic Analysis

The animals were first tested at 4 weeks of age, before treatment initiation. A creatine enriched-diet was started at 5 weeks of age, and the behavioral tests (including beam walk, motor swimming, foot printing, and SHIRPA protocol) were performed every 2 weeks until the animals reached 24 weeks of age (in the case of PCT1) and 34 weeks of age (in the case of PCT2). Detailed protocols for these behavior paradigms can be found in the Supplementary Methods.

Molecular Analysis

Western Blot

Brain tissue was homogenized in cold 0.1 M Tris Hidrochloride (Tris-HCl), pH 7.5, 0.1 M Ethylenediaminetetraacetic acid (EDTA), and a mixture of protease inhibitors (Complete; Roche, Switzerland). Protein concentration was determined using the Bradford assay (Bio-Rad Laboratories, Hercules, California). For each sample, 15 μg of total protein were loaded into sodium dodecyl sulfate (SDS)-Page gels and then transferred to nitrocellulose membranes (Biorad). After incubation with the primary antibodies—rabbit anti-ataxin-3 (1:10.000), mouse sequestrome 1 (p62) (1:1000, Abnova), rabbit light chain 3 (LC3) (1:1000, Novus Biologicals), rabbit anti glial fibrillary acidic protein(anti-GFAP) (1:500, Dako), mouse anti-α-tubulin (1:200, DSHB), and mouse anti-β-actin

(1:200, DSHB)—the secondary antibodies were incubated at the following dilutions: anti rabbit (1:10.000, Biorad) and anti mouse (1:10.000, Biorad). Antibody affinity was detected by chemiluminescence (Clarity Western ECL, Biorad). Band quantification was performed using the Image Lab software according to the manufacturer's instructions.

Quantitative Reverse-Transcriptase–PCR

Total ribonucleic acid (RNA) was isolated from CMVMJD135 mouse tissues using TRIZOL (Invitrogen, Carlsbad, California) according to the manufacturer's protocol. First-strand complementary DNA (cDNA), synthesized using oligo-dT (Biorad), was amplified by quantitative reverse-transcriptase polymerase chain reaction (PCR) (qRT-PCR) according to the guidelines (Biorad). Human and mouse ataxin-3 primers were used for transgene expression quantification.[27] The primers used were based on the literature[21] or designed using PRIMER-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) on the basis of the respective GenBank sequences. All accession numbers and primer sequences are available on request. qRT-PCR was performed on a CFX 96TM real-time system instrument (Bio-Rad Laboratories, Hercules, California), with the SoFast Eva Green RT-PCR reagent kit (Bio-Rad) according to the manufacturer's instructions using equal amounts of RNA from each sample. Product fluorescence was detected at the end of the elongation cycle. All melting curves exhibited a single sharp peak at the expected temperature.

Neuropathology and Immunohistochemistry

Transgenic and wt littermate mice were deeply anesthetized and transcardially perfused with phosphate-buffered saline followed by 4% paraformaldehyde in phosphate-buffered saline. The brains were postfixed overnight in fixative solution and embedded in paraffin. Slides with 4-μm-thick paraffin sections were stained with cresyl violet or processed for immunohistochemistry. For immunohistochemistry and quantification details, see the Supplementary Methods.

Statistical Analysis

The experimental unit used in this study was a single animal. Power analysis was used to determine the sample size as previously described.[21] The estimates of the required number of CMVMJD135 animals for specific behavioral tests and time-points of analysis are described in Teixeira-Castro and colleagues.[23] Continuous variables with normal distributions (Kolmogorov-Smirnov test $P > .05$) were analyzed with the Student's t test or repeated-measures 2-way analysis of variance (factors were genotype and treatment, post-hoc Tukey's for multiple comparisons). Behavioral data were subjected to the nonparametric Mann–Whitney U test when variables were noncontinuous or when a continuous variable did not present a normal distribution (Kolmogorov-Smirnov test $P < .05$). Categorical variables in the SHIRPA protocol were analyzed by nonparametric test Kruskal-Wallis H test. All statistical analyses were performed using SPSS 22.0 (IBM Corp., Armonk, New York). A critical value for the significance of $P < .05$ was used throughout the study. Although we did not specifically define a primary outcome for these preclinical trials, in our previous studies with this animal model we have consistently considered that a compound with a significant beneficial effect in the balance beam test and in the motor swimming test is a "therapeutically effective" compound, leaving the other tests as "secondary outcomes," and the same was assumed for the current study.

Results

In this study, we performed 2 independent preclinical trials using creatine food supplementation in the CMVMJD135 mouse model. In the first study (PCT1, Fig. S1A), we used a group of animals with milder disease severity and performed a shorter treatment (19 weeks). Given the promising results obtained in this trial, we performed a second independent trial (PCT2, Fig. S1B) using a cohort of animals with a longer CAG repeat and, consequently, a higher disease severity, and performed a longer treatment with creatine (29 weeks). The mean CAG repeat size ($\pm$ standard deviation) for all mice used was 133 ± 1 in PCT1 and 139 ± 4 for PCT2 ($U = 144.5$, $P < .0001$; Fig. S1C), with no statistically significant differences between the treated and untreated mice within each trial ($t_{30} = 0.748$, $P = .46$ for PCT1; $U = 73.5$, $P = .23$ for PCT2).

The amount of food intake per mouse in PCT1 and PCT2 was 3 g per day, with no significant difference between the treated and untreated mice (Fig. S1D and E). In both trials, the animals did not present any detectable side effects until the end of the preclinical trials, namely, neither body weight deficits related to creatine consumption nor visible aspects of discomfort (wounds, loss of fur, excessive grooming). All of the animals were alive by the end of both experiments.

PCT1

Creatine Supplementation improves Muscular Strength and Motor Deficits of CMVMJD135 Mice and Decreases ATXN3 Nuclear Inclusions in Affected Brain Regions

A loss of muscular strength is the first disease symptom observed in CMVMJD135 mice, and it has shown to be very difficult to improve with all the compounds tested in this model so far.[21,23-26] Yet in this

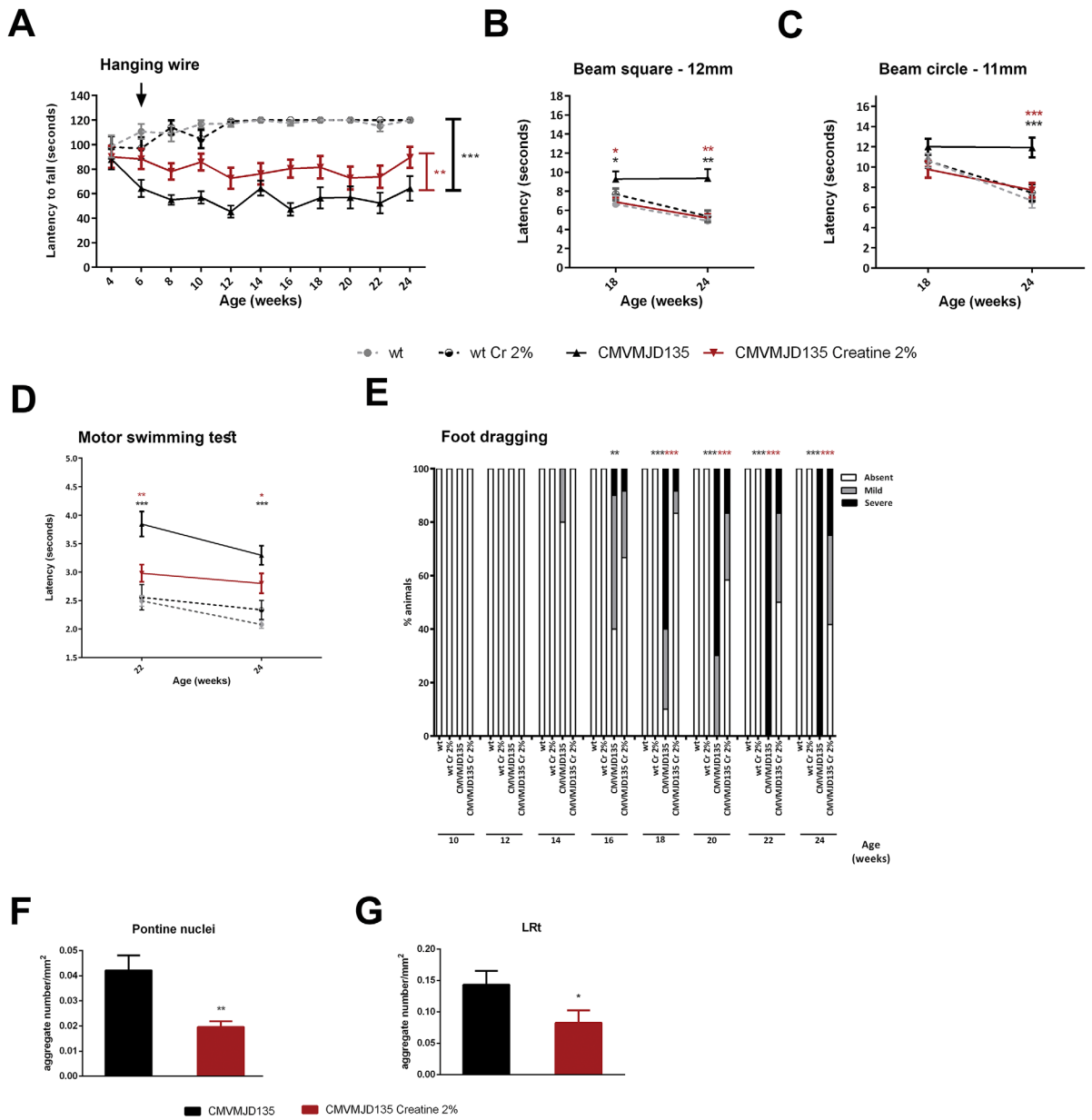

FIG. 1. Preclinical trial 1. Effect of creatine treatment during 19 weeks on CMVMJD135 mice. (**A**) The hanging wire test was performed to evaluate limb strength, and the phenotype onset occurred at 6 weeks of age. Creatine 2% improved this phenotype since the onset of the phenotype until the end of this study. CMVMJD135-treated mice had a better performance on the beam walk test both in the (**B**) square and in the (**C**) round beams. In the motor swimming test (**D**), an improvement was also observed at both time points tested, and (**E**) the foot dragging phenotype started being detectable at 16 weeks of age and progressed as animals aged; creatine supplementation improved this phenotype since 18 weeks of age until the end of this preclinical study; n = 10 for each group used. Symbols represent mean ± standard deviation of the different groups.*, **, *** represent the P <.05, .01, and .001, respectively. The abundance of ataxin-3 positive nuclear inclusions was reduced in the CMVMJD135 treated with ceatine 2% in the (**F**) pontine nuclei and (**G**) in the lateral reticular nucleus (LRt). n = 4 for each group (4 slides per animal). Symbols represent mean ± standard deviation of the different groups.*, **, represent the P <.05 and .01, respectively. [Color figure can be viewed at wileyonlinelibrary.com]

preclinical trial, creatine 2% prevented the loss of muscular strength during disease progression until the end of the trial ($F_{3,34} = 60.69$, $P < .001$; CMVMJD135 vs CMVMJD135-treated $P = .003$; Fig. 1A). The beam walk test was performed at 2 time-points (18 and 24 weeks of age). Creatine 2% treated mice clearly showed an improvement of the coordination deficits in the 12-mm square beam ($F_{3,35} = 8.38$, $P < .0001$; CMVMJD135 vs CMVMJD135-treated $P = .001$; Fig. 1B). Also, in the 11-mm circle beam, creatine 2% treatment significantly improved the phenotype of CMVMJD135 animals ($F_{3,33} = 6.81$, $P = .001$; CMVMJD135 vs CMVMJD135-treated $P = .001$; Fig. 1C). Motor coordination and strength were also evaluated in the motor swimming test. In accordance with the previous results, creatine 2% treatment significantly improved performance of transgenic mice in this test at both time points analyzed ($F_{3,32} = 21.96$,

$P < .001$; CMVMJD135 vs CMVMJD135-treated $P = .002$; Fig. 1D). The foot-dragging phenotype was also improved by creatine 2% treatment from 18 to 24 weeks of age ($H_3 = 27.97$, $P < .001$; CMVMJD135 vs CMVMJD135-treated $P = .001$; Fig. 1E). Next, we quantified the ATXN3 positive nuclear inclusions in the brain stem of the CMVMJD135 animals under standard and creatine diets. Interestingly, creatine 2% treatment significantly decreased the aggregate load in the pontine nuclei ($t_{11} = 5.25$, $P = .003$) and lateral reticular nucleus ($t_6 = 3.48$, $P = .013$) of the CMVMJD135 animals (Fig. 1F and G, respectively).

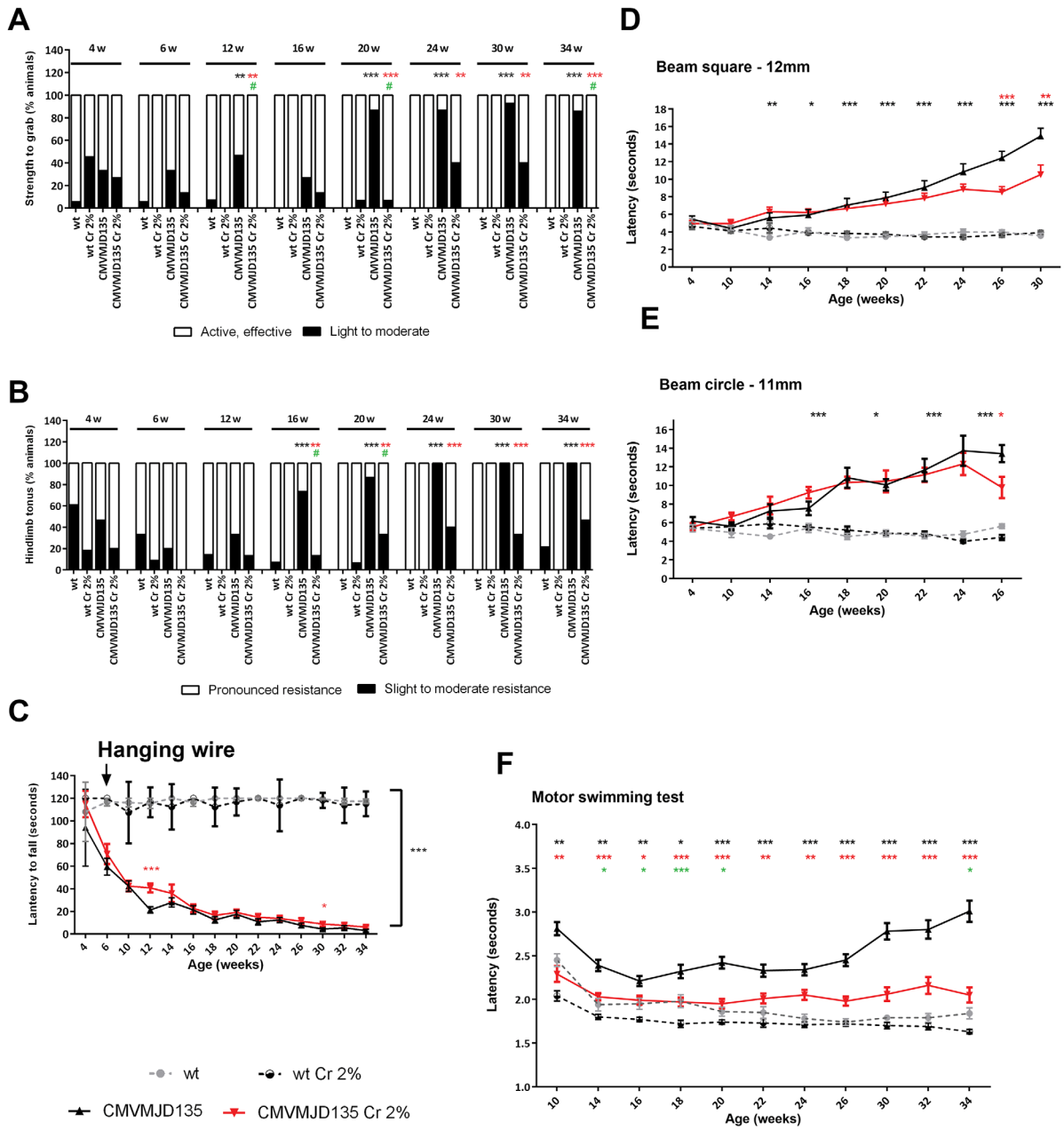

FIG. 2. Preclinical trial 2. Muscular strength, motor, and balance deficits are improved by creatine. (**A**) The animal's strength to grab a grid evaluates the forelimb strength. CMVMJD135 mice started to lose forelimb strength at 12 weeks of age, and creatine 2% treatment delayed disease onset by 8 weeks and improved this loss of strength until the end of the study. (**B**) The hindlimb tonus was evaluated and creatine 2% treatment delayed the onset of the phenotype by 4 weeks; this improvement was maintained until the end of the trial (**C**) The hanging wire test was performed to evaluate limb strength, and the phenotype onset occurred at 6 weeks of age. Creatine 2% supplementation initiated at 5 weeks had no major impact on this test, except for very transient improvements. CMVMJD135-treated mice showed a better performance on the beam walk test both in the (**D**) square and in the (**E**) round beams. In the motor swimming test (**F**), this improvement was even more pronounced during disease progression until the end of the trial at 34 weeks of age. Wild-type mice under creatine 2% diet also improved their performance in this test when comparing to wild-type mice under standard diet (green asterisks). n = 15-17 for each group used. Symbols represent mean ± standard error of the mean of the different groups. *, **, *** represent the P<.05, .01, and .001, respectively. Green represents the phenotype onset delay. [Color figure can be viewed at wileyonlinelibrary.com]

PCT2

Creatine Supplementation on CMVMJD135 Animals Improves Behavioral Phenotype

To validate the encouraging observations described previously, we performed a second trial (PCT2) using the same dosage of creatine in a larger group of animals with a more severe presentation of the disease. All of the animals were tested before any treatments were initiated, at 4 weeks of age, and the body weight (Fig. S2A), muscular strength (Fig. 2A), and motor coordination (Fig. 2D, E) were not different between the groups. Although the wt and CMVMJD135 animals presented significant differences in body weight gain ($F_{3,55} = 40.78$, $P < .001$) as expected, creatine treatment had no impact within transgenic groups (CMVMJD135 vs CMVMJD135-treated $P = .9276$; Fig. S2A). Interestingly, creatine ameliorated the brain atrophy seen in transgenic animals at 34 weeks of age. CMVMJD135 animals exposed to standard diet showed a 10% reduction in brain weight at this time-point ($F_{3,13} = 23.51$, $P < .001$; wt vs CMVMJD135 $P = .001$) whereas creatine-treated mice showed 6% of reduction when compared with the wt mice at the same age ($P = .042$; Fig. S2B).

The CMVMJD135 mice treated with creatine also showed improved forelimb strength ($H_3 = 138.06$, $P < .0001$; CMVMJD135 vs CMVMJD135-treated $P < .0001$) and hindlimb muscular tonus ($H_3 = 124.56$, $P < .0001$; CMVMJD135 vs CMVMJD135-treated $P < .0001$; Fig. 2A and B, respectively) until the end of the preclinical trial, and the onset of these deficits was delayed by 8 weeks. However, the overall muscular strength deficits observed in the CMVMJD135 mice ($F_{3,52} = 12.52$, $P < .0001$; wt vs CMVMJD135 $P = .002$), measured by the hanging wire test, showed only a transient amelioration by creatine at 12 weeks of age and was no longer observed as the disease progressed ($F_{3,52} = 12.52$, $P < .0001$; CMVMJD135 vs CMVMJD135-treated $P = .741$; Fig. 2C).

To assess motor coordination, the beam walk and the motor swimming tests were performed. In the 12-mm square beam, an improvement by creatine was observed at 26 and 30 weeks of age ($F_{3,46} = 49.39$, $P < .0001$; CMVMJD135 vs CMVMJD135-treated $P = .049$, Fig. 2D). When difficulty was increased by using a smaller and round beam (11 mm), the CMVMJD135 mice under a standard diet were only able to perform the task until 26 weeks of age (Fig. 2E), whereas the creatine-treated animals showed a more prolonged improvement (Fig. 2E). Although the creatine-treated animals improved in this test as the animals aged, we were not able to further pursue it given that from 30 weeks onward the untreated CMVMJD135 mice were no longer able to traverse the beam. Regarding the motor swimming test, an impaired performance was observed in the untreated CMVMJD135 mice since 10 weeks of age ($F_{3,51} = 45.34$, $P < .0001$; wt vs CMVMJD135 $P < .0001$, Fig. 2F). Creatine treatment significantly improved the coordination deficits at the onset of the phenotype until the end of the preclinical trial ($F_{3,51} = 45.34$, $P < .0001$; CMVMJD135 vs CMVMJD135-treated $P < .0001$). Not unexpectedly, the wt animals with the creatine 2% diet also performed better at some time-points when compared with the wt animals with the standard diet, although no significant changes were found throughout time (wt vs wt-treated $P = .094$, Fig. 2F).

Gait was qualitatively assessed by observing the animals freely moving in an open arena. Creatine treatment improved and delayed the transgenic animals' perceived gait deficits until 34 weeks of age ($H_3 = 293.62$, $P < .0001$; CMVMJD135 vs CMVMJD135-treated $P < .0001$; Fig. 3A). Unexpectedly, other (more quantitative) parameters used to assess gait, such as foot dragging (of early onset) and stride length (of later appearance), were not significantly improved by creatine treatment (Fig. S3A and B, respectively).

Additional tests were performed to evaluate spontaneous exploratory activity, tremors, and limb clasping. The CMVMJD135 mice travelled significantly less in the arena from 18 weeks of age on ($H_3 = 225.77$, $P < .0001$; wt vs CMVMJD135 $P < .0001$; Fig. 3B). Creatine slightly improved this phenotype and only later in the treatment ($U = 47$, $P = .013$). Regarding vertical movements, the CMVMJD135 mice started to explore significantly less at the age of 22 weeks, and only at 29 weeks did creatine ameliorate their performance (Fig. S3C). Of notice, in the last time-point of analysis, the untreated CMVMJD135 mice did not make any vertical movement during testing, whereas the treated animals showed some (albeit low) exploratory behavior (Fig. S3C).

Remarkably, creatine treatment delayed the appearance of limb clasping by 8 weeks and was able to improve it until the end of the preclinical trial ($H_3 = 313.30$, $P < .0001$; CMVMJD135 vs CMVMJD135-treated $P < .0001$; Fig. 3C). The effect of creatine treatment was even more pronounced concerning tremors because it completely abolished the onset of this symptom ($H_3 = 74.91$, $P < .0001$; CMVMJD135 vs CMVMJD135-treated $P < .0001$ and wt vs CMVMJD135-treated $P = .506$; Fig. 3D).

Effect of Creatine Supplementation on Mutant ATXN3 Aggregation, Calbindin Expression, and Astrogliosis

We measured the abundance of ATXN3 positive nuclear inclusions in the brain stem and in the deep cerebellar nuclei of the cerebellum. As seen in PCT1,

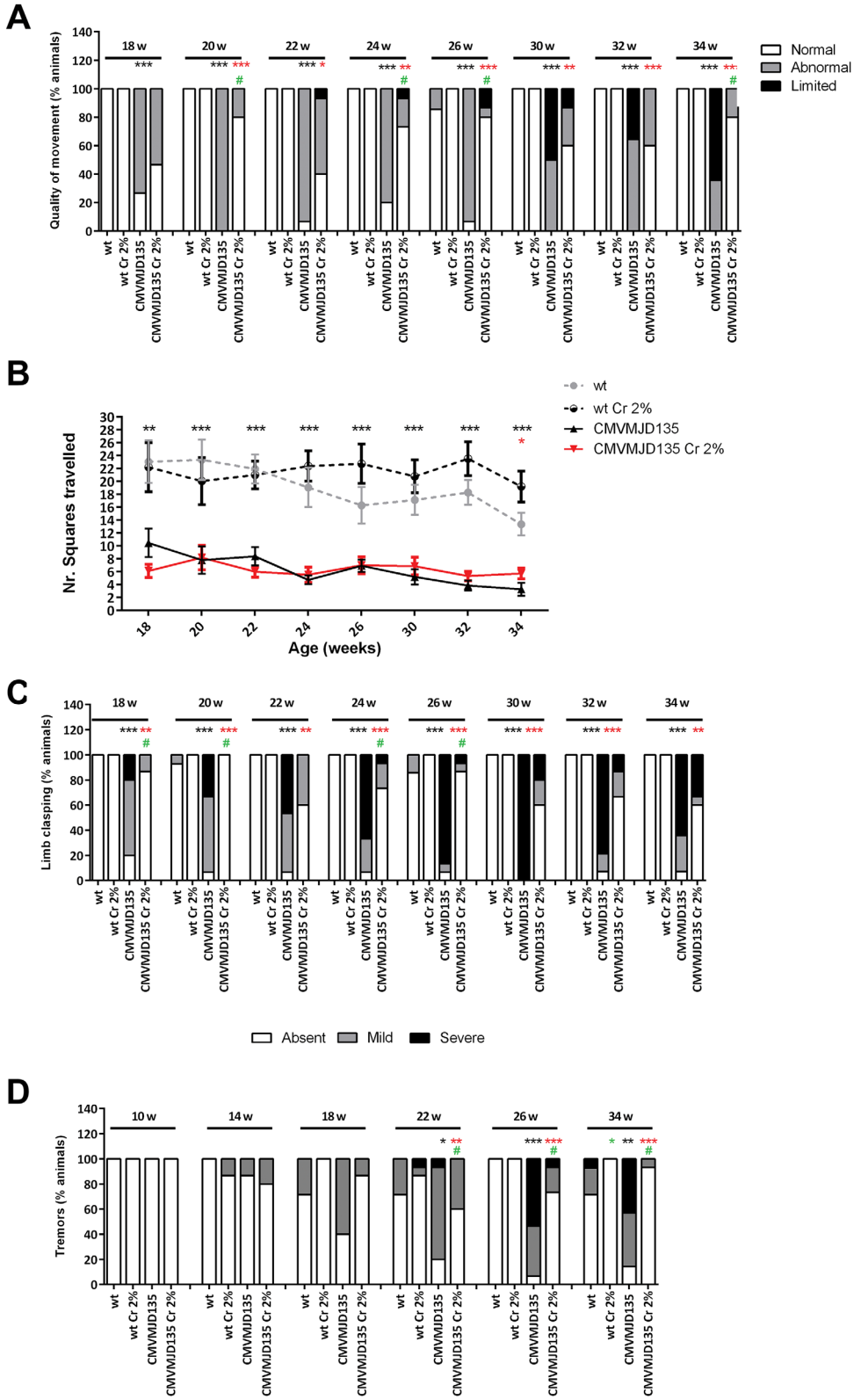

FIG. 3. Preclinical trial 2. Creatine food supplementation improved gait quality, limb clasping, and tremors. (**A**) Gait quality was evaluated in an open arena being the onset of abnormal gait observed at 18 weeks. Creatine 2% improved this phenotype parameter until 34 weeks of age. (**B**) The spontaneous activity measured by the number of squares travelled in an open arena for 1 minute was improved by creatine 2% after a long period of treatment. The limb clasping (**C**) and tremors (**D**) observed in the CMVMJD135 mice were significantly improved by creatine treatment; the phenotype onset was delayed by 8 weeks on the limb clasping and completely abolished in the case of tremors. n = 15-17 for each group used. Symbols represent mean ± standard deviation of the different groups. *, **, *** represent the $P < .05$, .01, and .001, respectively. Green represents the phenotype onset delay. [Color figure can be viewed at wileyonlinelibrary.com]

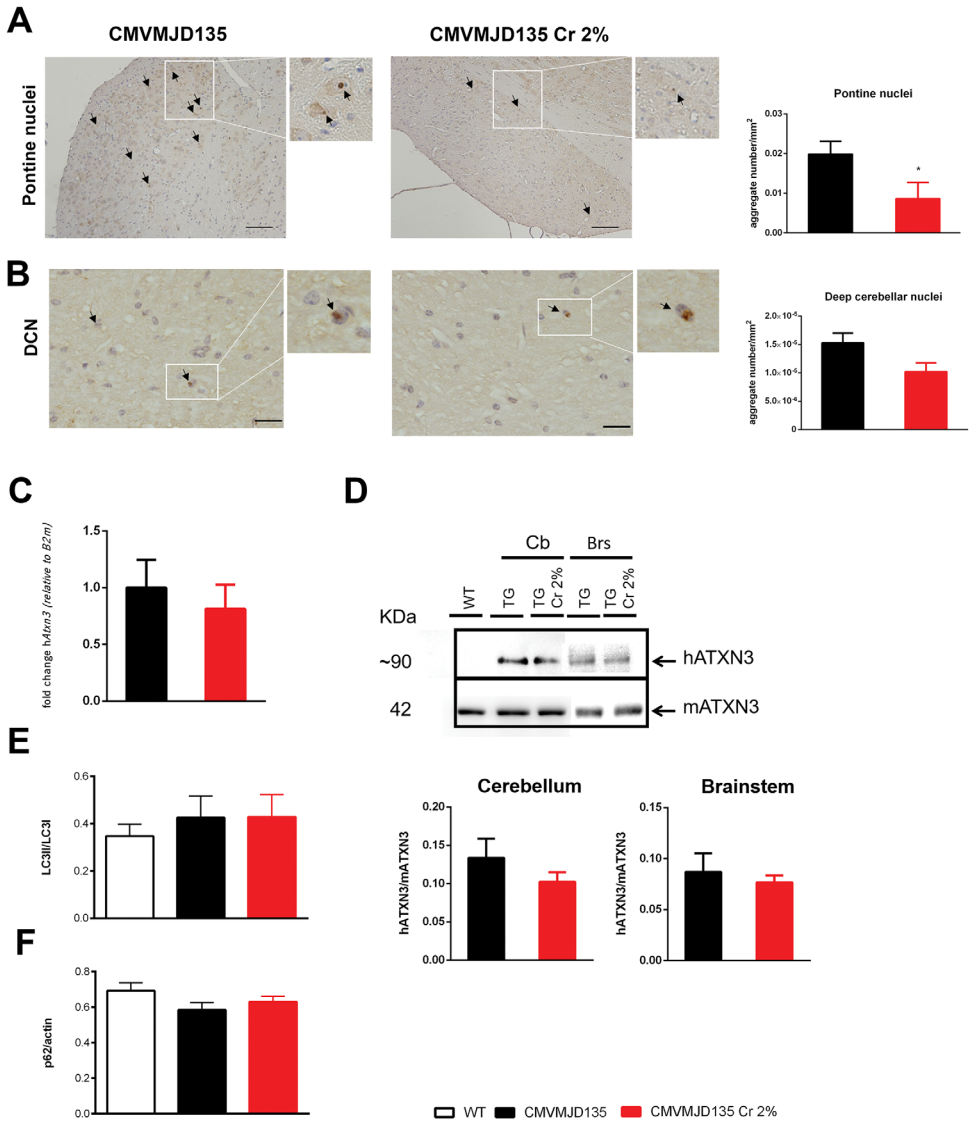

FIG. 4. Preclinical trial 2. Ataxin-3 positive nuclear inclusions in CMVMJD135 mouse brains were reduced on creatine treatment. Neuronal inclusions were counted in the (**A**) pontine nuclei and (**B**) deep cerebellar nuclei of 34-week-old animals under normal diet or creatine 2% supplementation (n = 3 for each condition). Four slides of each animal were used for the analysis. (**C**) Quantitative reverse-transcriptase–PCR analysis of human ataxin-3 (*ATXN3*) mRNA expression levels (n = 5 and 2 technical replicates were performed) showed no differences on creatine 2% treatment. Fold change (ΔΔCT method) is represented using *B2m* as the housekeeping gene (**D**). Anti-ataxin-3 Western blot of 34-week-old CMVMJD135 mice (n = 4 for each condition; at least 3 technical replicates were performed) in the whole cerebellum (Cb) and brain stem (Brs) showed that creatine 2% had no effect on the protein levels of total mutant ataxin-3 (hATXN3). Endogenous ataxin-3 protein (mATXN3) was used as loading control. hATXN3 has a molecular weight of approximately 90 kDa and mATXN3 42 kDa. (**E**) Anti-LC3 Western-blot of 34-week-old CMVMJD135 mice (n = 4 for each condition; at least 3 technical replicates were performed) in the whole brain stem. LC3II was normalized to LC3I. (**F**) Anti-p62 Western-blot of 34-week-old CMVMJD135 mice (n = 4 for each condition; at least 3 technical replicates were performed) in the whole brain stem. p62 was normalized to β-actin. Values are presented as mean ± standard of the mean. B2m, beta-2-microglobulin; LC3I, light chain 3 I; LC3II, light chain 3 II; *P <.05. Scale bar 200 μm. [Color figure can be viewed at wileyonlinelibrary.com]

creatine treatment significantly decreased the aggregate load in the pontine nuclei of the CMVMJD135 animals ($t_4 = 3.78$, $P = .0194$; Fig. 4A), and a trend toward a decrease was found in the deep cerebellar nuclei ($t_6 = 1.803$, $P = .1$; Fig. 4B). However, we found no differences in the levels of mutant ATXN3 messenger RNA (mRNA) or total protein levels upon treatment in the brain stem (Fig. 4C and D, respectively) or in the cerebellum (Fig. 4D). Mechanistically, creatine does not seem to be inducing the degradation of mutant/aggregated ATXN3 through autophagy, because light chain 3 (LC3) and p62 (autophagy markers) were not altered on treatment (Fig. 4E and F, respectively).

Regarding neuropathology, we found that creatine treatment increased calbindin Calbindin- D28-K (D28-K) staining in the Purkinje cell layer of the cerebellum when compared to untreated animals at 34 weeks of age (PCT2; $P = .009$; Fig. 5A and B), suggesting functional preservation of these neurons. Astrogliosis, evident in the brain stem of the CMVMJD135 mice at 34 weeks[20,22] ($F_{2,16} = 5.57$, $P = .014$; wt vs CMVMJD135 $P = .015$),

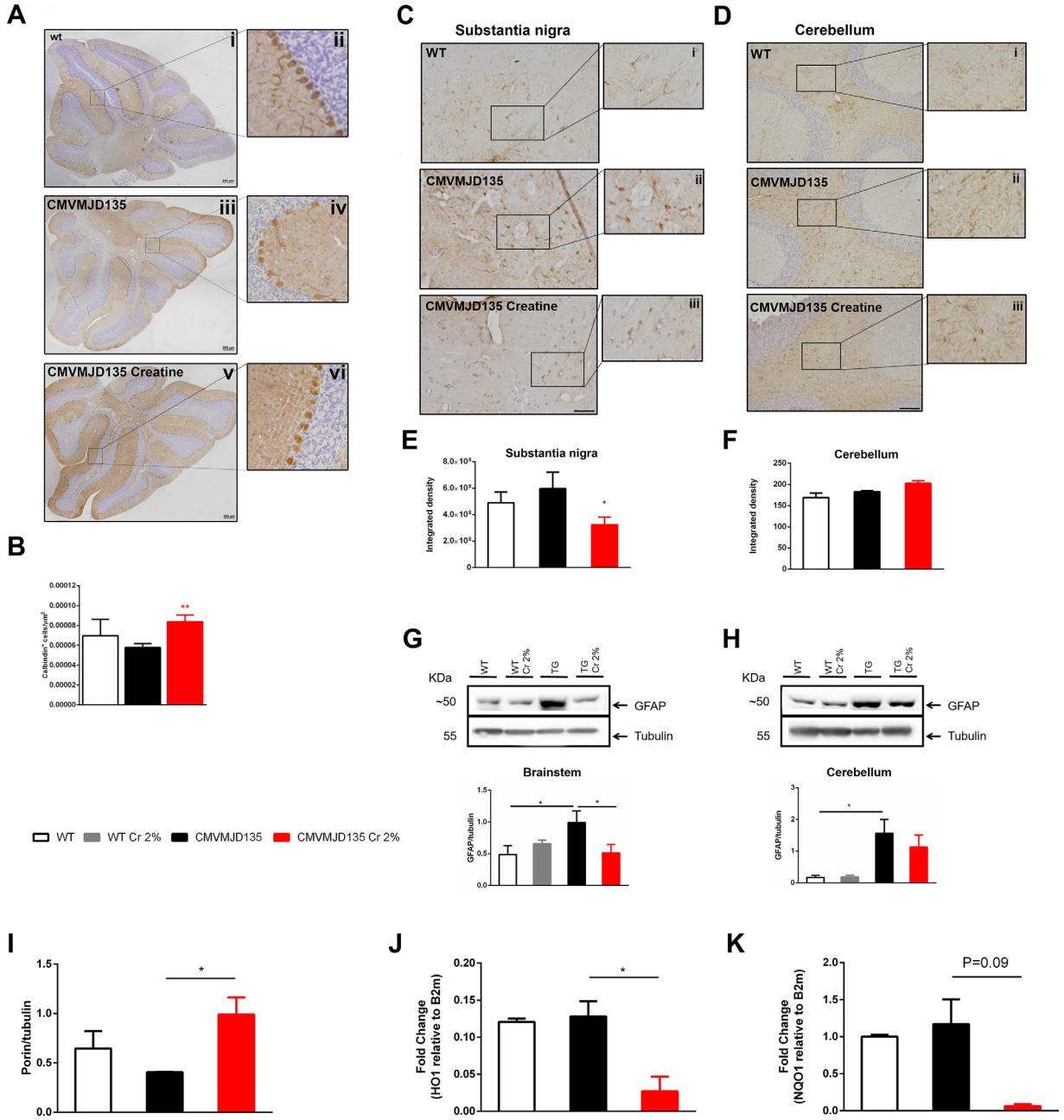

FIG. 5. Preclinical trial 2. Creatine treatment increases calbindin staining in the cerebellum and decreases astrogliosis in the brain stem and cerebellum of the CMVMJD135 mice. (**A**) Representative images of calbindin D28-K staining in wild-type (i-ii), CMVMJD135 (iii-iv), and CMVMJD135-treated animals (v-vi; n = 4 per group, 4 slides per animals were analyzed) and (**B**) quantitative analysis of calbindin D28-K staining. The calbindin D28-K positive cells were counted using the Fiji software (Image J). Scale bar 200 μm. Creatine was able to reduce GFAP staining in the substantia nigra and to a lesser extent in the cerebellum of the CMVMJD135 mice (n = 4 per group, 4 slides per animals were analyzed). Representative images of GFAP immunohistochemistry analysis in the (**C**) substantia nigra and (D) cerebellum at 34 weeks of age of (i) wild-type, (ii) CMVMJD135, and (iii) CMVMJD135 treated mice. The GFAP staining intensity was measured using the Fiji software (Image J) in the substantia nigra (**E**) and in the cerebellum (**F**). Scale bar 200 μm. Anti-GFAP Western-blot of 34-week-old CMVMJD135 mice (n = 4 for each condition; at least 3 technical replicates were performed) in the whole brain stem (**G**) and in the cerebellum (**H**) showed that creatine 2% reduced GFAP protein levels. Creatine treatment impacts on mitochondria and oxidative stress markers. (**I**) Western-blot analysis of Porin showed that creatine was able to restore its levels in the brain stem of CMVMJD135 animals (n = 4 per group, at least 3 technical replicates were performed). Quantitative reverse-transcriptase–PCR analysis of (**J**) *Ho1* and (**K**) *Nqo1* mRNA expression levels (n = 5 and 2 technical replicates were performed) showed to be reduced by creatine 2% treatment in the brain stem of the CMVMJD135 animals. Fold change (ΔΔCT method) is represented using *B2m* as the housekeeping gene. Values are presented as mean ± standard error of the mean. *, **, represent the $P < .05$ and .01, respectively. [Color figure can be viewed at wileyonlinelibrary.com]

was normalized by chronic creatine treatment ($F_{2,16} = 5.57$, $P = .014$; CMVMJD135 vs CMVMJD135-treated $P = .05$; Fig. 5C, E, and G), an effect that was less evident in the cerebellum (Fig. 5D, F, and H). Creatine treatment also restored the expression of the mitochondrial mass marker Porin ($F_{2,5} = 9.76$, $P = .018$; CMVMJD135 vs CMVMJD135-treated $P = .023$; Fig. 5I) and reduced expression of antioxidant enzymes Heme oxygenase 1

(*HO1*) ($F_{2,7} = 9.397$, $P = .01$; CMVMJD135 vs CMVMJD135-treated $P = .012$) and NAD(P)H Quinone Dehydrogenase 1 (*NQO1*) ($F_{2,6} = 3.848$, $P = .084$; CMVMJD135 vs CMVMJD135-treated $P = .09$; Fig. 5J and K, respectively) in the brain stem, suggesting a beneficial effect at the level of mitochondria and oxidative stress.

Discussion

The results of this study show that creatine 2% food supplementation (500 mg/Kg) during a shorter (19 weeks) or longer period of time (29 weeks) was safe and well tolerated and had a significant impact on the neurological phenotype of the CMVMJD135 mice. The dosage herein used was previously described to be the most effective in a mouse model of HD,[15] corresponding (according to the human equivalent dose formula[28] to a consumption of 3 g per day in humans, which is below the dosages used in the sports context and tested so far in human neurodegenerative diseases.[19,29] Creatine treatment was consistently able to delay disease onset in this animal model of SCA3, as assessed by several behavior parameters such as muscular strength, gait, limb clasping, and tremors, the latter symptom being completely abolished. Furthermore, in the first preclinical trial in which the mean CAG repeat length was lower and disease severity less, other phenotypic aspects of the disease developed by CMVMJD135 mice were also ameliorated, such as gait and body weight.

Creatine supplementation effects on muscle strength and growth are largely documented in the context of sports.[30,31] Loss of muscular strength is the first disease sign in the CMVMJD135 animals (by 6 weeks of age), and it progresses very fast and severely. In the first preclinical trial performed here, creatine supplementation had a significant impact on muscular strength deficits, the treated CMVMJD135 mice always performing better when compared with the untreated animals. Yet in PCT2, using more severely affected animals, creatine had a very limited effect on the hanging wire test performance, seen only at 12 weeks of age. Previous preclinical trials performed in the CMVMJD135 model to address the effect of other candidate therapeutic compounds, such as 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG),[21] lithium chloride,[26] or citalopram,[23] also showed no effect on this test, showing that this symptom is very difficult to revert, probably because of its early onset and severity. An earlier treatment would probably be needed to rescue this aspect of the phenotype. Nevertheless, creatine treatment did have a strong impact on other muscular strength parameters, such as hindlimb tonus and forelimb strength (assessed qualitatively within the SHIRPA protocol), even in PCT2. The motor performance of the CMVMJD135 animals was significantly improved by creatine treatment in both the beam walk and motor swimming tests, which measure 2 key aspects of the SCA3 phenotype: balance and motor coordination. Accordingly, in HD mouse models, the motor performance on the rotarod was ameliorated by creatine 2% treatment.[12,15] In contrast, the studies showed no effect of creatine 2% on motor performance in spinocerebellar ataxia type 1 (SCA1) and amyotrophic lateral sclerosis (ALS) mouse models.[32,33] Not unexpectedly, our results showed that creatine 2% supplementation also had an impact on the motor performance of wt mice, namely in the motor swimming test, showing that the effects of creatine are not limited to the disease state. Creatine supplementation can have beneficial effects on healthy people,[34] and, in conjugation with exercise, was shown to improve muscle performance in elderly men and women.[35,36] In addition, 1% creatine supplementation increased the lifespan of aged wt C57Bl/6J mice and improved their performance in several neurobehavioral tests. This suggests that creatine has a globally positive impact on the health and longevity of mice.[37] Nevertheless, and despite this lack of specificity, if the compound indeed shows efficacy in delaying symptoms in SCA3 patients that would be of great value given the current lack of therapeutic alternatives for this disorder.[38]

Creatine-fed CMVMJD135 mice showed a mild improvement on gait quality, but no improvement in stride length, a parameter that is consistently diminished in these mice after a certain age. The lack of efficacy of creatine treatment on gait was also shown in a SCA1 mouse model study in which creatine-fed mice did not show improved gait width despite the preservation of Purkinje cells in the cerebellum.[32] Interestingly, in the case of our study, the CAG repeat tract length (and the resulting phenotypic severity) seems to impact the likelihood of improvement of gait abnormalities because in the first preclinical trial that we performed, the foot-dragging phenotype of the CMVMJD135 animals was significantly improved throughout the trial, whereas in PCT2 a very limited improvement was observed and only at 14 weeks of age. Of notice, the appearance of this symptom in PCT1 was observed at 16 weeks of age, whereas in PCT2 it appeared 1 month earlier (12 weeks of age), which may explain the different results.

Consistent with a connection between symptom onset and the likelihood of rescue, in both trials creatine-treated animals did show a significant improvement in limb clasping, an abnormal reflex that is observed in several brain and spinal cord pathologies, when lesions are present in the cerebellum, basal ganglia, and neocortex[39] and that appears later than strength and gait alterations in the CMVMJD135 mice.

Although tremors are a rare event in SCA3 patients,[40] they are quite evident in CMVMJD135 mice; interestingly, they were completely abolished by creatine 2% treatment, as this symptom remained absent in treated animals until the end of PCT2.

In fact, and although creatine is well known for its muscle-related effects, in the case of this SCA3 model, our data lead us to believe it is providing actual neuroprotection. In accordance with previous reports in an HD mouse model,[12,15] creatine 2% treatment had a protective effect regarding brain atrophy in the CMVMJD135 mice. In addition, our results suggest a functional preservation of Purkinje cells of the cerebellum reflected by the normal expression of the calcium-handling protein calbindin.

Creatine 2% treatment has previously been shown to reduce striatal huntingtin-positive nuclear aggregates.[15] Accordingly, we found a reduction of ATXN3 aggregate load in the pontine nuclei of creatine-fed mice at 34 (PCT2) and 24 weeks of age (PCT1), respectively, with no differences being found on total mutant ataxin-3 protein and mRNA levels. Although the mechanism by which creatine decreases the aggregate load was not addressed in the present study and is also not described in other studies,[11,15] our results suggest that creatine does not act by increasing mutant ATXN3 degradation, namely, through autophagy. Alternatively, the energy-dependent protein folding/refolding systems challenged by mutant ATXN3 may become more effective in the presence of creatine supplementation, thus leading to increased solubility of the mutant protein, a hypothesis that requires further studies.

An interesting study by Yang and colleagues[41] demonstrated the neuroprotective effects of combined creatine 2% and coenzyme 10 1% in animal models of PD and HD and showed the additive effects of both compounds when administered together. It would be interesting to test the combination of creatine 2%/coenzyme 10 1% in CMVMJD135 animals, as this combination might potentiate the effects observed for creatine alone and could eventually further improve the motor benefits that were so evident in this preclinical trial.

In conclusion, the present findings support creatine treatment as a novel therapeutic strategy to delay disease progression and improve symptoms in SCA3, and it would be important to evaluate its effects on SCA3 patients. Independently of their applicability in the human disease, recently challenged by negative results in HD patients,[20] our findings support the concept that an energy deficit contributes to neuronal pathology in SCA3 and that rescuing this energetic deficit has a beneficial effect, decreasing mutant protein aggregation and the neuronal dysfunction associated with it. The discrepancy between preclinical and clinical findings regarding the effect of creatine may be related to the moment of initiation of the treatment, suggesting that perhaps in humans a presymptomatic/prophylactic approach will be required. ■

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Supporting Data

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.