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General Article

GENERAL ARTICLE

Curcumin dietary supplementation ameliorates disease phenotype in an animal model of Huntington's disease

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Abstract

Huntington's disease (HD) has traditionally been described as a disorder purely of the brain; however, evidence indicates that peripheral abnormalities are also commonly seen. Among others, severe unintended body weight loss represents a prevalent and often debilitating feature of HD pathology, with no therapies available. It correlates with disease progression and significantly affects the quality of life of HD patients. Curcumin, a naturally occurring polyphenol with multiple therapeutic properties, has been validated to exert important beneficial effects under health conditions as well as in different pathological settings, including neurodegenerative and gastrointestinal (GI) disorders. Here, we investigated the potential therapeutic action that curcumin-supplemented diet may exert on central and peripheral dysfunctions in R6/2 mice, a well-characterized HD animal model which recapitulates some features of human pathology. Maintenance of normal motor function, protection from neuropathology and from GI dysfunction and preservation of GI emptying and conserved intestinal contractility, proved the beneficial role of life-long dietary curcumin in HD and corroborated the potential of the compound to be exploited to alleviate very debilitating symptoms associated with the disease.

Introduction

Huntington's disease (HD) is an autosomal-dominant inherited neurodegenerative disorder caused by a CAG trinucleotide repeat expansion in the HTT gene (1), which encodes for huntingtin (Htt) protein. The resulting polyglutamine (polyQ)-stretch destabilizes the protein and confers toxic properties to it, ultimately resulting in a broad array of molecular and cellular dysfunction in both neuronal and non-neuronal cells (1, 2). The polyQ expansion causes conformational changes within Htt protein and makes it prone to misfolding and oligomerization.

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For many years, research into HD has conventionally focused on neurodegeneration, neurological symptoms and overall brain pathology; however, a growing body of evidence indicates that peripheral dysfunctions also occur in the disease.

Peripheral defects sometimes appear early in the disease course and can eventually contribute to both morbidity and mortality (3–7). Among all non-neurological features, the progressive early unintended weight loss is one of the most common and serious peripheral abnormalities that affects nearly all individuals with HD (4, 8, 9).

Severe body weight loss is a recurrent symptom in both humans and animal models of HD. It has been reported to worsen other aspects of the disease and to occur despite an adequate caloric intake. Loss of weight begins early, even before disease symptoms appear (10), and ends with profound cachexia in advanced disease stage (11). Such a gradual physical decline is also associated with progressive gut motility failure and subsequent chronic constipation, which becomes a very invalidating and unmanageable condition in HD patients over the time (12).

The molecular mechanism underlying weight loss in HD is still elusive; however, a possible relationship with dysfunction in the gastrointestinal (GI) tract and changes in the metabolism has been suggested to exist (13, 14). Work performed in HD R6/2 mice, one of the most used HD transgenic animal model that recapitulates many features of human pathology (15–17), and in humans has demonstrated that GI tract is precociously affected in the disease (14, 18, 19). Thus, protective treatment may need to be started long before the overt onset of clinical manifestation.

Curcumin, a natural dietary polyphenol derived from the *Curcuma longa* plant, with outstanding safety profile and multiple pharmacological properties ranging from anti-inflammatory to anti-oxidant through neuroprotective and anti-aggregation action, has been shown to be helpful in different degenerative brain pathologies (20–23) as well as in several peripheral illness (24) and GI dysfunction in metabolic settings (25, 26).

Besides all well-known pharmacological properties (27), curcumin stimulates appetite (28), promotes normal food digestion either by regulating digestive hormones, bile and gastric acids or by modulating smooth muscles functions (29–31) and significantly increases bowel motility in humans (32).

Recent data demonstrated that HD may also benefit from curcumin properties (23, 33, 34). Importantly, life-long dietary curcumin, started from conception or at very early developmental stages, has significant beneficial consequence on neuropathology and phenotypic manifestation in HD experimental models (33, 34).

The therapeutic efficacy of curcumin in HD has been mostly investigated on brain pathology, whereas its therapeutic value on peripheral perturbations has never been explored so far.

Curcumin has been widely described to act as protectant agent for GI dysfunction and to activate bowel motility in different disease conditions (25, 26).

In this study, besides consolidating the therapeutic effect of curcumin in brain pathology in HD, we tested the hypothesis that curcumin supplementation could be exploited to maintain GI homeostasis in the disease and to eventually prevent the associated dysfunctions.

R6/2 mice were chronically administered with the compound, and any disease phenotypic change, associated with life-long curcumin supplemented dietary, was assessed.

The overall purpose of this study was to address the impact of early intervention in these mice with the aim to counteract the toxic effect of mutant Htt (mHtt) and eventually to mitigate the development of classically associated pathological signs in both central and peripheral regions.

Our data corroborated the evidence of neuroprotective effect of curcumin in HD brain pathology, and for the first time, demonstrated that chronic administration of curcumin resulted in an overall health benefit in R6/2 offspring.

In particular, besides protecting the brain from neuropathological changes and from all the associated phenotypic complications, curcumin supplementation completely prevented body weight loss **in** R6/2 mice and preserved the normal intestine homeostasis and function in these mice.

Results

Life-long dietary curcumin preserves motor performance in R6/2 mice

Dietary curcumin supplementation has been associated with beneficial effects in different HD experimental models (33, 34).

Here, we demonstrate that life-long dietary curcumin had an overall beneficial effect in R6/2 mice. It prevented the classical motor deficit associated with disease progression in these mice, when fed from conception. HD-treated mice performed significantly better than untreated littermates over the entire period of observation (Fig. 1A). Curcumin-fed R6/2 mice did not even develop the characteristic hindlimb clasping behavior throughout the disease course (Fig. 1B).

Curcumin-supplemented diet evokes the activation of pro-survival pathways in the striatum of R6/2 mice

Evidence demonstrates that curcumin has neuroprotective effect in animal models of several types of neurodegenerative disorders, including HD [35–37]. Here, we investigated whether early supplementation of curcumin might prevent neurological changes that classically appear in R6/2 mice as the disease progresses. Curcumin-fed HD mice were significantly protected from brain weight loss when compared with unsupplemented mice (Fig. 2A). The neuroprotective effect of life-long dietary curcumin in these mice was associated with increased levels of striatal dopamine- and cAMP-regulated protein 32 (DARPP-32) (Fig. 1B), a specific marker of medium spiny neurons (35), whose downregulation is related to neurodegenerative processes in HD (36). Importantly, curcumin supplementation also evoked the phosphorylation of AKT and ERK (Fig. 2C–D and Supplementary Material, Fig. S1), two kinases with pro-survival function (37) in HD mice. While phosphorylation of AKT was clearly detectable in both striatum and cortex of treated mice (Fig. 2C and Supplementary Material, Fig. S1A), activation of ERK was only found in the striatum (Supplementary Material, Fig. S1B). No biochemical changes were observed in wild-type (WT) mice after treatment (Supplementary Material, Fig. S2).

Curcumin stabilizes levels of brain-derived neurotrophic factor in both striatal and cortical tissues from R6/2 mice

Reduced levels of brain-derived neurotrophic factor (BDNF) is one of the most common hallmarks of HD, and interventions aimed at modulating its production have been largely proposed as potential therapeutic approach for the disease (38). Evidence shows that dietary curcumin supplement increases BDNF levels in pre-clinical models of several pathological conditions (38). Here, in order to further support the neuroprotective properties of curcumin, brain tissues from supplemented mice were analyzed.

As reported in Figure 3, curcumin preserved normal levels of BDNF in the cortex (Fig. 3A) and, importantly, also in the striatum

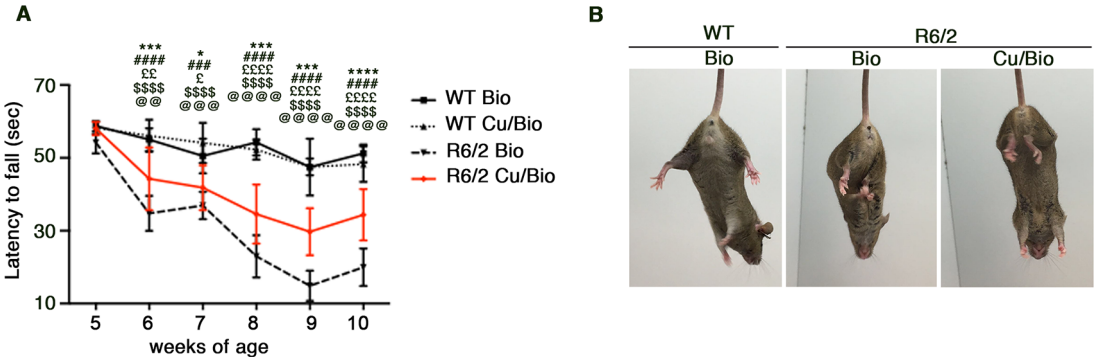

Figure 1. Administration of curcumin from conception preserves motor function in R6/2 mice. (**A**) Motor performance assessed by Rotarod. WT, $N = 5 + 5$; R6/2, $N = 8 + 8$. Values are represented as mean ± SD. @@, $P < 0.01$; @@@, $P < 0.001$; @@@@, $P < 0.0001$ (Cu/Bio-treated WT versus Cu/Bio-treated R6/2 mice). £, $P < 0.05$; ££, $P < 0.01$; ££££, $P < 0.0001$ (Bio-treated WT versus Cu/Bio-treated R6/2 mice). *$P < 0.05$; ***$P < 0.001$; ****$P < 0.0001$ (Bio-treated R6/2 versus Cu/Bio-treated R6/2 mice). ###, $P < 0.001$; ####, $P < 0.0001$ (Bio-treated WT versus Bio-treated R6/2 mice). $$$$, $P < 0.0001$ (Cu-treated WT versus Bio-treated R6/2 mice) (two-way ANOVA with Bonferroni post-test). **(B)** Limb-clasping response in 10-week old R6/2 mice. Bio: Bioperine/DMSO; Cu/Bio: Curcumin/Bioperine/DMSO.

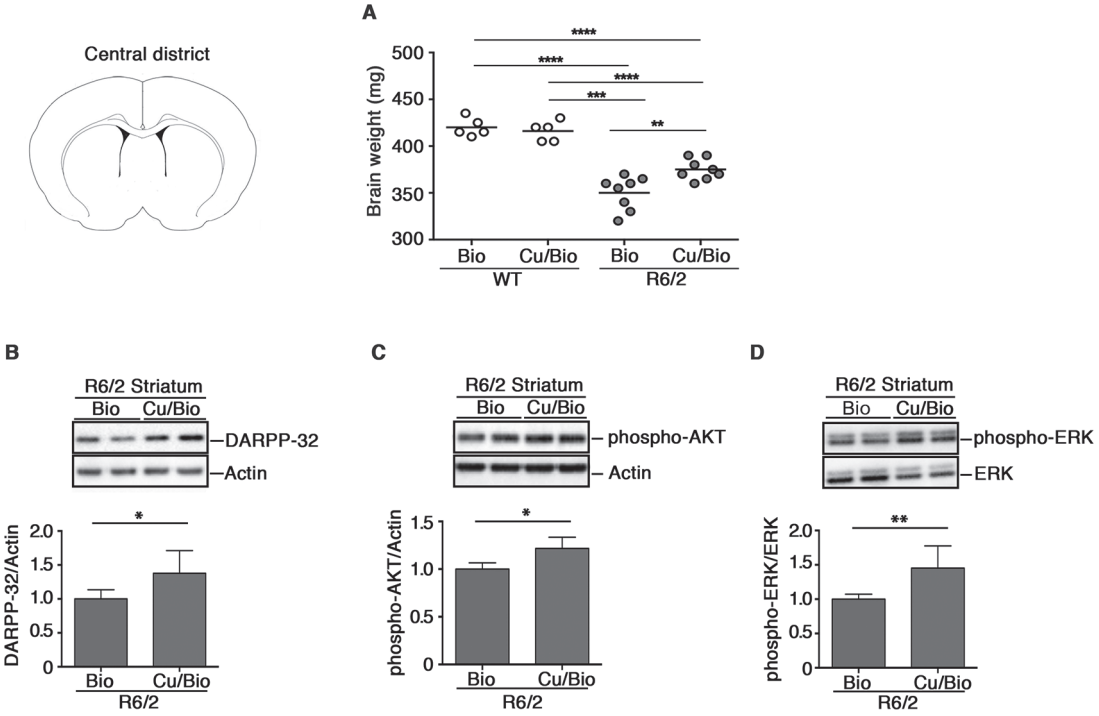

Figure 2. Life-long curcumin-supplemented diet preserves total brain weight and evokes the activation of pro-survival pathways in HD striatum. (**A**) Mouse brain weight. WT, $N = 5 + 5$; R6/2, $N = 8 + 8$. **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$ (one-way ANOVA with Tukey post-test). Representative immunoblottings and densitometric analysis of DARPP-32 (**B**), phospho-AKT (**C**) and phospho-ERK (**D**) in striatal tissues from Bio- and Cu/Bio-treated HD mice. $N = 6$–7. Values are represented as mean ± SD. *$P < 0.05$; **$P < 0.01$ (unpaired *t*-test).

(Fig. 3B) of HD mice. As expected, no effect was detected in WT mice after treatment (Supplementary Material, Fig. S3).

Life-long dietary curcumin reduces mHtt aggregation in brain tissues from R6/2 mice

mHtt aggregation is a major pathological feature that may conceivably account for cytotoxicity and neuronal dysfunction in HD (39, 40). Curcumin possesses anti-aggregation properties and its supplementation has been earlier found to mitigate cytotoxicity in different neurodegenerative conditions (41–43). Immunohistochemical staining of striatal sections from R6/2 mice showed lower number of EM48-positive ($EM48^+$) mHtt aggregates that were also significantly smaller in size when in contrast with those seen in control R6/2 mice (Fig. 4A–C). This result was further confirmed by immunoblotting analysis. $EM48^+$ SDS-insoluble aggregates were barely detectable in both striatal and cortical lysates from HD-supplemented mice (Fig. 4D and Supplementary Material, Fig. S4).

Curcumin-supplemented diet prevents body weight loss and ensures intestinal homeostasis in R6/2 mice

Loss of body weight is a severe peripheral complication in HD human patients and animal models, including R6/2 mice (4, 14). Evidence suggests that GI dysfunctions may contribute to this phenomenon (14). Curcumin dietary supplement preserves

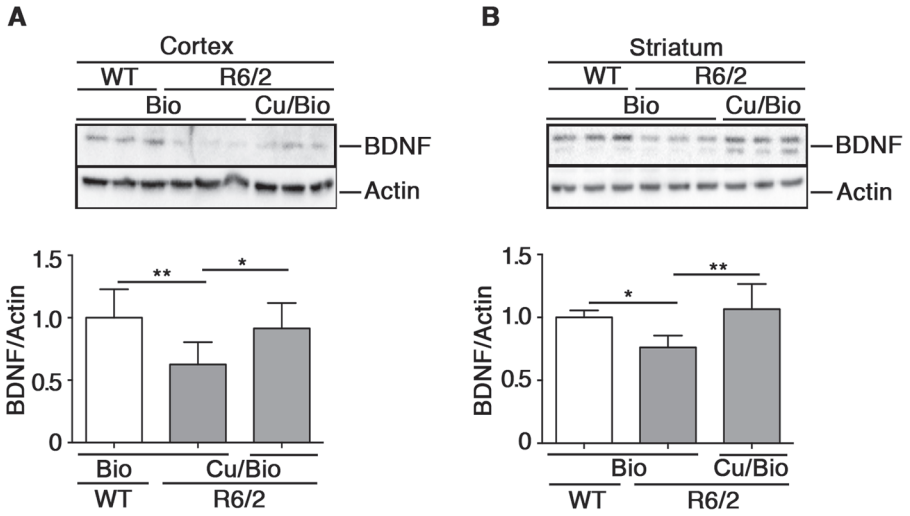

Figure 3. Curcumin restores normal levels of BDNF in HD mouse brain. Representative cropped immunoblottings of BDNF in cortex (**A**) and striatum (**B**) from Bio- and Cu-treated mice. $N = 6–7$. Values are represented as mean ± SD. $^*P < 0.05$ (unpaired *t*-test).

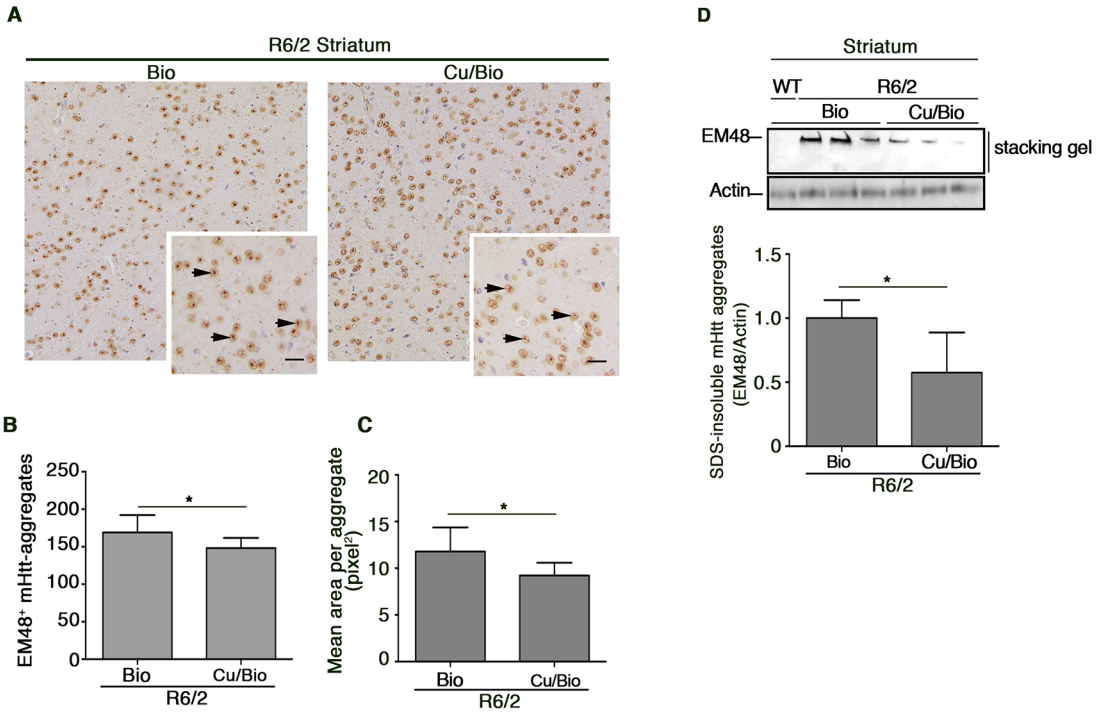

Figure 4. Curcumin-supplemented diet is associated with reduced mHtt aggregation. (**A**) Representative micrograph and (**B**) analysis of the number and (**C**) area of $EM48^+$ mHtt aggregates in the striatum of Bio- and Cu/Bio-treated R6/2 mice at 10 weeks of age. Arrows indicate mHtt aggregates. Scale bar represents 100 µm. Values are represented as mean ± SD. $^*P < 0.05$ (unpaired *t*-test). (**D**) Cropped immunoblotting of EM48-positive mHtt aggregates in striatal lysate from Bio- and Cu/Bio-treated R6/2 mice at 10 weeks of age. $N = 6–7$. Values are represented as mean ± SD. $^*P < 0.05$ (unpaired *t*-test).

metabolic health and stabilizes body weight in different disease conditions including GI disorders (25, 26).

Supplementation of curcumin completely prevented body weight loss in R6/2 mice to the extent to make them indistinguishable from WT littermates (Fig. 5A). Furthermore, curcumin increased food consumption in treated mice (Fig. 5B) and maintained the overall normal intestinal function by stimulating intestinal emptying as demonstrated by increased amount of collected stool over a period of 24 h (Fig. 5C).

Defective GI motility is known to be impaired in neurological disorders (44). Recent evidence demonstrates that curcumin has direct effect on intestinal contractility and significantly improves intestinal propulsion rate in functional GI disorders (45). Here, we demonstrated that administration of curcumin evoked changes in the smooth muscle contractility in both WT and HD mice (Fig. 5D and E). *Ex vivo* analysis of colonic ring contraction in response to KCl revealed barely detectable intestinal contractility in untreated HD mice (Fig. 5D, R6/2 Bio). Curcumin-supplemented R6/2 mice showed a significant difference in the mean contractile tension during KCl depolarization when in contrast with untreated R6/2 mice (Fig. 5D). Interestingly, curcumin preserved intestinal smooth muscle function in R6/2 mice to the

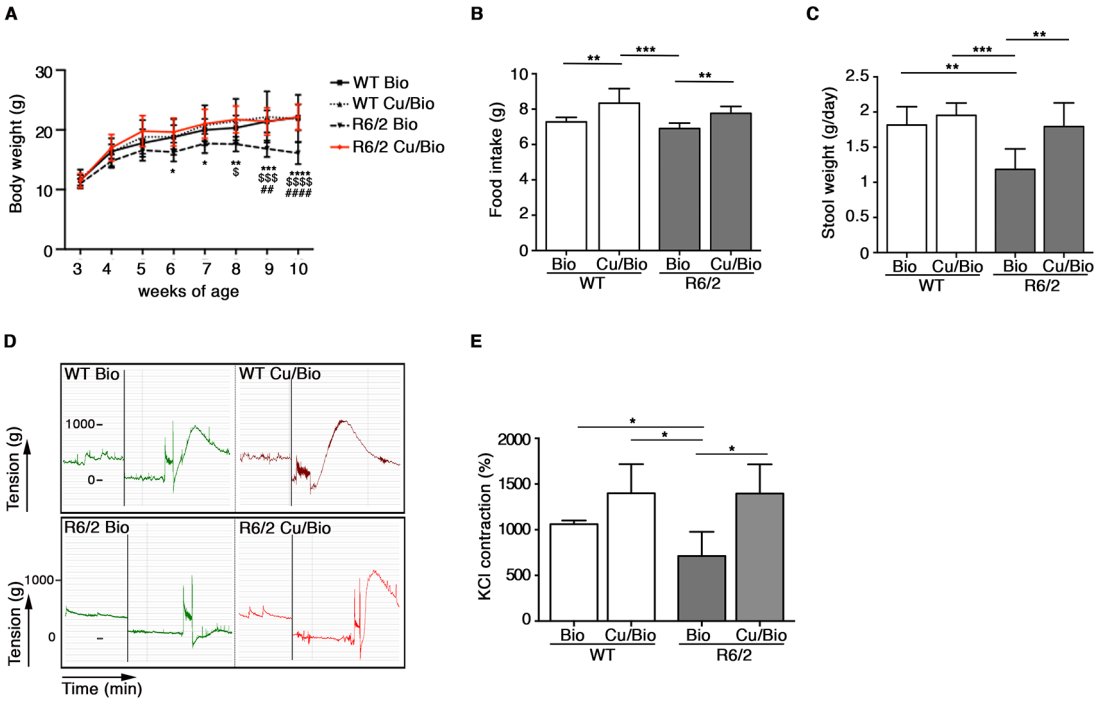

Figure 5. Curcumin-supplemented diet completely prevents the loss of body weight and maintains normal intestinal activity in HD mice. (**A**) Mouse body weight weekly measured during the entire period of the treatment. WT, $N = 5 + 5$; R6/2, $N = 8 + 8$. Values are represented as mean ± SD. (Bio-treated R6/2 versus curcumin-treated R6/2 mice). ##, $P < 0.01$; ####, $P < 0.0001$ (Bio-treated WT versus Bio-treated R6/2 mice). $, $P < 0.05$; $$, $P < 0.01$; $$$$, $P < 0.0001$ (Cu/Bio-treated WT versus Bio-treated R6/2 mice) (two-way ANOVA with Bonferroni post-test). (**B**) Food intake and (**C**) stool weight analysis assessed in Bio- and Cu/Bio-treated WT and R6/2 mice before sacrifice. (**D**) Representative contractility curves and (**E**) KCl-contraction percentage quantitation for Bio- and Cu/Bio-treated WT and R6/2 mice. $N = 4$ for each group of mice. Values are represented as mean ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (one-way ANOVA with Tukey post-test).

extent to make their contractile profile curve indistinguishable from that observed in WT mice (Fig. 5D, R6/2 Cu/Bio). No changes in the expression of peripheral neuronal markers were observed (Supplementary Material, Fig. S5).

Curcumin preserves normal GI tissue organization in R6/2 mice

Examination of the GI tract further corroborated the beneficial effect that curcumin exerts on GI dysfunction associated with HD. Curcumin-supplemented R6/2 mice did not show any pathological changes in the morphology of the GI tract as was, instead, clearly detectable in untreated HD mice (Fig. 6A). GI tracts of treated HD mice were indistinguishable in morphology from the WT group (Fig. 6A).

Morphometric analysis of small intestinal tract revealed that curcumin dietary supplement protected R6/2 mice from reduced villi length and atrophy that has been previously described to occur as the disease progresses (14) (Fig. 6B–C). No difference between R6/2 mice fed with curcumin and WT was observed (Fig. 6B and C).

Interestingly, colon cross-sections from curcumin-treated R6/2 mice showed significant reduction in the number of periodic acid–Schiff (PAS)-positive (PAS^+) goblet cells, which also appeared less dense of mucin, in the Lieberkühn crypts when in contrast with untreated R6/2 mice (Fig. 6D–E).

Positive modulation of gene expression of intestinal barrier proteins after curcumin supplementation further corroborated the protective effect that curcumin may exert on the GI tract in HD and consolidated its overall beneficial effect on physiological homeostasis (Fig. 7 and Supplementary Material, Figs S6 and S7).

Discussion

Since many years, curcumin, a component of turmeric is used worldwide in many different forms for its multiple potential health benefits in both preclinical animal models and human patients suffering from pathological conditions, ranging from the brain to GI disorders (26, 45, 46). Among the numerous therapeutic properties, the natural neuroprotective and anti-amyloid action makes curcumin a promising molecule for the treatment of several neurodegenerative diseases (46). After oral administration, curcumin readily crosses the blood brain barrier (47); however, the beneficial effect is best achieved when it is combined with bioavailability-enhancing agents such as black pepper extracts, like bioperine, that normally acts as an inhibitor of hepatic and intestinal glucuronidation, the metabolic pathway of curcumin. In rodent models of Alzheimer's disease (AD), administration of curcumin reduced plaque burden and protected against *β-amiloid* (A*β*)-toxicity *in vitro* and *in vivo* (42, 48, 49), thereby improving cognitive function (49, 50). Evidence indicates that administration of curcumin attenuated neuropathology and transcriptional deficits, including reduction levels of protein aggregates also in HD (34).

HD is conventionally defined as a neurological disorder; however, peripheral pathology, including GI perturbation, is increasingly becoming recognized as an important factor that may contribute to the complexity of the disease in both patients and animal models (2, 3, 14).

In this study, we reported for the first time that early intervention with oral curcumin mitigates the neuropathological disturbances as well as gastropathy in R6/2 mice, an HD transgenic animal model that displays some of the clinical features seen in HD patients (14, 15).

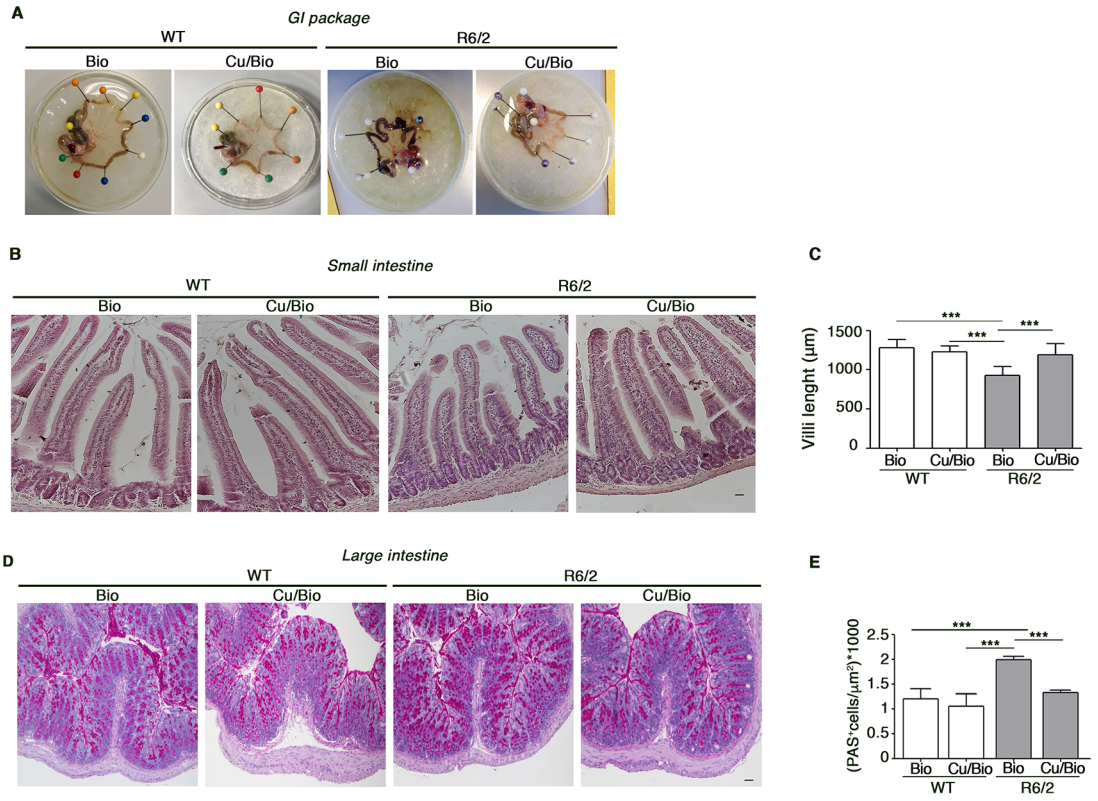

Figure 6. Life-long curcumin-supplemented diet preserves normal GI tissue organization in HD mice. (**A**) Representative micrographs of intestinal package from Bio- and Cu/Bio-treated WT mice and R6/2 mice at 10 weeks of age. (**B**) Representative hematoxylin and eosin-stained micrographs of small intestine and (**C**) analysis of their villi length of Bio- and Cu/Bio-treated WT mice and R6/2 mice at 10 weeks of age. (**D**) Representative PAS-stained micrographs of large intestine and (**E**) semi quantitative analysis of PAS^{+} cells in Bio- and Cu/Bio-treated WT mice and R6/2 mice at 10 weeks of age. Values are represented as mean ± SD. $^{***}P < 0.001$ (one-way ANOVA with Tukey post-test). Scale bar represents 100 µm.

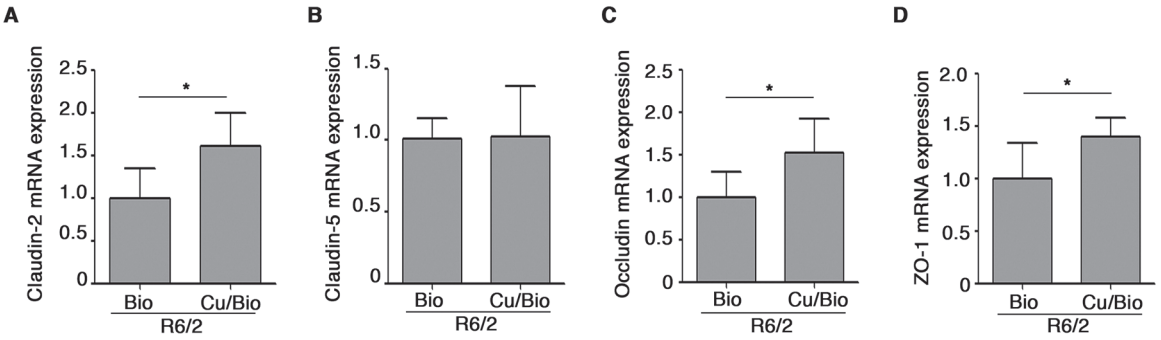

Figure 7. Curcumin increases expression of intestinal barrier genes. Quantitative PCR analysis of Claudin-2 (**A**), Claudin-5 (**B**), Occludin (C) and ZO-1 genes in Bio-and Cu/Bio-treated R6/2 mice at 10 weeks of age. $N = 5–6$. Data are represented as mean ± SD. $^{*}P < 0.05$ (unpaired *t*-test).

Life-long curcumin supplementation attenuated motor dysfunction in R6/2 mice and protected the brain from the classical atrophy occurring in these mice as the disease progresses. It evoked activation of pro-survival kinases AKT and ERK in brain tissues of these mice and stabilized the levels of BDNF in both cortex and striatum. Increased bioavailability of the neurotrophin in the striatum may likely depend on the effect that curcumin may have to support normal axonal transport between cortex and striatum. Unperturbed expression of BDNF in the striatum of treated mice may conceivably protect the tissue from loss of neurons, as revealed by increased levels of DARPP-32, whose downregulation is a clear sign of neuronal suffering in HD. In line with a neuroprotective action, curcumin also produces substantial decrease in EM48-positive misfolded Htt aggregates.

In addition to a protective effect of curcumin on brain pathology, we found that curcumin supplementation preserved GI homeostasis in R6/2 mice, which ultimately displayed a body weight indistinguishable from WT controls. Although the underlying molecular mechanism needs to be further investigated, we believe that the intestinal function and its preserved contractility after treatment may likely depend on activation of intestinal smooth muscle cells by curcumin rather than stimulation of peripheral neurons. The overall effect of curcumin in conserving intestinal morphology and villi length in treated mice resulted in an adequate absorption surface and nutrient uptake that could also explain the phenomenon. Reduced nutrient absorption along the GI tract has been already reported in R6/2 mice, and it is supposed to contribute itself

to the weight loss occurring during the progression of the disease (14).

The therapeutic effect of curcumin on GI dysfunction was further supported by quantitative PCR (qPCR) analysis that highlighted modulation of gene expression of proteins normally involved in the intestinal barrier integrity.

Our data corroborated the evidence of impaired intestinal contractility in R6/2 mice and highlighted, for the first time, an increased number of mucin dense goblet cells along GI tract that clearly indicates a perturbed intestinal physiology. Life-long treatment with curcumin normalized both intestinal contractility profile and the number of mucin-secreting cells in R6/2 mice to the extent of making them indistinguishable to WT littermates.

In line with previous evidence (28, 29, 31), curcumin-supplemented diet stimulated appetite and regularized the overall function of GI tract in treated R6/2 mice.

All these findings clearly indicated a role of dietary curcumin in inhibiting GI complication and preserving the homeostasis of intestine in HD mice. This result likely suggests that curcumin supplementation may be exploited to reduce paracellular permeability and malabsorption in HD.

The efficacy of curcumin in several pre-clinical trials for neurodegenerative diseases has created considerable excitement mainly due to its lack of toxicity and low cost. Taking into consideration our data and making use of the existing literature in support of the use of curcumin as a beneficial supplementation in humans, we believe that it would be worth investigating the potential therapeutic effect that curcumin administration may have in HD patients and its clinical significance.

Whether the effectiveness of curcumin supplementation in R6/2 mice can also be confirmed in HD patients needs to be tested; however, if this was the case, from our perspective, patients could greatly benefit from it, and although only speculative by now, it might be thought to start the supplementation even at pre-symptomatic stage of the disease.

Collectively, results from our study suggest that curcumin could be a worthy candidate for nutraceutical intervention in the disease and represent a new choice for HD patients, as it bears a therapeutic potential to treat both neurologic and GI abnormalities. However, further clinical investigation is still required.

Materials and Methods

Animal model, husbandry and treatment

Breeding pairs of the R6/2 line of transgenic mice [strain name: B6CBA-tgN (HDexon1) 62Gpb/1 J] with $\sim 160 \pm 10$ (CAG) repeat expansions were purchased from the Jackson Laboratories. All procedures on animals were approved by the IRCCS Neuromed Animal Care Review Board and were conducted according to EU Directive 2010/63/EU for animal experiments. Mice were housed in a temperature and humidity-controlled room under 12 h light–dark cycle. Mice were given *ad libitum* access to food and water. Male R6/2 mice were crossed with female B6CBA WT mice, and the resultant WT and R6/2 mice were used for all the experiments performed in this study. Breeding pairs were checked daily for litters and two rounds of breeding were used to generate mice for preclinical studies. Female breeding WT mice were fed normal cow supplemented with curcumin (25 mg/kg/day)/bioperine (1 mg/kg/day)/DMSO (Cu/Bio) or bioperine (1 mg/kg/day)/DMSO (Bio). One week before breeding, WT females ($n = 8$) were divided into two groups and the oral gavage administration of Bio or Cu/Bio was started. Females were then crossed with R6/2 males fed with normal cow. Dietary supplementation of Cu/Bio in breeding females was performed for the entire period of gestation and for 3 weeks after the offspring birth. Treatment continued in weaned pups for 7 weeks (Supplementary Material, Fig. S8).

In order to assess any possible effect of bioperine, a pilot study was performed on a small group of both WT and HD mice. To this purpose, bioperine dissolved in DMSO and diluted in saline (vehicle) was daily administered by gavage at a dose of 1 mg/kg per day and motor function was assessed. Control mice (WT and R6/2) were daily fed with the same volume of vehicle-containing DMSO. As reported in the Supplementary Material, Fig. S9, no effect was detected in any of the groups.

A total of 43 litters (17 WT: 8 Bio and 9 Cu/Bio; 26 R6/2: 13 Bio and 13 Cu/Bio) were generated and used in this study. Food consumption in adult progeny was determined over the course of a 24 h observation period. Mice were housed separately to permit each animal's food consumption to be calculated from the difference in weights of the food supply at the beginning and at the end of the observation period.

General health monitoring and motor behavior tests

The overall animal health was monitored every day. Body weight was recorded in the offspring once per week starting from 4 weeks of age. Motor performance and abnormalities were assessed by Rotarod tests and hindlimb clasping behavior, respectively, as previously described (51, 52). Mice were habituated to the testing rooms for 15–20 mins prior to testing. All tests took place during the light phase of the light–dark cycle, and littermates were tested for the entire period of the treatment at the indicated time points. All analyses were carried out blinded to genotype and treatment.

Measurement of gastric emptying by 24 h stool collection

For the assessment of gastric emptying, a single-housed mouse was placed in a separate clean cage and fecal pellets were collected after a 24 h period in 1.5 ml eppendorf tubes. Tubes were weighed to obtain the wet weight of the stool.

Measurements of intestinal contractility

At a designated time point, animals were sacrificed by cervical dislocation and the peritoneal cavity was opened via a U-shaped incision based in the lower abdomen for complete removal of the abdominal package. Colonic segments were then isolated, and contractility was measured in organ baths containing Krebs-Ringer buffer at 37°C and aerated with 95% O_2 and 5% CO_2. Colonic segments were stretched gradually to 400 g tension and challenged with 10 mM potassium chloride (KCl). Changes in isometric tension were measured and recorded by LabScribe 2 software (BIoseb).

Histological and immunohistochemical analysis of intestine

After sacrifice, small and large intestine segments were dissected out, gently flushed with cold sterile saline solution to remove intestinal contents and immediately placed in 10% neutral buffered formalin for no longer than 48 h and then processed for histology. Intestinal segments were then cut into 5 µm coro-

nal sections on an RM 2245 microtome (Leica Microsystem) and stained with hematoxylin/eosin and PAS for the assessment of villi length and detection of the secretory cells lining the GI tract and the goblet cells, respectively. Four mice per group were used and six coronal sections for each animal were scanned. For each coronal section, a total number of 2 fields at 10× magnification were analyzed. Images were acquired with Nikon Eclipse Ni motorized microscope. All stained sections were examined on a blinded basis.

Villi length. Only well-orientated villi indicating complete longitudinal sectioning were selected for the analysis. The measurements were taken linearly at the center of the villus from the basis at the crypt-villus junction to the villus apex. The average of villi length per small intestine section was quantified by NIS-Elements AR Analysis. Mean villus length per segment was estimated as the mean of the tallest four to seven villus profiles measured per location.

PAS$^+$ goblet cells. Paraffin embedded 5 µm colon specimen sections were stained with PAS-hematoxylin system (Sigma-Aldrich) to assess the positive goblet cell population (53). Results were expressed as '(PAS positive cells/µm^2)*1000'.

RNA extraction from intestinal samples and qPCR

Total RNA from small intestine was extracted using RNeasy kit (Qiagen) according to the manufacturer's instructions. Of total RNA, 1000 ng was synthesized using Super Script III reverse transcriptase (Invitrogen) and the resulting cDNA was then amplified by quantitative RT-qPCR to determine the mRNA expression levels of intestinal barrier genes, using specific primers: mouse Claudin-1 FW: 5′-CTGGAAGATGATGAGGTGCAGAAGA-3′; mouse Claudin-1 RV: 5′-CCACTAATGTCGCCAGACCTGAA-3′; mouse Claudin-2 FW: 5′-TGAACACGGACCACTGAAAG-3′; mouse Claudin-2 RV: 5′-TTAGCAGGAAGCTGGGTCAG; mouse Claudin-5 FW: 5′-CAGTTAAGGCACGGGTAGCA-3′; mouse Claudin-5 RV: 5′-GGCACCGTCGGATCATAGAA-3′; mouse Claudin-15 FW: 5′-GCAGGGACCCTCCACATA-3′; mouse Claudin-15 RV: 5′-GACGGCG TACCACGAGATAG-3′; mouse Occludin FW: 5′-AGACCTGATGAAT TCAAACCCAAT-3′; mouse Occludin RV: 5′-ATGCATCTCTCCGCCA TACAT-3′; mouse ZO-1 FW: 5′-TTCTTCGAGAAGCTGGATTCCT-3′; mouse ZO-1 RV: 5′-TCTGGCAACATCAGCTATTGGT-3′; mouse Cyclophilin A FW: 5′-TCCAAAGACAGCAGAAAACTTTCG-3′; mouse Cyclophilin A RV: 5′-TCTTCTTGCTGGTCTTGCCATTCC-3′. RT-qPCR was performed on a CFX Connect RT-PCR Detection System (Bio-Rad Laboratories) by SYBR-Green mix (Lifetech, Cat. no.: 4367659). Cyclophilin A was used as a housekeeping gene for normalization of mRNA expression results.

Brain pathology and immunohistochemistry

After sacrifice, the brains were pulled out of the skull and trimmed by removing the olfactory bulbs and spinal cord. The remaining brain was then weighed, processed for histology and embedded in paraffin wax for microtome cutting. Four mice per group were used, and four coronal sections spread over the anterior–posterior extent of the brain (200–300 µm intersection distance) were scanned. For each 10 µm coronal section, a total number of 5 fields at 20× magnification were analyzed. Immunostaining for mHtt aggregates was carried out by using EM48 antibody (1:200) (Millipore). The number of striatal mHtt inclusions as well as their average size of per brain section were quantified by ImageJ software.

Brain lysate and immunoblottings

After sacrifice, some of the mouse brains were snap-frozen in liquid N2 and pulverized in a mortar with a pestle. Pulverized tissue was then homogenized in lysis buffer containing 20 mm Tris, pH 7.4, 1% Nonidet P-40, 1 mm EDTA, 20 mm NaF, 2 mm Na3VO4 and protease inhibitor mixture (Santa Cruz, Cat. N. sc-29 131), sonicated with 2 × 10s pulses and then centrifuged for 10 min at 10 000*g*. Protein concentration was determined by Bradford method. Protein lysates (20 µg) were resolved on 10% SDS-PAGE and immunoblotted with the following antibodies: anti-phospho-AKT (1:1000) (Immunological Sciences, Cat. N. AB-10521); anti-phospho-ERK (1:1000) (Cell Signaling, Cat. N. #9101); anti-ERK (1:1000) (Immunological Sciences, Cat. N. AB-82379); anti-DARPP-32 (1:1000) (Cell Signaling, Cat. N. #2302); anti-BDNF (1:1000) (Santa Cruz, Cat. N. sc-546); and anti-actin (1:5000) (Sigma Aldrich, Cat. N. A5441). For the analysis of SDS-insoluble mHtt aggregates, cell lysates were resolved on 10% SDS-PAGE; the entire gel, including the stacking portion, was transblotted overnight on a 250 mV in 0.05% SDS and 16% methanol-containing transfer buffer (51). Membrane was blocked in 5% non-fat dry milk TBST for 1 h and successively immunoblotted with EM48 antibody (1:1000). Immunoblots were then exposed to specific HRP-conjugated antibodies (Santa Cruz, Cat. N. sc-2004 and sc-2005). Protein bands were visualized by ECL and quantitated with Quantity One software (Bio-Rad Laboratories).

Statistics

Two-way ANOVA followed by Bonferroni post-test was used to compare experimental groups for Rotarod tests and for mouse body weight analysis. One-way ANOVA and two-tailed unpaired *t*-test were used in all other experiments as indicated. All data were expressed as mean ± SD.

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