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The autophagy-enhancing drug carbamazepine improves neuropathology and motor impairment in mouse models of Machado–Joseph disease

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Abstract

Aims: Machado–Joseph disease (MJD), or spinocerebellar ataxia type 3 (SCA3), is the most common autosomal dominantly-inherited ataxia worldwide and is characterised by the accumulation of mutant ataxin-3 (mutATXN3) in different brain regions, leading to neurodegeneration. Currently, there are no available treatments able to block disease progression. In this study, we investigated whether carbamazepine (CBZ) would activate autophagy and mitigate MJD pathology.

Methods: The autophagy-enhancing activity of CBZ and its effects on clearance of mutATXN3 were evaluated using *in vitro* and *in vivo* models of MJD. To investigate the optimal treatment regimen, a daily or intermittent CBZ administration was applied to MJD transgenic mice expressing a truncated human ATXN3 with 69 glutamine repeats. Motor behaviour tests and immunohistology was performed to access the alleviation of MJD-associated motor deficits and neuropathology. A retrospective study was conducted to evaluate the CBZ effect in MJD patients.

Results: We found that CBZ promoted the activation of autophagy and the degradation of mutATXN3 in MJD models upon short or intermittent, but not daily prolonged, treatment regimens. CBZ up-regulated autophagy through activation of AMPK, which was dependent on the myo-inositol levels. In addition, intermittent CBZ treatment improved motor performance, as well as prevented neuropathology in MJD transgenic mice. However, in patients, no evident differences in SARA scale were found, which was not unexpected given the small number of patients included in the study.

Conclusions: Our data support the autophagy-enhancing activity of CBZ in the brain and suggest this pharmacological approach as a promising therapy for MJD and other polyglutamine disorders.

KEYWORDS
ataxin-3, autophagy, carbamazepine, Machado–Joseph disease, neuroprotection

INTRODUCTION

Machado–Joseph (MJD), also known as spinocerebellar ataxia type 3 (SCA3), is a fatal neurodegenerative disorder, characterised by a wide range of clinical manifestations, including limb and gait ataxia, progressive external ophthalmoplegia, pyramidal and extra pyramidal signs (such as dystonia with rigidity), and distal muscular atrophy [1, 2]. MJD is caused by the expansion of the CAG repeat region in the *ATXN3* gene, which translates into an expanded polyglutamine tract within the protein ataxin-3 (ATXN3) [3]. Mutant ATXN3 (mutATXN3) becomes prone to misfolding, aggregation and acquires toxic properties, which lead to neuronal dysfunction and cell death [4, 5]. Since mutant and aggregated ATXN3 is insoluble and cannot be efficiently degraded by the ubiquitin-proteasome system, autophagy is an alternative pathway for degradation.

Autophagy is a major clearance pathway in which dysfunctional proteins and organelles are transported to lysosomes for degradation [6–8]. This clearance pathway contributes to the normal turnover of intracellular components, playing a key role in cellular quality control.

Extensive evidence supports a crucial role for autophagy in preventing neurodegeneration. Studies using knockout mice for *Atg5* or *Atg7* showed that impairment of autophagy led to the accumulation of ubiquitin-positive inclusions and to the development of a neurological phenotype with neurodegeneration [9, 10]. Moreover, the presence of an abnormal number of autophagosomes in affected neurons of different neurodegenerative conditions indicates altered function of the autophagy pathway [11–13]. Dysfunction in autophagy has been linked to the pathogenesis of numerous neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease and MJD, as we previously reported [14, 15]. We demonstrated that stimulation of this pathway by overexpression of beclin-1 or ULK1 [14, 16, 17] could mitigate the disease, supporting the idea that activation of autophagy may be a suitable strategy for treatment.

Although gene therapy with viral vectors for beclin-1 or ULK1 overexpression was promising in restoring autophagy in specific CNS regions, less invasive strategies, able to reach of the brain should be explored. Various pharmacological strategies aimed at induction of autophagy in neurodegenerative disorders have been investigated in recent years. Effective activation of autophagy can be achieved through inhibition of mTOR [18–21]; however, the long-term use of drugs such as rapamycin or rapalogues may lead to complications associated with their immunosuppressive activity and pleiotropic effects. For this reason, molecules that trigger mTOR-independent autophagy have emerged as promising alternatives [22–26].

Some evidence suggests that carbamazepine (CBZ), an anticonvulsant and mood-stabilising drug, signals through the phosphoinositol pathway, lowering inositol and IP3 levels, and enhancing autophagy [27–30]. CBZ is a drug approved for clinical use, able to cross the blood brain barrier, and therefore a natural candidate to promote autophagy in the brain. Therefore, the main goal of this study was to investigate whether the autophagy-enhancing activity of CBZ would alleviate MJD.

Key points

- Carbamazepine (CBZ) stimulates autophagy by a mechanism dependent on the myo-inositol levels and AMPK activation.
- The autophagy-enhancing activity of CBZ leads to the degradation of mutATXN3 in *in vitro* and *in vitro* models of Machado–Joseph disease (MJD).
- Intermittent CBZ treatment improves MJD-associated motor deficits and prevents cerebellar degeneration in transgenic mice, which suggests that CBZ may be a promising therapeutic approach for MJD.

Using in vitro and *in vivo* models of the disease, we found that CBZ was able to increase autophagy and promote degradation of mutATXN3, through AMPK activation. Moreover, intermittent CBZ treatment alleviated the motor defects and neuropathology of MJD transgenic mice. This suggests that an optimal CBZ treatment regimen might be a powerful therapeutic approach to alleviate MJD and possibly other diseases characterised by misfolding and toxicity of aggregate-prone proteins.

MATERIALS AND METHODS

Viral production, purification and titre assessment

Lentiviral vectors encoding for (i) mutATXN3 (LV-PGK-ATXN3 72Q) [31]; (ii) mCherry-EGFP-LC3 (LV-CMV-mcherry-EGFP-LC3) were produced in the Human Embryonic Kidney cells 293T with a four-plasmid system, as previously described [32]. Lentiviral particles were suspended in sterile 0.5% bovine serum albumin diluted in PBS. The viral particle content of each batch was evaluated by assessing the HIV-1 p24 antigen levels by ELISA (Retro Tek, Gentaur), in accordance with the manufacturer's instructions.

Neuro2a cell culture

The Neuro2a cells were (i) infected with lentiviral vectors encoding for mCherry-EGFP-LC3B (LV-CMV-mcherry-EGFP-LC3B) or (ii) transfected with the plasmid encoded for truncated mutATXN3 with 69 glutamines (TmutATXN3) [29], with a mixture of DNA/polyethylenimine complexes (MW40000, PolySciences).

At 2 weeks post-infection and 24-h post-transfection, cells were treated with CBZ, rapamycin and/or chloroquine. Further information can be found in the supporting information Materials and Methods section.

Fluorescent detection of mCherry-EGFP-LC3B and quantitative analysis

Cell cultures were washed with PBS and fixed with 4% paraformaldehyde in PBS. Cells were incubated for 5 min with DAPI (1:5000, Sigma), washed and mounted in fluorescence mounting medium (Dako). Direct fluorescence of mCherry-GFP-LC3B was visualised using a Zeiss LSM 710 confocal microscope (Carl Zeiss MicroImaging), equipped with Plan-Apochromat 63x/1.40 Oil DIC M27 (420782-9900) objective and Zen Black 2012 Image software.

To quantify autophagosomes/autolysosomes, six images were randomly acquired, and 10 cells selected per condition. The total yellow and red puncta were then manually counted using ZEN software.

Subjects and data collection

All subjects included in this study are genetically confirmed MJD patients and were followed-up at the Department of Neurology of the University of Campinas, São Paulo, Brazil. To obtain data from the identified subjects, available clinical records from January 2011 until December 2015 were retrospectively inspected. Demographic data—sex, age and age of disease onset—were collected. The usage of CBZ treatment and the Scale for the Assessment and Rate of Ataxia (SARA) scores were compiled for each clinical assessment, when available. Other variables, such as the number of clinical visits and the follow-up time were assessed after all data collection.

The study design consisted of two arms defined in accordance with the usage of CBZ: one arm constituted by the patients who took CBZ, and the second arm consisting of those that did not take CBZ. No interindividual distinction was made regarding dose (200 to 600 mg/day) and time of CBZ intake. SARA scores were collected only during the period of treatment (6–42 months) of each patient.

Clinical visits with no information regarding SARA score were excluded from analysis.

Individuals with no or only one visit in the defined study timeframe were excluded from the analysis, given that it was not possible to evaluate the evolution of the SARA score. Likewise, individuals whose CBZ intake was not known were also excluded.

Animals

The lentiviral-based MJD mouse model was generated as previously established in our laboratory [31]. C57BL/6 mice from Charles River Laboratories were used. The detailed protocol is described in the next section.

The transgenic mouse model used was developed by Hirokazu Hirai group, Kanazawa University, Japan [33, 34] and a colony of these transgenic mice was established in the licensed animal facility (International Animal Welfare Assurance number 520.000.000.2006) at the Center for Neuroscience and Cell Biology (CNC) of the University of Coimbra, Portugal. The transgenic mice express N-terminal truncated human ATXN3 in the cerebellum, with 69 CAG repeats together with an N-terminal HA Epitope, driven by a L7 promoter. The genotype was confirmed by PCR. Animals were housed in groups (2–5 per cage) in plastic cages (365 × 207 × 140 mm) with food and water ad libitum and maintained on a 12-h light/dark cycle at a room with constant temperature (22 ± 2°C) and humidity (55 ± 15%).

In vivo injection of viral vectors

Six-week-old WT mice were anaesthetized by intraperitoneal injection of a mixture of ketamine (80 mg/kg) and xylazine (4 mg/kg). Mice were then stereotaxically injected unilaterally in the striatum with lentiviral vectors at a 400 ng of p24 antigen concentration and encoding for the mutATXN3 with 72Q, at the following coordinates: +0.6 mm rostral to lambda, −1.8 mm midline, and −3.3 mm ventral to the skull surface, with the mouth bar set at 0.

Carbamazepine treatment

In the first and second *in vivo* experiments, CBZ (Sigma, St. Louis, USA) was administered at 50 mg/kg/day in 1% Tween 80/saline by i.p. injection for 1 and 4 weeks, respectively, and the equivalent volume of the vehicle was given to the control group. In the third *in vivo* experiment, mice received 50 mg/kg of CBZ or equivalent volume of the vehicle, 3 times per week for 10 weeks.

Behavioural assessment

Transgenic mice were subjected to locomotor tests at 2, 4, 6 and 8 weeks of CBZ treatment. Animals were acclimatised for 1 h to a quiet room with controlled temperature and ventilation, dimmed lighting, and were handled prior to behavioural testing. Rotarod and footprint tests were used and are described in the supporting information Materials and Methods section.

Protein isolation and western blot analysis

Protein was extracted from brain tissue and cells and western blot was performed as detailed in the supporting information Materials and Methods section.

Tissue preparation

Mice were sacrificed with an overdose of Xylazine/Ketamine (8/160 mg/kg body weight, intraperitoneally). Perfusion with PBS and fixation with 4% paraformaldehyde (Sigma-Aldrich) were performed transcardially. Brains were collected and post-fixed in 4% paraformaldehyde for 24 h and were then cryoprotected/dehydrated by

immersion in 25% sucrose in PBS for 36 to 48 h. Brains were frozen at −80°C and 30 μm sagittal sections were sliced using a cryostat (LEICA CM3050 S, Leica Microsystems) at −21°C. Slices were collected in anatomical series and stored in 48-well plates as free-floating sections in PBS supplemented with 0.05% (m/v) sodium azide, at 4°C until immunohistochemical procedures, described in the supporting information Materials and Methods section.

mRNA expression analysis and quantitative real-time PCR

mRNA from mice samples was extracted with the Nucleospin RNA kit (Macherey-Nagel), preceded by homogenisation of the tissue with TRIzol™ Reagent (Invitrogen) and d-chloroform. Quantitative real-time PCR (qPCR) was performed to evaluate the levels of LC3B, *C/EBPβ*, *Arg1*, *IL-1β and IL-6*, and RT^2 profiler PCR array was used for the analysis of 84 autophagy-associated genes, as described in detail in the supporting information Materials and Methods section.

Statistical analysis

Results are presented as mean ± standard error of mean (SEM) of at least three independent experiments. Statistical analysis was performed with unpaired Student's *t* test to compare means between two groups. One-way ANOVA, followed by Dunnett's post hoc test was used to perform comparisons between multiple conditions for a single control, Tukey's post hoc test was used when testing for all possible variable pairings within an experiment, and Šidák's post hoc test when a more conservative method was adequate. Significant thresholds were set at $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, as defined in the text.

In clinical data, longitudinal variations were determined over the longest available extent of time and then converted to annual coefficients.

All statistical analysis was conducted in GraphPad Prism version 6.01 for Windows (GraphPad Software) and R (R Core Team).

RESULTS

Carbamazepine increases autophagy in neuronal models

CBZ has been proposed to be a potent autophagy-enhancing drug in different diseases [27–29, 35]. To investigate whether CBZ would activate autophagy in neuronal cells, we used lentiviral vectors to generate a Neuro2a cell line stably expressing mcherry-EGFP-LC3B [36]. This assay allowed monitoring of the distribution of LC3B, and formation of autophagosomes and autolysosomes, given by quantification of yellow puncta (fluorescence for both mCherry and GFP) and red puncta (fluorescence of mCherry alone due the decline of GFP fluorescence in acidic lysosomal environment), respectively. A robust increase in the number of autophagosomes and autolysosomes were observed in cells treated with CBZ and with the positive control, rapamycin, when compared to control cells, suggesting an increase in autophagic flux (Figure 1A–D).

These observations were further confirmed by western blot analysis (Figure 1E). To measure autophagic activity, we used a lysosomotropic reagent, chloroquine, which blocks degradation of LC3BII and allows enhanced detection of the LC3B that is delivered to lysosomes for degradation [37, 38]. We found increased levels of LC3BII in cells treated with CBZ, as well as with rapamycin, compared to basal conditions (Figure 1F). These results were also accompanied by a decrease of p62 levels in cells treated with CBZ and rapamycin, when compared to control, demonstrating an efficient turnover of protein cargo (Figure 1G).

We further evaluated whether CBZ would have the same effect when administrated *in vivo*. We performed experiments using two animal models: (a) a lentiviral mouse model [31, 39] expressing full-length mutATXN3 (72CAG) and pre-symptomatic at the time of the treatment initiation, and (b) a transgenic mouse model expressing a truncated fragment of mutATXN3 with 69 CAG (HA-tagged), symptomatic and with cerebellar atrophy at the time of treatment initiation [34]. Mice were treated for 1 week with 50 mg/kg/day of CBZ or vehicle, and autophagy activation was evaluated by measuring LC3B and p62 levels. In both animal models, we observed a significant decrease of p62 levels in animals treated with CBZ, compared to controls (Figures 1H,I and 1K,L), which was accompanied by an increase of *LC3B* mRNA expression (Figures 1J and 1M). Moreover, using a qPCR array that allowed the evaluation of 84 key genes involved in autophagy and autophagy-related processes (Autophagy RT^2 Profiler PCR Array; Qiagen), we found that 4 weeks of treatment with CBZ in transgenic mice promoted up-regulation of important genes involved in autophagy such as *LC3B*, *Atg4a* or *Atg7* (Figure S1).

These results indicate that CBZ mediates activation of autophagy *in vitro* and in the brains of two mouse models of MJD.

Carbamazepine promotes clearance of mutATXN3 in Machado–Joseph disease cellular and mouse models

Having found evidence of CBZ-mediated activation of autophagy in the different models of MJD, we then evaluated whether this effect would result in the clearance of mutATXN3. For this purpose, we treated Neuro2a cells transfected with TmutATXN3 [34] with different concentrations of CBZ (10–100 μM) for 24 h. Western blot analysis revealed a drastic reduction of the high molecular weight (MW) species of mutATXN3 after the treatment with 50 and 100 μM of CBZ (Figure 2A,B). Soluble TmutATXN3 was also significantly decreased when cells were treated with 100 μM of CBZ (Figures 2A and 2C), which indicates this dose as the most effective to promote clearance of mutATXN3.

We further evaluated whether the treatment of animal mouse models under the same previous conditions could lead to a decrease

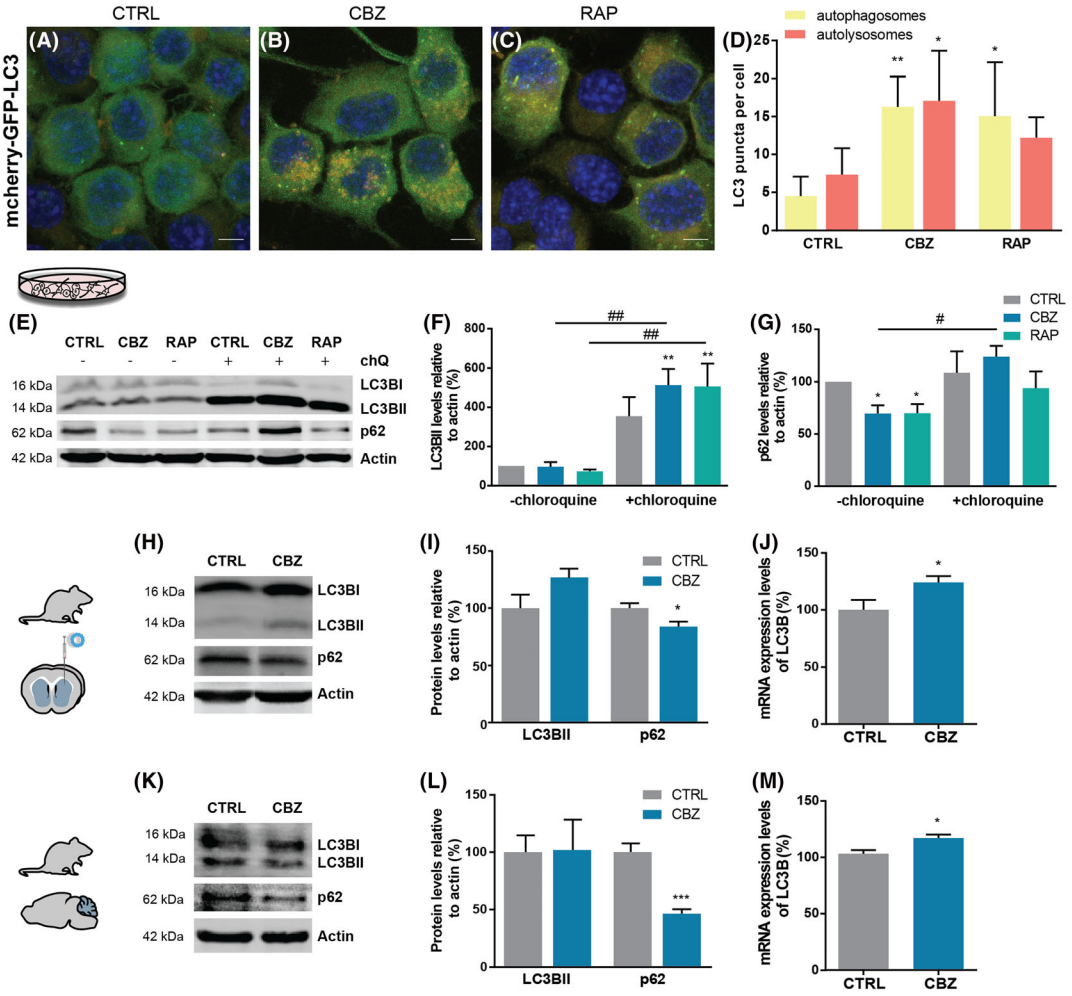

FIGURE 1 CBZ increases autophagy in neuronal models. (A–D) confocal microscopy analysis of Neuro2a cells expressing mcherry-EGFP-LC3B treated with CBZ (50 μM, 6 h) and rapamycin (100 nM, 2 h). CBZ significantly increases the number of autophagosomes (yellow dots) and autolysosomes (red dots). Values are expressed as mean ± SEM of $n = 4, 5$. One-way ANOVA with Dunnett's post hoc test was used to compare Neuro2a cells expressing mcherry-EGFP-LC3B treated with CBZ and rapamycin relative to CTRL (*$P < 0.05$, **$P < 0.01$). (E–G) Western blot analysis of Neuro2a cells treated with CBZ and rapamycin in presence or absence of chloroquine (chQ, 100 μM). CBZ and rapamycin significantly increase LC3BII levels in the presence of chloroquine (F) and decreases p62 levels in the absence of chloroquine (G). Densitometric quantification of LC3B and p62 levels relative to actin. Values are expressed as mean ± SEM of n = 5. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test (LC3B and p62 analysis) and Dunnett's post hoc test (p62 analysis of Neuro2a cells treated with CBZ and rapamycin relative to CTRL in absence of chQ) (*$P < 0.05$, **$P < 0.01$, #$P < 0.05$, ##$P < 0.01$). (H–J) Mice injected with lentivirus encoding for mutATXN3 and (K-M) transgenic mice were treated with CBZ (50 mg/kg) or vehicle for 1 week. (I, L) Western blot analysis of p62 shows a significant decrease of p62 levels in CBZ treated mice, compared to controls. (J, M) relative mRNA expression of *LC3B* is significantly increased in CBZ treated mice, compared to controls. Densitometric quantification of LC3B and p62 levels relative to actin. Values are expressed as mean ± SEM of $n = 4$. Statistical analysis was performed with unpaired Student's *t* test (*$P < 0.05$, ***$P < 0.001$). CTRL—control; CBZ—carbamazepine; RAP—rapamycin; chQ—chloroquine

of mutATXN3 levels upon activation of autophagy. Striatal and cerebellar lysates of mice were analysed by western blot to measure the levels of mutATXN3. After 1 week of treatment, we observed a decrease in the levels of the high MW species of mutATXN3 in lentiviral (though not reaching significance) and transgenic mouse models, when compared to control group (Figures 2D,E and 2G,H).

These results suggest that CBZ promotes degradation of mutATXN3 in the *in vitro* and in the two *in vivo* mouse models of MJD after a short-term treatment.

Carbamazepine enhances autophagy through activation of AMPK

Different studies have suggested that CBZ, similarly to other mood-stabilising drugs, induces autophagic activity through the phosphoinositol pathway, lowering inositol and IP3 levels [27, 29, 40]. To investigate whether CBZ acts through the same signalling pathway in our neuronal models, we used Neuro2a cells transfected with TmutATXN3 that were treated in the presence or absence of CBZ

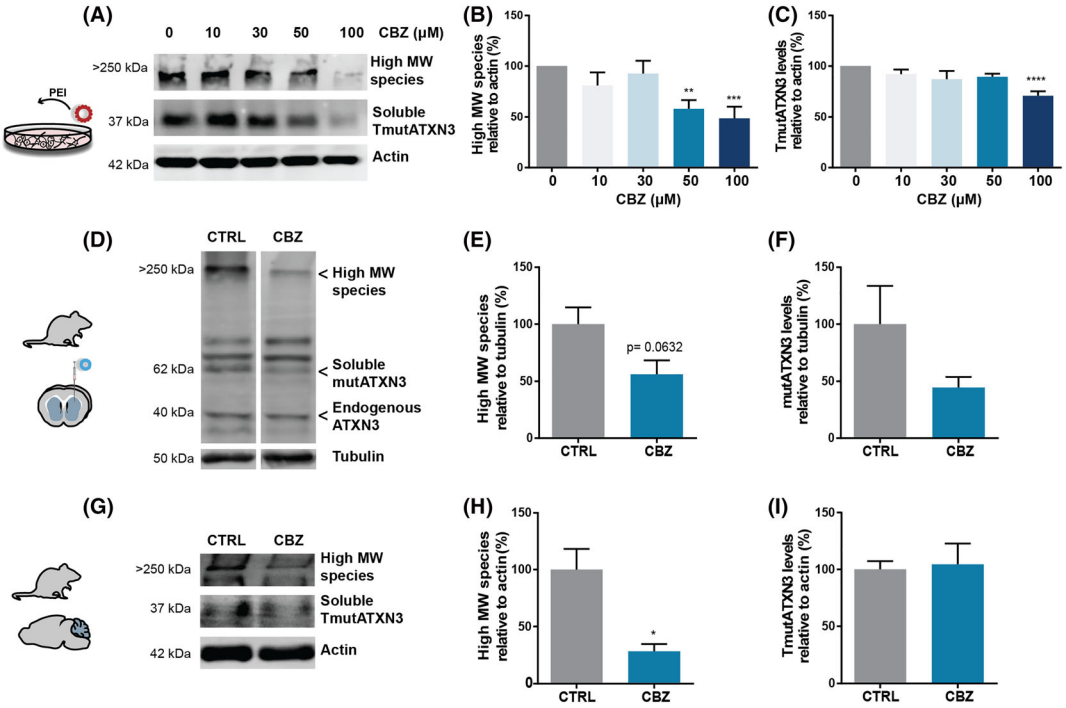

FIGURE 2 CBZ promotes clearance of mutATXN3 in Machado–Joseph disease (MJD) cellular and mouse models after short-term treatment. (A–C) Western blot analysis of Neuro2a cells expressing mutATXN3 treated with CBZ (10 to 100 μM, 24 h). CBZ significantly decreases the high MW species (B) and soluble TmutATXN3 (C) from 50 μM. Densitometric quantification of mutATXN3 levels relative to actin. Values are expressed as mean ± SEM of $n = 5–14$. One-way ANOVA with Dunnett's post hoc test was used to compare Neuro2a cells treated with different doses of CBZ relative to CTRL (**$P < 0.01$, ***$P < 0.01$, ****$P < 0.0001$). (D–F) Mice injected with lentivirus encoding for mutATXN3 and (G–I) transgenic mice were treated with CBZ (50 mg/kg) or vehicle for 1 week. (E, H) Western blot analysis of mutATXN3 shows a decrease of the high MW species in CBZ treated mice, compared to controls. Densitometric quantification of mutATXN3 relative to tubulin (D) and actin (G). Values are expressed as mean ± SEM of $n = 4$. Statistical analysis was performed with unpaired Student's t test (*$P < 0.05$). CBZ—carbamazepine; CTRL—control; MW—molecular weight

and myo-inositol. As predicted, CBZ led to a significant decrease in the levels of the high MW species of mutATXN3. However, when an excess of myo-inositol was applied to the cells, CBZ no longer promoted clearance of the mutant protein (Figure 3A). Accordingly, the increase in autophagic flux ((LC3BII + ChQ) − (LC3BII − ChQ)) observed in CBZ-treated cells was lost after myo-inositol treatment (Figure 3B), indicating that excess of myo-inositol blocked the ability of CBZ to enhance autophagy and promote clearance of mutATXN3.

mTOR is a major regulator of the initiation of autophagy [41, 42] and the AKT pathway is an upstream key modulator of mTOR [43]. To investigate the involvement of CBZ in these pathways, we evaluated AKT and mTOR activation *in vitro* by western blot analysis (Figure 3C). As expected, no differences were observed in phospho-AKT or mTOR levels, suggesting that CBZ is acting through an mTOR independent mechanism. In comparison, AMPK, previously shown to be involved in CBZ-mediated autophagy activation [29, 30], showed increased levels of phosphorylation in Neuro2a cells treated with CBZ (Figure 3C), as well as in CBZ-treated mice (Figure 3D), compared to controls.

These results suggest that CBZ induces autophagy through activation of AMPK, which is dependent on the myo-inositol levels.

Four weeks of daily carbamazepine administration does not promote mutant protein degradation in transgenic mice

After demonstrating that a short period of CBZ treatment (1 week) was sufficient to promote the clearance of TmutATXN3, we then investigated whether a longer period of treatment would result in a more effective degradation of the different toxic species of mutATXN3. Transgenic mice received a daily dose of 50 mg/kg CBZ or vehicle for 4 weeks, and mice cerebellar lysates were evaluated by western blot. After 4 weeks of treatment, no differences were observed in the levels of the high MW species or soluble mutATXN3, compared to controls (Figure 4A–C). Moreover, contrary to the previous experiment, p62 levels did not decrease (Figures 4D and 4F), which suggest that autophagy flux was not enhanced in this CBZ treatment regimen. In accordance, no differences in the phosphorylation status of AMPK were observed in animals treated with CBZ, compared to controls (Figure 4G).

Taking into account the possible involvement of CBZ in neuroinflammation [44–47], we evaluated markers of inflammation in the two treatment regimens (1 and 4 weeks). After 1 week of CBZ

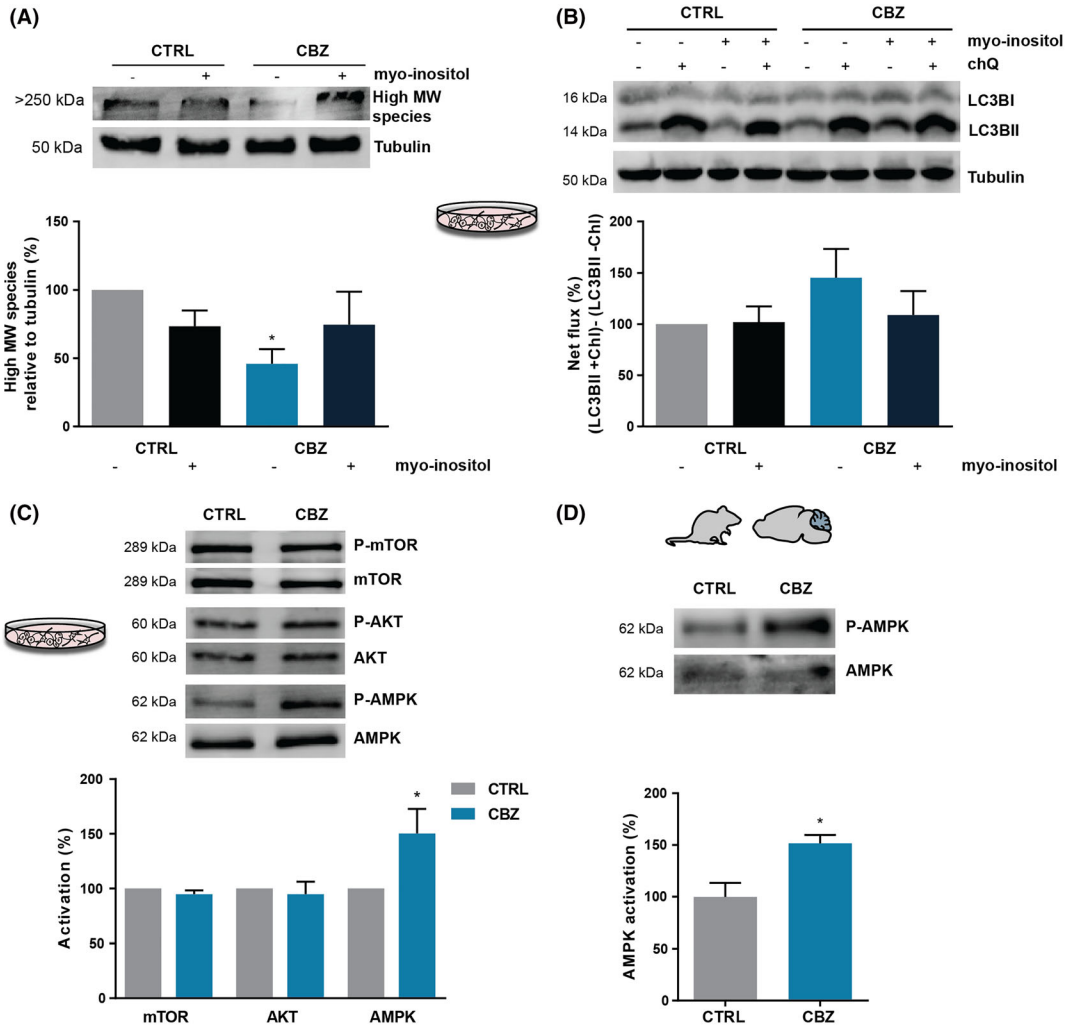

FIGURE 3 CBZ enhances autophagy through activation of AMPK. (A-C) Neuro2a cells expressing mutATXN3 were treated with or without CBZ (50 μM, 24 h), myo-inositol (1mM, 24 h) and chloroquine (chQ, 100 μM, 6 h). (A) Wester blotting analysis of mutATXN3 shows that ability of CBZ to promote clearance of the high MW species of mutATXN3 is supressed after the increment of myo-inositol levels. (B) Western blot analysis of LC3B. LC3BII net flux was determined by subtracting the densitometric value of LC3BII amount in samples nontreated with chloroquine (LC3BII − ChQ) from the corresponding sample treated with chloroquine (LC3BII + ChQ). The increase of LC3B net flux meditated by CBZ is reverted after myo-inositol treatment. (C) CBZ treatment leads to a significant increase AMPK activation, but it did not alter phosphorylation status of mTOR and AKT. Densitometric quantification of mutATXN3 and LC3B relative to tubulin (A, B) and P-mTOR, P-AKT and P-AMPK relative to respective total levels (C). Values are expressed as mean ± SEM of $n = 8$ (A), $n = 7–8$ (B), $n = 4–13$ (C). Statistical analysis was performed using one-way ANOVA followed by Šidák's post hoc test (A, B) and unpaired Student's *t* test (C). (D) Western blot analysis of AMPK phosphorylation in transgenic mice after 1 week of treatment (50 mg/kg) reveals a significant increase of AMPK activation. Densitometric quantification of P-AMPK relative to total AMPK levels. Values are expressed as mean ± SEM of $n = 4$. Statistical analysis was performed with unpaired Student's *t* test (*$P < 0.05$). CBZ—carbamazepine; CTRL—control; MW—molecular weight; chQ—chloroquine

treatment, we found that *C/EBPβ*, an important transcription factor that regulates genes involved in immunological and inflammatory responses, and *Arg1*, a common anti-inflammatory marker, were up-regulated in CBZ-treated mice, compared to controls (Figure S2A). In contrast, after 4 weeks of treatment, *C/EBPβ* and *IL-6*, a pro-inflammatory cytokine, were significantly increased in CBZ-treated mice, compared to controls (Figure 4H), suggesting a potential increase in neuroinflammation.

We further evaluated whether CBZ treatment could affect liver function. Biochemical analysis of the liver enzymes, alanine transaminase and alkaline phosphatase, indicates that none of CBZ treatment regimens caused hepatic toxicity (Figure S2B).

These results suggest that CBZ did not lead to severe toxic effects, but may play opposite roles in inflammatory response, depending on drug treatment regimen. Nevertheless, further studies are needed to confirm this hypothesis.

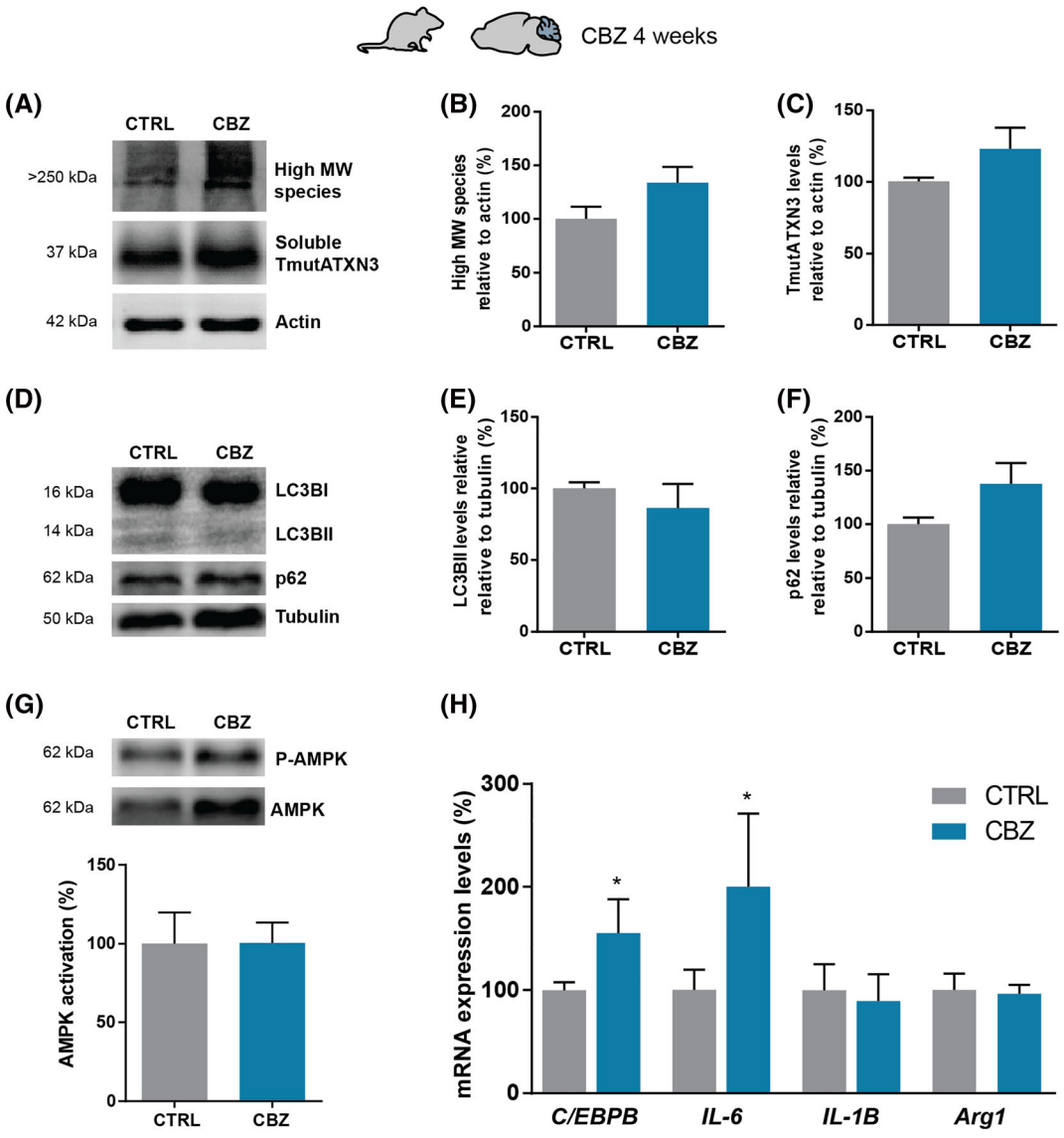

FIGURE 4 Four weeks of daily CBZ administration does not promote mutant protein degradation in transgenic mice. (A–H) Transgenic mice were treated with CBZ (50 mg/kg/daily) or vehicle for 4 weeks. (A-C) Western blot analysis of mutATXN3 shows no clearance of the high MW species (B) or soluble TmutATXN3 (C) in CBZ treated mice, compared to controls. (D-F) Western blot analysis of LC3B (E) and p62 (F) shows no alteration of these autophagy markers after 4 weeks of CBZ treatment. Densitometric quantification of TmutATXN3 relative to actin (A) and LC3B and p62 relative to tubulin (D). (G) Western blot analysis of AMPK phosphorylation reveals no alteration of phosphorylation status of AMPK. Densitometric quantification of P-AMPK relative to total AMPK levels. (H) Relative mRNA expression of *C/EBPβ* and *IL-6*, a pro-inflammatory cytokine, is significantly increased after 4 weeks of CBZ treatment. Values are expressed as mean ± SEM of n = 4–5. Statistical analysis was performed with unpaired Student's *t* test (*$P < 0.05$). CBZ—carbamazepine; CTRL—control; MW—molecular weight

Intermittent carbamazepine treatment improves motor performance and alleviates neuropathology in Machado–Joseph disease transgenic mice

Previous studies have demonstrated that intermittent treatment with autophagy-enhancing drugs are sufficient to promote degradation of mutant proteins [19, 48, 49].

Therefore, we next investigated whether intermittent CBZ treatment would improve the severe motor deficits of transgenic mice. The study in this transgenic model allowed an evaluation of the disease rescue after the onset of the disease, since at the time of initiation of CBZ treatment mice already had a profound cerebellar atrophy and severe motor deficits [34]. Animals received a CBZ dose of 50 mg/kg, three times per week, for 10 weeks from 4 weeks of age.

In the rotarod test, mice were forced to walk on a rotarod apparatus on a constant speed. Animals treated with CBZ had a strong tendency for improvement in the latency to fall in the rotarod from 4 weeks of treatment, particularly significant after 8 weeks of treatment, suggesting a rescue of balance and motor coordination (Figure 5A,B).

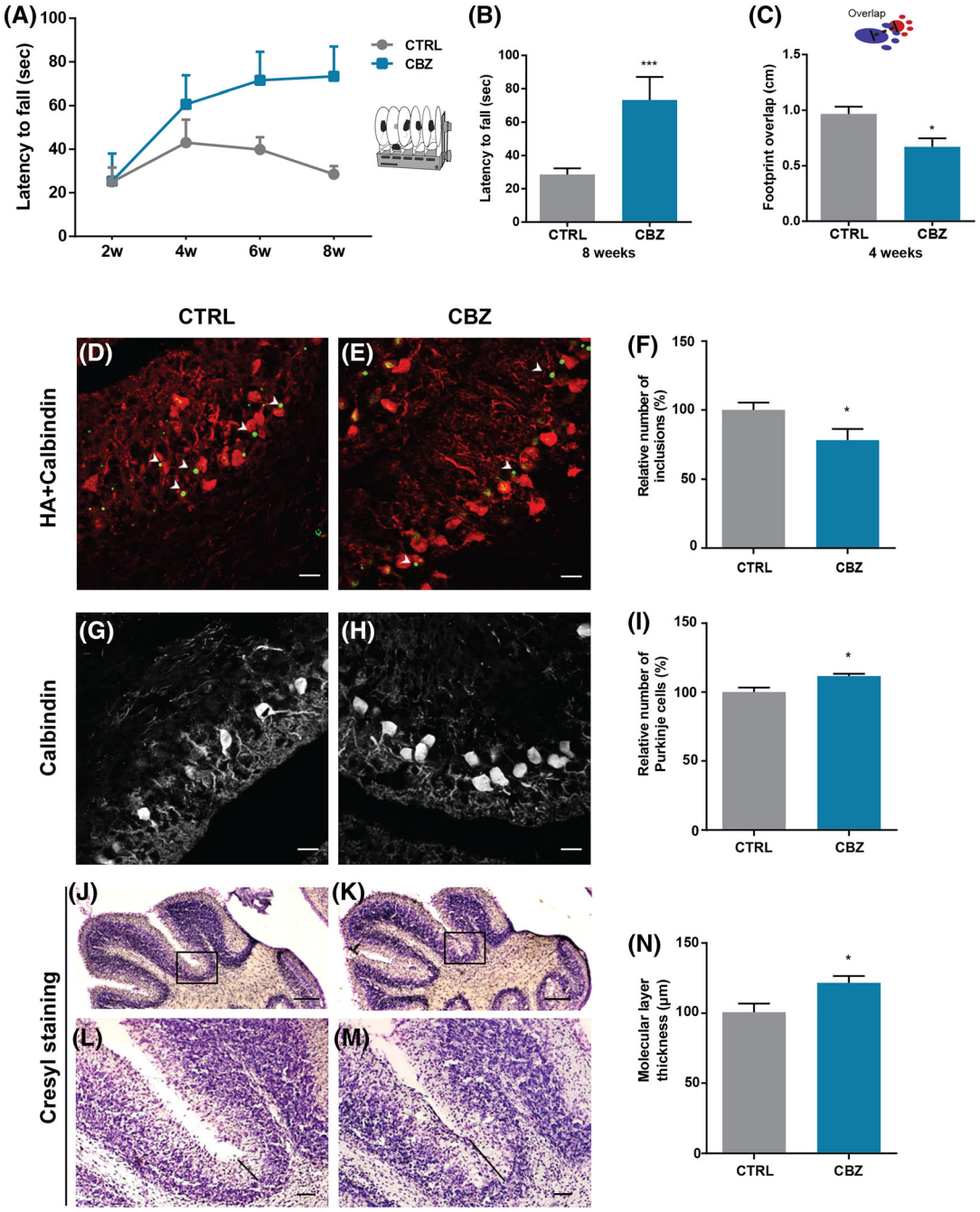

FIGURE 5 Intermittent CBZ treatment improves motor performance and alleviates neuropathology in Machado–Joseph disease (MJD) transgenic mice. (A-C) Behavioural assessment of transgenic mice treated for 8 weeks with 50 mg/kg 3 times per week of CBZ or vehicle. Transgenic mice treated with CBZ revealed an improvement in motor performance compared to control mice, observed in the stationary rotarod test (A), with a significant increase of latency to fall at 8 weeks of treatment (B) and footprint test, with improvement of overlap measurement at 4 weeks of treatment (C). Values are expressed as mean ± SEM of $n = 10$. Statistical analysis was performed with unpaired Student's t test (*$P < 0.05$, ***$P < 0.001$). (D–H) Immunohistochemical analysis of mutATXN3 inclusions, detected by HA antibody, in the Purkinje cells, detected by calbindin antibody, shows a decrease in the number of HA-positive inclusions in transgenic mice treated during 10 weeks with 50 mg/kg/3 times per week (E), compared to control transgenic mice (E). (G–I) Immunohistochemical analysis of calbindin-positive cells indicates a preservation of Purkinje cells in CBZ treated mice (H), compared to control transgenic mice (G), which was confirmed by quantification of the relative number of Purkinje cells (I). (J–M) Cresyl violet staining sections from transgenic mice. Representative images of molecular layer from control transgenic mice (J, L) and CBZ treated mice (K, M) show that CBZ treatment prevented the reduction of the thickness of the molecular layer, as confirmed by quantification in (N). Values are expressed as mean ± SEM of $n = 4–6$. Statistical significance was performed with unpaired Student's t test (*$P < 0.05$). Scale bar: 20 µm (D and E; G and H); 200 µm (J–M). CBZ—carbamazepine; CTRL—control

Since MJD, similar to other SCAs, is characterised by the development of ataxic gait [50], we analysed the footprint patterns of mice. Footprint overlap revealed a significant improvement after 4 weeks of treatment with CBZ, as compared to the control group (Figure 5C), suggesting an amelioration of the ataxic gait.

To investigate whether the improvement of behavioural performance correlates with a prevention of neurodegeneration, we evaluated cerebellar neuropathology in the transgenic mice by immunohistochemistry. Because intranuclear inclusions are a hallmark of MJD pathology [5], we first analysed the number of HA-positive inclusions in Purkinje cells. We observed a significant decrease in the number of inclusions in the animals treated with CBZ, compared to the control group (Figure 5D–F), suggesting that CBZ mediated the clearance of mutATXN3.

This transgenic mouse model is characterised by profound cerebellar atrophy, specially affecting Purkinje cells, which are also affected in MJD [51, 52]. Thus, we next evaluated Purkinje cells by calbindin immunohistochemical labelling. We observed that CBZ-treated mice exhibited significantly higher number of these neurons, when compared to controls (Figure 5G–I). Furthermore, by cresyl violet staining, we found a preservation of the thickness of the molecular layer in the cerebellar cortex of CBZ-treated mice, compared to control mice, suggesting a prevention of neurodegeneration (Figure 5J–N).

Importantly, intermittent long-term CBZ treatment did not increase reactive microgliosis in WT or transgenic mice (Figure S3). After 10 weeks of treatment with CBZ, microglia activation was assessed by immunohistochemical analysis of Iba-1. No differences were detected, as quantified in Figure S3E,F. This suggests that the tested intermittent CBZ treatment regimen does not promote a neuroinflammatory response.

Overall, these results indicate that CBZ ameliorates neuropathology, which correlates with the improvement of motor performance.

Retrospective study of carbamazepine treatment in Machado–Joseph disease patients

Cramps are a frequent and disabling symptom in MJD [53, 54]. This feature has been attributed to the increase of persistent sodium currents in the peripheral motor axons that lead to axonal hyperexcitability. Hence, sodium channel blockers, such as CBZ, have been successfully used to treat patients with muscle excitability abnormalities [53, 55].

To study the effect of CBZ in MJD progression, a retrospective analysis was performed using clinical records from MJD patients taking CBZ for cramps (CBZ group) and MJD patients without CBZ treatment (CTRL group). As MJD is a progressive disease, patients at different stages of disease evolve differently. To reduce this bias, the two study groups were established considering the baseline values for SARA score, age, age of onset and number of follow-up visits variables (Figure 6A–D). After establishing the groups, statistical analysis confirmed homogeneity for all these defined variables in both groups ($p > 0.05$).

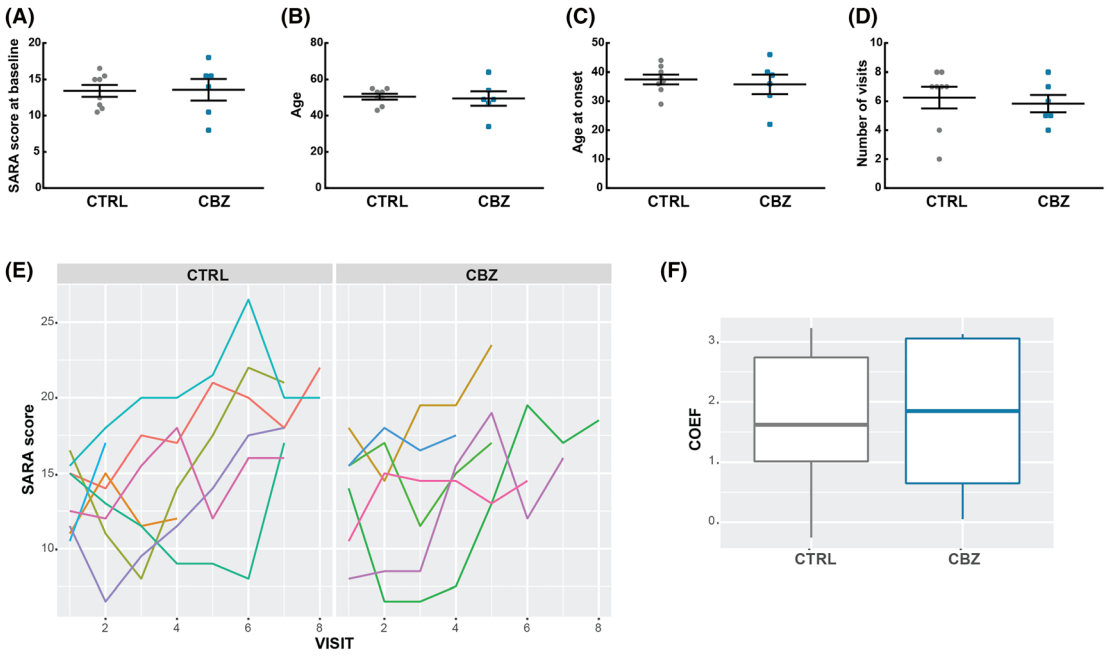

FIGURE 6 Retrospective study of CBZ treatment in Machado–Joseph disease (MJD) patients. (A–D) MJD patients were followed-up for 5 years. CTRL and CBZ-treated groups were established based on baseline values for SARA score, age, age of onset and number of follow-up visits, to reduce heterogeneity between groups. (E and F) For each study subject, a coefficient was calculated for the annual rate of progression of the SARA score. Median and mean annual progression rates of both groups indicate no significant differences between CBZ-treated and CTRL patients. In (A)–(D) values are expressed as mean ± SEM and in (F) boxplots represent median, 25%–75% interquartile range ($n = 8$ CTRL, 6 CBZ). Statistical analysis was performed with unpaired Student's t test ($P > 0.05$). CBZ—carbamazepine; CTRL—control

We further calculated, for each study subject, a coefficient for the annual rate of progression of the SARA score. Based on median (CTRL: 1.62 versus CBZ: 1.85) and mean (CTRL: 1.63 versus CBZ: 1.76) annual progression rates of both study groups, no differences were detected, suggesting that CBZ had no evident effect in the studied conditions (Figure 6E,F). Nevertheless, a larger prospective cohort would be necessary to assess the potential beneficial effect of CBZ in MJD patients.

DISCUSSION

We provide evidence in model systems that pharmacological activation of autophagy using the drug CBZ may be a promising avenue for therapy for MJD. CBZ is a low molecular weight drug used in the clinic for decades, and whose safety and tolerability profiles in humans are already known.

Repurposing CBZ for the treatment of different clinical conditions characterised by the accumulation of aggregate-prone proteins has been suggested in the last years [27–30, 35, 40, 49, 56]. The autophagy-enhancing activity of CBZ was shown to be beneficial in some neurodegenerative disorders, however, the effect of CBZ in polyglutamine disorders has not been elucidated. In this study, we provide the first *in vivo* evidence that CBZ-mediated autophagy activation leads to the clearance of the polyglutamine expanded protein ATXN3 and to the improvement of associated motor deficits.

First, we confirmed the ability of CBZ to activate autophagy in different neuronal models. Using one *in vitro* and two *in vivo* models, we showed that CBZ was able to increase LC3B levels, while diminishing p62 protein levels, which is in accordance with previous studies [30, 49]. Consistent with this, the same regimen of treatment led to the clearance of the mutATXN3 forms, suggesting a protective effect of CBZ in MJD. These observations are in line with previous studies showing that CBZ-mediated autophagy activation leads to the decrease of antitrypsin Z, TAR DNA-binding protein 43 and superoxide dismutase 1 aggregation [27, 30, 49]. In particular, we observed that CBZ preferentially removed the aggregated forms of mutATXN3 compared to soluble protein, which is consistent with a role of autophagy as a major route for the degradation of aggregate-prone proteins [57]. Similar observations were reported in a study using two autophagy-enhancing drugs, shown to mediate the preferential degradation of insoluble species of tau and α-synuclein [48].

In this study, we also explored the mechanism of action of CBZ. Consistent with previous reports, we confirmed that CBZ-mediated autophagy activation and subsequent clearance of mutATXN3 is dependent on the inositol levels [29, 58]. Moreover, we found that CBZ does not affect the levels of p-mTOR or p-AKT, but leads to the increase of AMPK activation, which was observed in *in vitro* and *in vivo* models of MJD. This is in agreement with independent studies, showing that the decrease of IP3 levels is correlated with the enhancement of AMPK activity [29, 30, 59].

With the aim of achieving a larger effect, a 4-week treatment regimen was evaluated in a transgenic mouse model. Strikingly, contrary to what we had observed after 1 week of treatment, no differences on the levels of mutATXN3 or autophagy markers were detected. This was supported by the loss of AMPK activation. Although the same CBZ dosage (50 mg/kg i.p.) was previously reported in different studies carried out in mice [29, 49], we hypothesised that these observations may be due to the CBZ treatment regimen.

Toxicological studies of CBZ have established a median toxic dose (TD50) between 33–76 mg/kg following a single i.p. administration in mice [60–63]. Since some drug remains in the body 24 h after i.p. administration [64], the cumulative dose resulting from a daily treatment might contribute to different effects of CBZ. Moreover, the increase in systemic bioavailability after i.p. administration can result in transient exposures of the mice to the drug at high concentration, which might cause side-effects. In our study, the dose of CBZ administered to mice (human equivalent dose of 243 mg) [65] is much lower than the one normally seen in the clinic, which has been shown to be safe in humans. Nevertheless, further pharmacokinetics and brain distribution studies would be important to evaluate drug's efficacy and toxicity.

Importantly, the biochemical analysis of the liver enzymes did not reveal hepatic toxicity after 1 or 4 weeks of daily CBZ administration. On the other hand, we did observe a possible regimen-dependent effect of CBZ on neuroinflammation. CBZ was previously shown to be able to modify the expression of different cytokines, which may account for its neuroprotective effect, as previously suggested [45, 66]. In our study, we observed that CBZ increased mRNA levels of the transcription factor *C/EBPβ* in both regimens of treatment, while the anti-inflammatory cytokine *Arg1* and pro-inflammatory *IL-6* were significantly increased after 1 or 4 weeks of treatment, respectively. Several studies have argued for an important anti-inflammatory role of antiepileptic drugs, including CBZ [45, 67, 68]; nevertheless, their ability to enhance pro-inflammatory cytokines and chemokines was also described. In a study carried out in epileptic patients receiving CBZ treatment for 1 year, levels of IL1-α, IL-1β, IL-6, IL-2 and chemoattractant protein 1 were found increased [69]. These divergent observations support the need of a study of the differential immunological responses with the time, dosage and type of treatment, in order to establish the optimal conditions.

Taking all these into account, a third regimen of treatment, based on intermittent CBZ administration, was applied to evaluate the long-term effect of CBZ in transgenic mice that exhibits profound cerebellar atrophy and severe motor deficits [34]. Remarkably, animals treated with CBZ for 8 weeks demonstrated a significant improvement in motor performance and neuropathology. Moreover, after 10 weeks of treatment, we did not detect an increase in microgliosis, which supports the treatment with CBZ under these conditions. Importantly, the efficacy observed with this intermittent strategy suggests that a constitutive activation of autophagy may not be required nor desirable for long-term treatment. In accordance, different studies using intermittent treatment with CBZ, temsirolimus (a rapamycin ester) or felodipine have also reported promising results due to the autophagy-enhancing ability of these drugs [19, 48, 49].

In an attempt to evaluate the CBZ effect in MJD progression, we analysed retrospective data on the use of CBZ in MJD patients. CBZ

has been successfully used to treat abnormalities of muscle excitability, such as cramps, a common feature of MJD [53, 54]. Treatment of this clinical problem, along with the effect of CBZ as a mood-stabiliser, may provide additional benefits in MJD. Our analysis did not reveal a significant improvement in the SARA score. Nevertheless, these findings might be attributed to the (a) small number of patients in the study; (b) the variable disease stage between patients and/or (c) variable dosage and time of treatment. Thus, further prospective controlled studies are necessary to clarify the potential of CBZ to prevent and/or delay MJD progression.

In conclusion, we provide evidence that treatment with CBZ ameliorates motor deficits and neuropathology in mouse models of MJD by activation of autophagy. The encouraging results here described support further studies to fine-tune CBZ treatment conditions, aiming at developing a translational approach for MJD therapy and other polyglutamine disorders.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Conceptualization, L.P.d.A., A.V.F. and C.N.; Investigation, A.V.F., S.C.S. and J.M.C.; Data analysis, A.V.F., S.C.S, A.R.M.M, M.C.F.J. and P.S., Writing – Original Draft, A.V.F; Writing Review & Editing, L.P.d.A. and C.N; Funding Acquisition, L.P.d.A; Resources, L.P.d.A. and M.C.F.J.; Supervision, L.P.d.A. and C.N.

ETHICS STATEMENT

Animal experiments were carried out in accordance with the European Union Community Council Directive (86/609/EEC) for the care and use of laboratory animals and were previously approved by the Responsible Organization for Animal Welfare of the Faculty of Medicine and CNC of the University of Coimbra (ORBEA and FMUC/CNC, Coimbra, Portugal; ORBEA_66_2015/22062015). Researchers received adequate training (Federation of European Laboratory Animal Science Associations [FELASA] certified course) and certification from Portuguese authorities (Direcção Geral de Alimentação e Veterinária [DGAV]) to perform the experiments.

Clinical data were collected after approval by the institutional ethics committee of the University of Campinas (Comitê de Ética em Pesquisa – UNICAMP, approval CAAE 83241318.3.1001.5404, PI: Prof. Marcondes França).

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/nan.12763

DATA AVAILABILITY STATEMENT

All data generated or analysed during this study are included in this published article [and its supporting information files].

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