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Divalproex sodium modulates nuclear localization of ataxin-3 and prevents cellular toxicity caused by expanded ataxin-3

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Summary

Background & Aims: Spinocerebellar ataxia type 3 (SCA3), also known as Machado-Joseph disease (MJD), is an autosomal dominantly inherited neurodegenerative disorder and the most common form of SCA worldwide. It is caused by the expansion of a polyglutamine (polyQ) tract in the ataxin-3 protein. Nuclear localization of the affected protein is a key event in the pathology of SCA3 via affecting nuclear organization, transcriptional dysfunction, and seeding aggregations, finally causing neurodegeneration and cell death. So far, there is no effective therapy to prevent or slow the progression of SCA3.

Methods: In this study, we explored the effect of divalproex sodium as an HDACi in SCA3 cell models and explored how divalproex sodium interferes with pathogenetic processes causing SCA3.

Results: We found that divalproex sodium rescues the hypoacetylation levels of histone H3 and attenuates cellular cytotoxicity induced by expanded ataxin-3 partly via preventing nuclear transport of ataxin-3 (particularly heat shock-dependent).

Conclusion: Our study provides novel insights into the mechanisms of action of divalproex sodium as a possible treatment for SCA3, beyond the known regulation of transcription.

KEYWORDS
divalproex sodium, HDAC inhibitors, nuclear localization, SCA3 treatment, transcriptional dysfunction

1 | INTRODUCTION

Spinocerebellar ataxia type 3 (SCA3), also known as Machado-Joseph disease (MJD), is an inherited neurodegenerative disorder and the most common form of autosomal dominant inherited ataxias worldwide.[1] Spinocerebellar ataxia type 3 (SCA3) is one of nine polyglutamine (polyQ) expansion diseases which form a group of hereditary neurodegenerative disorders: six types of spinocerebellar ataxias (SCA) such as SCA1, SCA2, SCA6, SCA7, SCA17, and SCA3, Huntington's disease (HD), dentatorubral-pallidoluysian atrophy (DRPLA), and spinal and bulbar muscular atrophy (SBMA).[2-9]

Spinocerebellar ataxia type 3 (SCA3) is caused by the expansion of a CAG repeat in the *ATXN3* gene encoding a polyglutamine-containing protein called ataxin-3. In general, ataxin-3 is a primarily cytoplasmic protein, although, depending on the cell type and level of expression, it may also be detected both in the nucleus and in the cytoplasm.[10-15] Beyond doubt, nuclear localization of ataxin-3 plays an important role in the pathology of SCA3, and ataxin-3 was shown to be more toxic in the nucleus than in the cytoplasm by disturbing nuclear organization, transcriptional function, and seeding aggregations.[11,16-18] In a previous in vivo study, we demonstrated that nuclear-localized expanded ataxin-3 aggravates the SCA3

phenotype in transgenic mice and increases the presence of aggregations. On the other hand, transgenic mice with an ataxin-3, which is kept in the cytoplasm, showed much milder symptoms and fewer inclusions.[19] This evidence demonstrates that nuclear ataxin-3 is required for the manifestation of a SCA3 phenotype and proved that strategically preventing nuclear translocation of ataxin-3 is a promising treatment for SCA3.

Emerging data indicate HDAC inhibitors as potential therapeutic compounds for the treatment of neurodegenerative diseases such as SCA3, Alzheimer's disease, Parkinson's disease, Huntington's disease, and Amyotrophic lateral sclerosis.[20-35] However, the exact mechanism of action of HDAC inhibitors is still widely unknown. HDACi include aliphatic acids (valproic acid [VPA], sodium butyrate [SB], phenylbutyrate), hydroxamate (trichostatin A [TSA], SAHA, CBHA, LAQ-824, PXD-101), cyclic peptide (depsipeptide), benzamide (MS-275), and others (Tubacin).[36] Phenylbutyrate has already entered a phase II clinical trial for the treatment of HD.[25] Recent studies show that VPA alleviates neurodegeneration and extends the life span of a Drosophila SCA3 model[35] and that VPA rescues hypoacetylated H3 and H4 levels in SCA3 cell models.[20] Valproic acid is found to ameliorate dopaminergic neurodegeneration and prolongs the life span in *Caenorhabditis elegans*.[37,38] Besides, safety and efficacy of VPA has been tested in patients with SCA3/MJD.[23] However, the effect of VPA on nuclear transport of ataxin-3 in a SCA3 cell model has never been tested. Divalproex sodium (or valproate semisodium, a mixture of VPA and sodium valproate) is a member of an aliphatic acids family of HDACi and is FDA-approved for the treatment of bipolar disorders, epilepsy, and migraine. Therefore, we decided to test whether divalproex sodium modulates the translocation of ataxin-3 and rescues cytotoxicity induced by the nuclear transport of expanded ataxin-3 in SCA3 cell models, providing novel clues for the potential mechanisms of HDAC inhibitors as a potential therapy for SCA3.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Human Embryonic Kidney 293 (HEK293) cells were maintained as previously described.[39] Chinese hamster ovary (CHO) cells were obtained from DiscoveRx (San Diego, CA, USA). Cells were cultured in F12 medium (Gibco, Life Technologies, Darmstadt, Germany) containing 10% fetal bovine serum (Gibco, Life Technologies), 1% penicillin/streptomycin (Gibco, Life Technologies), and 300 μg/mL Hygromycin B (Invitrogen, Carlsbad, CA, USA).

2.2 | Cell transfection

For cell transfection, human ataxin-3 was cloned into a pEGFP vector system. The constructs with three different CAG repeats stretches (15, 77, or 148 CAG) named as ataxin-3^{15CAG}, ataxin-3^{77CAG}, and ataxin-3^{148CAG} encode ataxin-3 containing 15Q, 77Q, or 148Q, respectively. These constructs were linearized by the ApaI restriction enzyme for stable transfection. CHO cells were stably transfected using FuGENE 6 (Roche, Basel, Switzerland) according to the manufacturer's recommendation. Ataxin-3^{15CAG}, ataxin-3^{77CAG}, and ataxin-3^{148CAG} were used for transient transfection into HEK293 cells using the Attractene Transfection Reagent (QIAGEN, Hilden, Germany) according to the manufacturer's description.

2.3 | Protein extraction

Subcellular fractionation was performed to separate the cytoplasmic and nuclear fractions. The cells were harvested, washed once with 5 mL PBS, and lysed in 100-200 μL cold cytoplasmic buffer (10 mmol/L HEPES pH 7.5, 10 mmol/L KCl, 0,1 mmol/L EDTA, 0,1 mmol/L EGTA, 1 mmol/L DTT, 0.5% NP40, Complete Protease Inhibitor Cocktail [Roche]). The lysates were placed on ice for 20 minutes, vortexed twice (5 seconds each), and centrifuged at 18 928 *g* at 4°C for 5 minutes in a tabletop centrifuge. The supernatant was stored as the cytoplasmic fraction. The pellet was resuspended in 200 μL washing buffer (cytoplasmic buffer with final concentration of 1% NP-40) and recentrifuged as before. The pellet was then again resuspended in 100 μL nuclear buffer (20 mmol/L HEPES pH 7.9, 400 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, Complete Protease Inhibitor Cocktail [Roche]), rotated for 30 minutes at 4°C, and centrifuged at 18 928 *g* for 20 minutes in a tabletop centrifuge. The supernatant was saved as the nuclear fraction.

For total protein extraction, cells were harvested, washed once with cold PBS, and centrifuged. The pellet was resuspended in 50 μL TNES (50 mmol/L Tris pH 7.5, 400 mmol/L NaCl, 0.5% Tween 20, Complete Protease Inhibitor Cocktail (Roche), incubated for 5 minutes on ice, and then centrifuged for 15 minutes at 4°C at 18 928 *g* in a tabletop centrifuge. The supernatant contained the total protein lysate.

2.4 | SDS PAGE and Western blot

The protein concentration was measured using a Bradford assay[40]; 30 μg of protein samples and the protein marker (Precision Plus Protein Standards, Bio-Rad Laboratories, Hercules, CA, USA) were loaded onto the gel (10%) and run as described previously (Schmidt et al[11]). Separated proteins were transferred onto nitrocellulose (NC) membrane (0.2 μm, GE Healthcare Life Sciences, Chicago, IL, USA). After transfer, the membrane was blocked in 1 × TBST (10 mmol/L Tris pH 7.5; 0.15 mol/L NaCl; 0.1% Tween 20) with 4% low fat dry milk (SlimFast, Allpharm Vertriebs GmbH) for 2 hours and incubated with the primary antibody diluted in 1 × TBST for 2 hours at room temperature or for overnight at 4°C. After washing three times with 1 × TBST, the blot was incubated with the secondary antibody diluted in 1 × TBST for 1 hour, washed again as before, detected with Amersham ECL Western Blotting Detection Kit (GE Healthcare Life Sciences), and processed using an Odyssey Fc system (LI-COR Biotechnology, Lincoln, NE, USA).

2.5 | Filter trap assay

Filter trap assays were used to detect the SDS-resistant insoluble aggregations of mutant ataxin-3.[41,42] Seventy-two hours after

transfection, HEK293 cells were harvested, centrifuged at 250 *g* for 10 minutes, and resuspended in 300 μL PBS with Complete Protease Inhibitor Cocktail (Roche). The lysates were sonicated for 1 minutes (30 seconds twice) for cell lysis and DNA fragmentation. A Bradford assay (see above) was performed to measure the protein concentration. The sample was then supplemented with 2% SDS for the filter trap assay; 100 μg of sample was loaded onto a cellulose acetate membrane (0.2 μm, GE Healthcare Life Sciences) on top of filter paper. The filter trap apparatus was run and washed twice with PBS. The membrane was then prepared for Western blot as described above.

2.6 | Cell viability assay

The PrestoBlue Cell Viability Reagent (Life Technologies, Darmstadt, Germany) is a resazurin-based reagent and was used to detect cell viability. Cells were plated at 5×10^4 cells per well of a 96 well plate. Cell viability was then assessed with the PrestoBlue Cell Viability Reagent after incubating for 10 minutes and measured by the fluorescent intensity using a Synergy HT microplate reader (BioTek, Bad Friedrichshall, Germany) (Excitation: 535 nm [25 nm bandwidth], Emission: 615 nm [10 nm bandwidth]).

2.7 | Statistical analyses

Data from multiple experiments were expressed as means ± standard error (SEM). Statistical significance was analyzed by Student's *t*-test for data with a normal distribution. The effect of multiple factors was determined by a two-way analysis of variance (ANOVA) test. Significance levels were described as follows: $P < 0.05$*; $P < 0.01$**; $P < 0.001$***, except where noted.

3 | RESULTS

3.1 | Divalproex sodium increases the acetylation level of histone H3

A growing number of evidence shows that the acetylation level of histones is reduced in polyQ diseases.[35,43] To determine whether divalproex sodium which is a mixture of sodium valproate and valproic acid indeed leads to an inhibition of histone deacetylation, the effect of divalproex sodium on levels of histone acetylation was investigated. Pronouncedly increased levels of acetylated histone H3 were observed in HEK293 cells expressing ataxin-3^{15Q} or ataxin-3^{77Q} treated with divalproex sodium for 24 hours compared to a control group treated with DMSO (Figure 1), and an increased tendency was observed after treatment of 6 and 12 hours. Thereby, we conclude that divalproex sodium increases levels of acetylated histone H3.

3.2 | Divalproex sodium does not alter soluble mutant ataxin-3 protein level

After confirming the effect of divalproex sodium on the histone H3 acetylation level, we wanted to know whether divalproex sodium also affects the protein level of ataxin-3. HEK293 cells

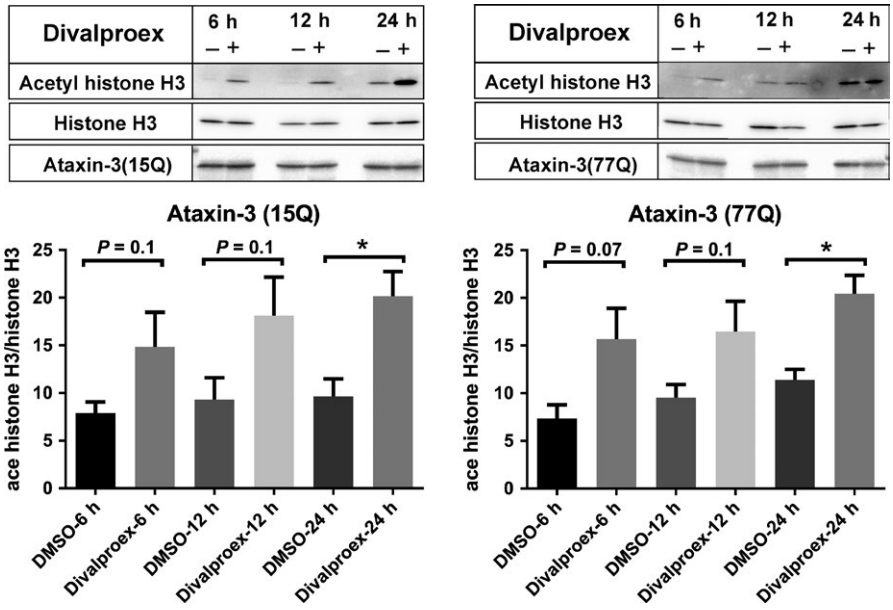

FIGURE 1 Divalproex sodium increases the level of acetylated histone H3 level in ataxin-3-transfected HEK293 cells. Normal (15CAG) or expanded (77CAG) ataxin-3-transfected cells were treated with divalproex sodium (Selleck chemical, 0.3 mmol/L in DMSO, (+)) or with DMSO alone (−) 48 h after transfection. The cells were treated with divalproex sodium or DMSO over 6, 12, and 24 h. The Western blot was probed with antiacetylated histone H3 (Abcam, Cambridge, UK), antihistone H3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and antiataxin-3 (1H9, Merck Millipore, Darmstadt, Germany). Antibodies include antihistone H3 and antiataxin-3 are used as controls. The *x*-axis shows the different treatment group. The *y*-axis represents the acetylated histone H3 values normalized to histone H3. Error bars represent the standard error of the mean. Data represent three independent experiments (n = 3). The data with a normal distribution were analyzed with Student's *t*-test. *$P < 0.05$

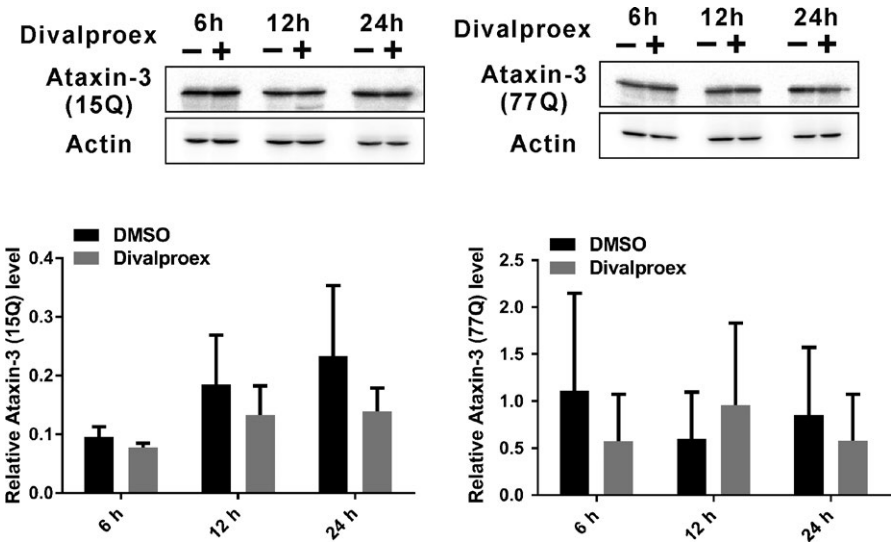

FIGURE 2 Divalproex sodium does not change the protein levels of ataxin-3 in HEK293 cells. HEK293 cells were transfected with ataxin-3^{15CAG} or ataxin-3^{77CAG}. 48 h after transfection, cells were treated with divalproex sodium (at 0.3 mmol/L in DMSO for 6, 12, or 24 h; +) or DMSO alone as control (−). Western blots were probed with antiataxin-3 (1H9, Merck Millipore) and antiactin (Sigma Aldrich, St. Louis, MO, USA) as a loading control. Error bars represent the standard error of the mean. Data represent three independent experiments (n = 3). The data in each time point with a normal distribution were analyzed with Student's *t*-test

expressing ataxin-3^{15Q} or ataxin-3^{77Q} were treated with divalproex sodium for 6, 12, and 24 hours and analyzed. Western blot analysis demonstrated that divalproex sodium does not change soluble mutant ataxin-3 (ataxin-3^{77Q}) and normal ataxin-3 (ataxin-3^{15Q}) (Figure 2).

3.3 | Divalproex sodium does not significantly affect aggregate formation of expanded ataxin-3

As the level of expanded ataxin-3 is not altered upon treatment with divalproex sodium, we evaluated whether protein aggregates formed by expanded ataxin-3 is reduced after treatment with divalproex sodium. We therefore used HEK293 cells expressing ataxin-3^{148Q} and treated them with divalproex sodium and DMSO as a control. We applied a filter trap assay to quantify the level of protein aggregates (Figure 3). The filter trap assay revealed that divalproex sodium exhibits a negative effect on the aggregates in comparison with the DMSO-treated group and with the untreated group.

3.4 | Divalproex sodium alleviates heat-shock-induced nuclear localization of ataxin-3

It is known that protein aggregates are easily formed when ataxin-3 is localized in the nucleus.[10,11,16-19] Although divalproex sodium had no influence on the aggregation, we next explore whether it affects the nuclear localization of ataxin-3 as the early pathological stage before aggregation forms. It has been shown that certain cellular stress such as heat shock promotes ataxin-3′s translocation into the nucleus.[44] Therefore, we asked whether this effect of cellular stress-induced nuclear presence of ataxin-3 can be directly alleviated using divalproex sodium. To do this, we stressed the cells by heat shock and investigated whether treatment with divalproex sodium would alleviate the nuclear localization of ataxin-3. CHO cells expressing ataxin-3^{77Q} were pretreated with divalproex sodium for 24 hours and then heat-shocked for 1.5 hours. Fractionation data indicated that heat shock increased the nuclear abundance of ataxin-3, whereas divalproex sodium alleviated the heat-shock-induced nuclear translocation of ataxin-3 (Figure 4).

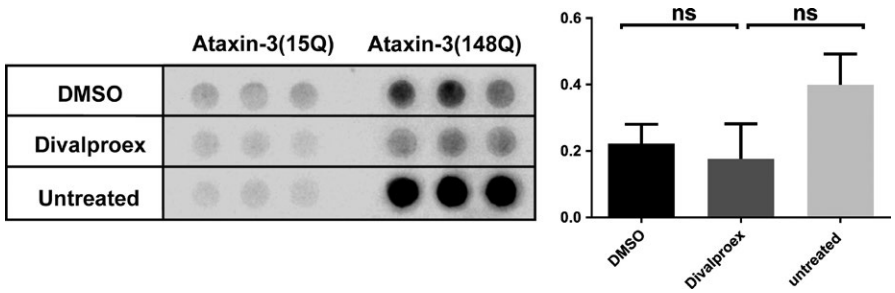

FIGURE 3 Divalproex sodium does not affect ataxin-3^{148Q} aggregation as measured by filter trap assay. Three days after transfection, HEK293 cells expressing ataxin-3^{148Q} or ataxin-3^{15Q} were treated with DMSO alone as a control and divalproex sodium at 10 μmol/L in DMSO for 24 h. The untreated cells were used as blank control. Error bars represent standard error of the mean over four independent experiments (n = 4). Values with a normal distribution were assessed by Student's *t*-test

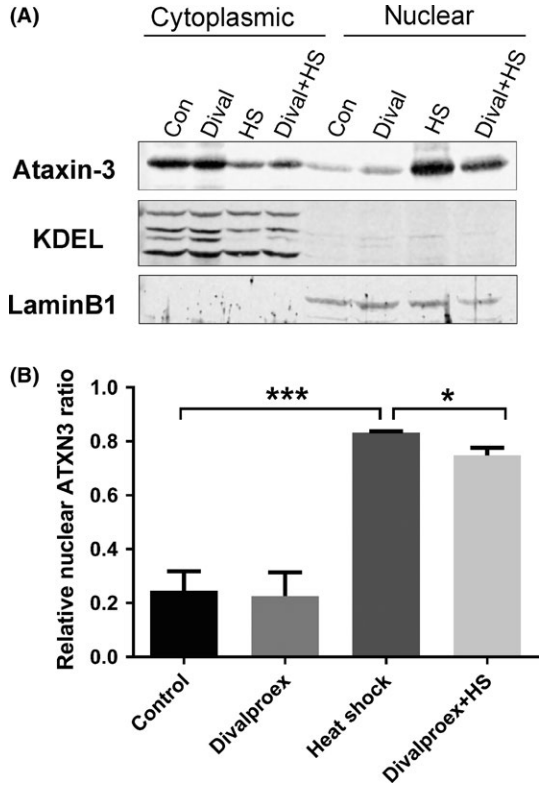

FIGURE 4 Divalproex sodium alleviates heat-shock-induced nuclear uptake of ataxin-3. A, Western blot of the subcellular fraction of CHO cells expressing ataxin-3^{77Q} was probed with antiataxin-3 (1H9, Merck Millipore), anti-KDEL (cytoplasmic marker, Enzo Life Sciences, Farmingdale, NY, USA), and antilamin B1 (nuclear marker, Santa Cruz Biotechnology). Four groups of cells with different treatments are shown. Those without heat shock were treated with either divalproex sodium (0.3 mmol/L, 24 h; Dival) or DMSO as a control (Con). The heat-shocked cells were pretreated with either divalproex sodium (0.3 mmol/L, 24 h; Dival + HS) or DMSO as control before heat shock at 43°C for 1.5 h (HS). B, Quantification of Western blot data showed that heat shock induces a strong shift of ataxin-3 from the cytoplasm to the nucleus and significantly reduced levels of nuclear ataxin-3 in cells pretreated with divalproex sodium before heat shock compared with the control. Error bars represent standard error of the mean in four independent experiments (n = 4). Values with a normal distribution were assessed by Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

3.5 | Divalproex sodium suppresses the cytotoxicity of expanded ataxin-3

PolyQ proteins commonly show stronger toxicity in the nucleus than in the cytoplasm.[19,28,45] If divalproex sodium prevents the stress-induced nuclear entry of expanded ataxin-3, we would expect an alleviated cellular viability of CHO cells expressing expanded ataxin-3 (77Q) upon divalproex sodium treatment. To assess a rescuing effect of divalproex sodium on the cytotoxicity caused by expanded ataxin-3, cell viability tests using PrestoBlue were performed. We observed that divalproex sodium successfully rescued the cytotoxicity induced by expanded ataxin-3 (77Q) at concentrations ranging from 100 to 1000 μmol/L; divalproex sodium on its own at 1000 μmol/L showed weak toxicity to the cells (Figure 5). However, the extent of toxicity was still weaker than the effect of DMSO, suggesting that the toxicity was induced by high concentration of DMSO rather than divalproex sodium. In addition, the rescuing effect was observed in cells expressing ataxin-3^{77Q} either at 2 hours or at 24 hours of treatment (Figure 5).

4 | DISCUSSION

Transcriptional disturbance has been suggested as a potential mechanism for the pathology of polyQ neurodegenerative diseases. Pathological polyQ protein or inclusions formed by expanded polyQ protein bind transcription factors, and these aberrant interactions cause transcriptional dysregulation, resulting in neuronal toxicity.[46-49] Moreover, histone acetylation and deacetylation, regulated by histone acetyltransferases and histone deacetylases, are responsible for the transcriptional regulation of eukaryotic cells. Histone acetylation activates transcription by facilitating more relaxed chromatin structure, and histone deacetylases suppress transcription by facilitating chromatin condensation.[50-53] A reduction of acetylated H3 and H4 induced by mutant Htt was observed in vitro and in vivo, reducing the histone acetyltransferases activity of the CREB-binding protein (CBP).[29,31,32] Therefore, HDAC inhibitors rescuing transcriptional dysfunction and rescuing the damage to histone acetylation homeostasis are proposed as a potential therapeutic strategy. In this study, we found that divalproex sodium increased the acetylation levels of histone H3. These results are consistent with previous studies and indicate that divalproex sodium prevents the cytotoxicity partly via rescuing hypoacetylation of histones in the polyQ diseases in combination with cell viability results.[29,35]

Previous studies indicated that none of the HDAC inhibitors could prevent aggregations of mutant Htt.[30-32] Similar observations were described for VPA tested in transgenic mouse models of SCA3.[35] In agreement with this, our study also found that divalproex sodium at the concentration of 10 μmol/L and 0.3 mmol/L (data not shown) both did not inhibit aggregation formation of mutant ataxin-3. Aggregations caused by the mutant ataxin-3 are a hallmark of SCA3/MJD disease; however, the effect of aggregation has been reported to be dichotomous.[54] Our results suggested they might mediate neuroprotective effects.

In addition, we evaluated the effect of divalproex sodium on the translocation of ataxin-3 and observed that divalproex sodium decreases heat-shock-induced nuclear accumulation of ataxin-3 as the early stage before aggregates forms. However, how does it achieve this effect? Proteins are transported between the cytoplasm and nucleus through the nuclear pore complex (NPC) which bridges the nuclear envelope, connecting the nucleus to the cytoplasm.[55,56] Only proteins less than around 40-60 kDa in size can be shuttled into nucleus via passive diffusion.[57,58] Other large proteins must enter the nucleus through the NPC via an active transport system regulated by a signaling pathway.[59] It is generally hypothesized that normal ataxin-3, which is around 42 kDa, can pass through NPC by passive diffusion, whereas expanded ataxin-3 is actively transported into the nucleus

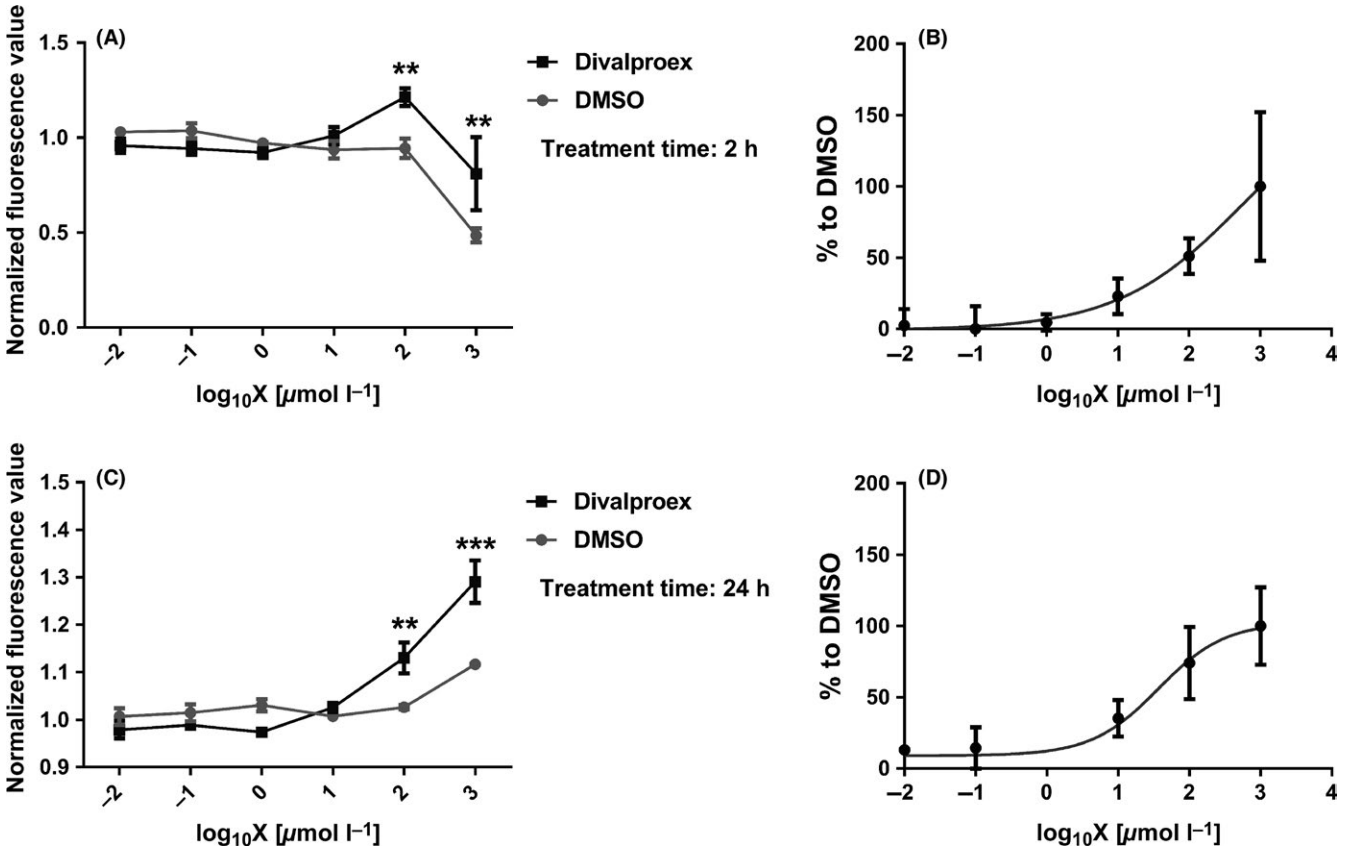

FIGURE 5 Divalproex sodium alleviates the cytotoxicity induced by expanded ataxin-3 as assessed by cell viability assay using the PrestoBlue cell viability reagent (Life Technologies). CHO cells expressing ataxin-3^{77Q} were seeded in a 96 well plate and treated with various concentrations of divalproex sodium for 2 h (A,B) and 24 h (C,D). The *x*-axis shows the $\log_{10}$ of concentration of divalproex sodium. The y-axes of the left diagrams (A,C) represent fluorescence values normalized to untreated cells. The diagrams on the right (B,D) show on the *y*-axis values normalized to the DMSO-treated group as sigmoidal curves. Error bars represent standard error of the mean in three independent experiments (n = 3). The data with a normal distribution were assessed by two-way ANOVA. $^{**}P < 0.01$, $^{***}P < 0.001$

required for transport proteins. Up to this study, no import protein was reported to shuttle ataxin-3 and only one export protein was indentified to modulate polyQ protein transport.[60]

Importins are a class of Karyopherins which modulate the nucleocytoplasmic transporting in eukaryotic cells through the NPC.[57,61,62] Acetylases can acetylate histones and also target other proteins. CBP, one acetylase, acetylates the nuclear import proteins (importins) such as importin-α2.[63] HDACi, like TSA, could increase this acetylation process and also regulate the localization of importin-α2.[63,64] These results indicate that nuclear import is likely to be regulated by acetylation and HDACi could modulate this process.

In our primary experiments (data not shown), over-expressing an importin isoform was found to increase the nuclear amount of ataxin-3, suggesting that it may be involved in the nuclear transport of ataxin-3. In addition, over-expressing the importin isoform also augmented the aggregation formation of mutant ataxin-3. The divalproex sodium-treated importin-expressing cells showed less tendency to form aggregations in comparison with the importin isoform coexpressing cells and had the same level of aggregations as DMSO-treated cells. These data indicate that this importin isoform may play a role in nuclear import of ataxin-3 and divalproex sodium prevents it to perform this function likely via arresting the localization of the importin subunit and subsequently alters the transport of ataxin-3. This suggests that divalproex sodium affects localization of ataxin-3, as mediated by this importin isoform.

More importantly, nuclear polyQ proteins are more toxic than those in the cytoplasm. Cell viability data demonstrated that divalproex sodium increased cell viability, suggesting it could prevent the cytotoxicity caused by nuclear expanded ataxin-3. Our finding supports that HDAC inhibitors reduce the polyQ toxicity[28,65,66] and also demonstrates that this beneficial effect may result from the inhibition of nuclear accumulation of mutant polyQ, subsequently reduction of nuclear-localized polyQ-induced toxicity. In addition to mutant polyQ-induced toxicity, another key mechanism of cell death in the polyQ diseases may be the cause of decreasing histone acetylation and HDACi can reduce this cytotoxicity by rescuing histone acetylation.[28,65]

5 | CONCLUSIONS

These results suggest divalproex sodium, as an HDAC inhibitor, not only rescues transcriptional dysfunction, but also affects nuclear

transport. Therefore, our data provide evidence for the mechanism of divalproex sodium (a HDAC inhibitor) as a promising therapeutic strategy for SCA3 treatment via regulating the translocation of ataxin-3 and preventing the cytotoxicity induced by expanded ataxin-3.

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CONFLICT OF INTEREST

The authors declares no conflict of interest.

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