Withaferin A Induces Heat Shock Response and Ameliorates Disease Progression in a Mouse Model of Huntington's Disease

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Abstract

Impairment of proteostasis network is one of the characteristic features of many age-related neurodegenerative disorders including autosomal dominantly inherited Huntington's disease (HD). In HD, N-terminal portion of mutant huntingtin protein containing expanded polyglutamine repeats accumulates as inclusion bodies and leads to progressive deterioration of various cellular functioning including proteostasis network. Here we report that Withaferin A (a small bioactive molecule derived from Indian medicinal plant, *Withania somnifera*) partially rescues defective proteostasis by activating heat shock response (HSR) and delay the disease progression in a HD mouse model. Exposure of Withaferin A activates HSF1 and induces the expression of HSP70 chaperones in an *in vitro* cell culture system and also suppresses mutant huntingtin aggregation in a cellular model of HD. Withaferin A treatment to HD mice considerably increased their lifespan as well as restored progressive motor behavioural deficits and declined body weight. Biochemical studies confirmed the activation of HSR and global decrease in mutant huntingtin aggregates load accompanied with improvement of striatal function in Withaferin A treated HD mice brain. Withaferin A treated HD mice also exhibit significant decrease in inflammatory processes as evident from the decreased microglial activation. These results indicate immense potential of Withaferin A for the treatment of HD and related neurodegenerative disorders involving protein misfolding and aggregation.

Introduction

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder characterized by motor deficits like chorea and fluctuations in gait, cognitive decline and psychiatric instabilities. Chorea or involuntary movement of extremities is the most prominent symptom afflicted by the patient with HD, although, impairment in voluntary movements is also can be observed in these patients[1, 2]. The HD is caused by an abnormal expansion of CAG triplets in the exon-1 of *Huntingtin* (*HTT*) gene resulting in translated abnormal polyglutamine (polyQ) stretch in the encoded protein[3]. The length of the CAG repeat in the *HTT* gene can be considered as an on or off switch for the development of the disease and the severity of the disease is directly proportional with the repeat length. Further, the polyQ containing segment of the mutant huntingtin is enzymatically cleaved into N-terminal fragments that are very unstable and eventually form insoluble aggregates in the nucleus, cytoplasm and neuronal processes[4–6]. As a result, there is selective dysfunction and degeneration of striatal neurons in the early stage followed by progressive degeneration of other brain regions[7, 6, 8, 9]. Neuropathological severity of the disease is categorized into five distinct scores with 0 (least display of micro- and macroscopic abnormalities) to 4 (noticeable alterations) in due course of the disease progression[10].

Formation of proteinaceous inclusion is a characteristic hallmark of most protein misfolding neurodegenerative diseases like HD and Alzheimer's disease [6, 11, 12]. In HD, polyQ containing misfolded mutant huntingtin fragments or their aggregates have been demonstrated to disrupt wide array of cellular functioning including disruption of protein quality control mechanisms in various model systems[11, 13–15]. Normally, cells have stringent protein quality control mechanisms that systematically employ

chaperone machinery, ubiquitin-proteasome system and autophagy to clear abnormal proteins. These components are necessary for maintaining cellular proteostasis and efficient cellular functioning[16]. However, in HD, all these proteostasis regulating mechanisms are progressively impaired[17–22, 13, 23]. Pharmacological inducers/activators targeting different components of proteostasis machinery have been reported to reduce aggregates load and slows the disease progression in various animal models of HD[24, 21, 22, 25, 26]. Since the function of various molecular chaperones as well as their master regulator, heat shock factor 1 (HSF1) are severely compromised, it is believed that up-regulation of HSF1 activity could a promising strategy to restore proteostasis and disease pathogenesis in HD[27–31]. Indeed, several HSF1 activators showed promising result in rescuing HD phenotypes in various animal models[28, 27, 32–35]. However, many of the pharmacological activator of HSF1 are toxic to the cell and therefore, identification of relatively safer activator is crucial for prolonged treatment.

In the present study, we dissected the potential role of Withaferin A (WA) in inducing heat shock response (HSR) through the activation of HSF1 and possible involvement of WA-mediated HSR in suppressing mutant huntingtin aggregation and rescuing neuronal dysfunction and disease progression using cellular and R6/2 transgenic mouse model of HD. WA, a small active principal component of Indian medicinal plant *Withania somnifera* (commonly known as Ashwagandha), has been shown to possess an anti-inflammatory, anti-cancer and neuroprotective properties[36, 37]. This molecule has been demonstrated to rescue neuronal dysfunction and neurodegeneration in various animal models of neurodegenerative disorders[38–42]. However, the molecular mechanism through which WA provides neuroprotective effect is still unclear and the effect of this molecule in animal model of HD is unexplored. Here, we demonstrate that the treatment of WA strongly activates HSF1, which could be mediated through its effect on thiol modification of various cellular proteins. Treatment of low doses of WA to HD150Q cells (a cellular model of HD) significantly reduces the mutant huntingtin aggregation. We also demonstrate that the prolonged treatment of WA from the early stage of disease progression significantly recover various motor behavioural deficits and extend the lifespan of HD mice.

Materials And Methods

Materials

Cell culture reagents including high glucose DMEM (Dulbecco's Modified Eagle Medium), BCA protein determination kit, MG132, primary antibodies such as mouse monoclonal anti- β-actin (A5316) anti-GFP (11814460001) and Withaferin A were procured from Sigma. Fetal bovine serum (FBS), trypsin, penicillin-streptomycin, Opti-MEM and Lipofectamine-2000 were purchased from Gibco/Thermo Fisher Scientific. Goat polyclonal anti-huntingtin (SC8767), rabbit polyclonal anti-DARPP32(SC11365), mouse polyclonal anti-HSP70 (SC-24) and mouse monoclonal anti-GAPDH (SC-32233) were purchased from Santa Cruz Biotechnology. Mouse monoclonal anti-1C2 (MAB1574) and Immobilon Western chemiluminescent HRP substrate were purchased from Millipore. Rabbit polyclonal anti-ubiquitin (Z0458) and anti Iba1 (019-19741) was purchased from Dako and Wako respectively. Horseradish peroxidase (HRP) conjugated and biotinylated secondary antibodies, VectaStain ABC kit, ImmPACT NovaRED HRP substrate kits were

purchased from Vector laboratories. Dual-Luciferase Reporter assay system (E1910) and plasmids pGL4.41[*luc2P*/HSE/Hygro] and pRL-SV40 were purchased from Promega. Ponasterone A (45-0478) and trizol reagent were procured from Invitrogen. For cDNA synthesis and semi-quantitative PCR TaKaRa RNA LA PCR™ Kit (RR012A) was used.

Cell culture, drug treatment, dual luciferase assay and mutant huntingtin aggregates counting

HT22 cells (Kindly provided by Dr. Dave Schubert, Salk Institute, United States) were cultured in DMEM supplemented with 10% heat inactivated FBS and penicillin-streptomycin. For immunoblot analysis, cells were routinely cultured in 6-well tissue culture plate at sub confluent density and after 24 h of plating, cells were treated with WA for different time periods. For dual luciferase assay, HT22 cells were plated in 24-well tissue culture plates at sub-confluent density. After 15-18 hours, cells were transiently transfected with pGL4.41-HSE Firefly luciferase reporter plasmid (containing 4 copies of HSE) and Renilla normalization plasmid (pRLSV40) using Lipofectamine 2000 as per manufacturer's protocol. After 18 hours of transfection, cells were treated with WA for 12 h. Cells were then lysed with the passive lysis buffer and subjected to dual luciferase assay. The ratio of Firefly to Renilla was calculated and data was expressed as fold change.

In another set of experiments, HD150Q cells (a stable and ecdysone-inducible cellular system that express truncated N-terminal huntingtin fused with enhanced green fluorescence protein (tNhtt-150Q-GFP) were cultured in DMEM supplemented with heat-inactivated 10% fetal bovine serum and antibiotics (0.4 mg/ml Zeocin and 0.4 mg/ml G418). Expression of tNhtt-150Q-GFP protein was induced with 1 μM of ponasterone A and simultaneously treated with different doses of WA for 48 h followed by preparation of cell lysate for immunoblot analysis. To count GFP-positive mutant huntingtin aggregates, HD150Q cells were seeded onto chamber slides and the aforementioned paradigm was followed for induction and treatment. After 48 hours, images were taken in a fluorescence microscope across regions of the chamber slide. Three independent experiments were done to calculate percentage of cells with aggregates.

Animals and drug treatment

Mouse model of Huntington's disease (strain B6CBA-Tg (HDexon1) 62Gpb/3J) was procured from The Jackson Laboratory. The animals were bred in the National Brain Research Centre animal facility. These transgenic animals (also referred as R6/2 line) carry the 5'-portion of the human *HTT* gene, containing the 120 CAG repeats. This model represents a rapidly progressive diseased phenotype. Animals had free access to pelleted diet and water *ad libitum*. All experiments were conducted in accordance to the strict guidelines outlined by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forestry, Government of India and were approved by the Institutional Animal Ethics Committee of the National Brain Research Centre (Protocol number

NBRC/IAEC/2019/154). After PCR based genotyping, female HD mice along with their wild type littermates were used for the present study. WA was dissolved in DMSO and diluted in saline (5% DMSO). Literature survey and pilot experiments of 5, 2 and 1 mg/kg body weight dose were carried out. Drug dose of 1 mg/kg body weight was found to be effective and safe and 15 doses were administered via intra-peritoneal route every alternate day starting at their age of 56 day till 84 day. Mice were constantly monitored for their behaviour, body weight and motor symptoms. At 84 day, animals were sacrificed, brains were collected, and different regions were dissected out and stored at -80ºC for further processing.

Behavioral study

Clasping test

Mice were suspended through their tails and the time was noted when they brought their limbs firmly together. For every experimental time point, three trials of 60 seconds each was performed for each mouse and clasping scores assigned accordingly. 0, no clasping in 60s; 1, clasping in 31-60s; 2, clasping in 16-30s; 3, clasping 11-15s, 4, clasping in 06-10s; 5, clasping 1-5s.

Gait analysis

Footprint gait analysis was done by immersing the hind and fore paws of the animal in different coloured non-toxic ink. Mice were trained for three days to walk inside a wooden tunnel (40x5 cm) lined with white paper and then assessed for three trials. The walking footprint obtained on the white paper was used to measure stride length and distance between the fore and hind paws.

Rotarod analysis

Mice were placed on the instrument with a rotating rod at 5rpm for training. After three days of training, they were assessed for 3 trials in each week at constant speed of 10rpm. A maximum of 60s was permissible per trial per mouse. Average time of three trials per week was tabulated and plotted.

Measurement of striatal volume

Mice were anaesthetized with xylazine (10mg/kg body weight) and ketamine (100mg/kg body weight) and then transcardially perfused using PBS followed by 4% paraformaldehyde (PFA). Brains were carefully dissected and immersion-fixed in 4% paraformaldehyde (pH 7.4) overnight at 4°C, rinsed thrice in PBS, and impregnated with sucrose gradient of 10%, 20%, 30% for 24 h each at 4°C. Leica CM3050 cryotome was used for cryo-sectioning of brain to 25 µm thick sections that were stored at 4°C in PBS with 0.02% sodium azide. Coronal sections were taken that spanned entire striatum area from the anterior to posterior region. For measuring striatal volume, serial sections (every fifth section) were Nissl stained.

Peripheral boundary of the striatum was carefully traced (taking reference from Allen Brain atlas) using stereo investigator software and the volume was calculated.

Immunostaining and aggregate counting

For immunostaining, cryo-sections from different experimental groups were placed onto the glass slide and first incubated with antigen masking solution for 40 minutes at 70°C for antigen retrieval. After washing with PBS, quenching solution (10% H_2O_2, 10% methanol in PBS) was added for removal of endogenous peroxidases for 15 minutes and then washed with PBS. Permeabilization was done with 0.3% tritonX-100 for 10 minutes followed by blocking with normal horse serum (3%), bovine serum albumin (1%) and triton-100(0.3%) for 2 h inside a humid chamber at room temperature. Sections were then incubated with huntingtin antibody (1:100 dilutions in blocking solution) for 16 hours. After washing with PBS, the sections were incubated with biotinylated secondary antibody at a dilution of 1:500 for 2 hrs. Next step was VECTASTAIN-Elite ABC solution incubation of 2 h. Staining was developed using ImmPACT Novared peroxidase substrate kit as per the manufacturer's protocol. Images were acquired by Leica DM RXA2 bright field microscope. The 40x images of motor cortex, striatum and hippocampal region of the brain were analysed for total number of huntingtin positive aggregates using ImageJ software.

Immunoblotting and dot blot assay

Mice were sacrificed and cortex and striatal regions were carefully dissected out and snap frozen in liquid nitrogen. Samples were placed in -80°C for storage. Tissues were lysed in RIPA lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 2.5 mM EGTA, 10 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 0.1 mM Na_2VO_5, 10 mM NaF, 5 mM Na_4P2O_7, and complete protease inhibitor cocktail) at 4°C using tissue homogenizer. Lysates were sonicated and centrifuged at 15,000 × g for 10 min. Supernatants obtained were collected and protein estimation was done using BCA method. The lysates were then mixed with sample buffer (1:1 ratio) and subjected to SDS-PAGE. Resolved proteins on the gel were transferred to nitrocellulose membrane that was followed by blocking with 5% non-fat skimmed milk for 2 h at room temperature. Next, the membrane was probed with different primary antibodies as followed: HSP70 (1:5000), GFP (1:5000), DARPP32(1:5000), 1C2(1:1000), Iba1 (1:500), β-actin (1:5000), GAPDH (1:5000). After primary incubation, membrane was washed with TBST and appropriate HRP or ALP tagged secondary antibody was added. The blot was developed using Millipore Immobilon Western chemiluminescent HRP substrate.

For dot blot assay, cortex samples were homogenized in ice cold RIPA buffer, sonicated on ice (pulse on time:1s, pulse off time:1s, total time 30s) followed by BCA method for protein estimation. An equal concentration of each sample was treated with DNAse I and then centrifuged at 15,000 x g for 15 min. The pellet was collected and treated with 2% SDS for 5 min at boiling temperature. An equal volume

(comprising equal concentration) of different samples was filtered through SDS wash buffer pre-equilibrated nitrocellulose membrane. Membranes were then washed with SDS wash buffer and processed for immunoblotting with 1C2 antibody.

Semi-quantitative PCR

Total RNA was extracted from HD150Q cells using Trizol reagent following manufacturer protocol. cDNA was then synthesized using cDNA synthesis kit from TaKaRa. The cDNA from HD150Q was processed for semi-quantitative PCR using GFP specific primers. The data obtained was analysed and expressed as fold change after normalization with GAPDH. Primer sequences for GAPDH and GFP are as follows: GAPDHF, 5'-GGTTGTCTCCTGCGACTTCA-3'; GAPDHR, 5'-TAGGGCCTCTCTTGCTCAGT-3'; GFPF, 5'-ACGTAAACGGCCACAAGTCC-3'; GFPR, 5'-TGTTCTGCTGGTAGTGGTCG-3'.

Statistical analysis

Statistical scrutiny was conducted with SigmaPlot software. The data was represented as mean ± standard deviation (SD). Values were analysed by using one or two-way ANOVA followed by Holm-Sidak post-hoc test. Survival data were inspected by Kaplan-Meier survival study and multiple curves were compared by log rank test. $P<0.05$ was considered statistically significant.

Results

WA induces HSR and reduces mutant huntingtin aggregation

Severe impairment of HSR in HD prompted us to screen and identify novel small molecule activator of HSF1 from the natural product that could offer potential therapeutic benefit. Initial screening was performed using a luciferase-based reporter assay where cells were transiently transfected with luciferase reporter vector containing heat shock elements (pGL4.41) along with pRLSV40 control plasmid, treated with different molecules followed by dual luciferase assay. Using this screening method, we have identified WA as one of the strong activator of HSF1 (Fig.1A). MG132 was used as positive control while topotecan did not affect HSF1 activity. We further characterized the HSF1 activating property of WA by checking the expression of HSP70 (one of the commonly known HSF1 induced chaperone). Treatment of WA dose and time dependently induced the expression of HSP70 (Fig.1B-D). We have also noticed that WA at the dose ranging from 1-5μM caused about 10% - 50% loss of cell viability when treated for 12 h duration. The WA-mediated HSF1 activation was partially prevented upon co-treatment of the cell with N-acetyl cysteine (NAC) indicating further that WA-induced thiol adduct formation could be playing an important role in this process (Fig.1E and F) WA-induced thiol modifications of various cellular protein could also be linked with proteasomal malfunction as evident from increased accumulation of

ubiquitinated proteins. These studies confirm that WA induces HSR through the activation of HSF1 and these results are in line with earlier studies[43,44].

Since, WA activates HSR and is relatively less toxic at lower doses (0.1-0.5μM), we further explored its effect on mutant huntingtin aggregation in a cellular model of HD (HD150Q cell) that inducibly expresses truncated N-terminal mutant huntingtin containing 150Q fused with GFP (tNhtt-150Q-GFP). HD150Q cells were simultaneously induced to express tNhtt-150Q-GFP with Ponasterone A and treated with different doses of WA and the rate of aggregate formation was monitored at different time points using fluorescence microscope. As shown in Figure 2A and B, exposure of WA for 48 h significantly reduced tNhtt-150Q-GFP aggregation. WA treatment also significantly reduced the soluble level of tNhtt-150Q-GFP protein along with the induction of HSP70 in HD150Q cells (Fig.2C and D). WA did not have any effect in the transcript level of tNhtt-150Q (Fig.2E).

Treatment of WA to HD mouse improved their motor behavioural deficits and survival rate along with restoration of striatal integrity

Since WA treatment reduced the aggregation of tNhtt-150Q protein in HDD150Q cells, we further investigated the effect of this molecule in rescuing HD phenotype using a R6/2 transgenic mouse model of HD. These HD mice exhibit observable motor behavioural deficits along with impairment of HSF1 activity from 8 weeks onwards. Widespread nuclear aggregates of mutant huntingtin are also detectable from this time point[29,45]. We started administering WA to HD mice at their 8 weeks of age. Initially, we tried to optimize the tolerable dose of WA using pilot experiment. At 5mg/kg body weight dose for alternate days, 50% of HD mice died after 5 doses, however, at 1mg/kg dose for 14 doses, all animals were healthy and even loss of body weight was partially improved. We then repeated the experiments with 1mg/kg dose and treatment was continued up to 12 weeks. Body weight and various motor behavioural tests were monitored in every week. In this dosing paradigm, WA administration significantly improved the progressively declined body weight of HD mice (Fig.3A). Interestingly, survival rate was considerably increased in this dosing schedule of WA (Fig.3B). Average lifespan of HD mice was increased about 25 days. In the rotarod performance test, WA treated mice performed much better than vehicle treated HD group, although, the efficiency was decreased with increasing age (Fig.4A). WA and vehicle treated wild type mice group performed well and did not fall down in this experimental condition. Clasping behavior of HD mice (one of the unique behavioural deficit in these mice) was progressively increased with age, which was significantly reduced upon WA treatment (Fig.4B). Similarly, in footprint gait analysis, WA treatment to HD mice significantly increased their stride length and decreased the distance between fore and hind paws (Fig.4C and D). All these tests clearly indicate that the treatment of WA considerably improved motor deficits in HD mice.

Since striatal degeneration is one of the distinctive features of HD and many of motor deficits are manifested due the striatal dysfunction, we investigated the striatal structure and volume in the drug

treated HD mice along with controls. We noticed a significant reduction in striatal volume in 12 weeks old HD mice and treatment of WA prevented the atrophy of the striatum (Fig.5). Size of lateral ventricles were enlarged due to the atrophy of the striatum in HD mice brain as evident from the Nissl-stained coronal brain section and treatment of WA for prolonged duration prevented the striatal atrophy.

WA treatment reduced the global aggregate burden and the soluble level of mutant huntingtin in HD mice brain along with increased HSR

HSR inducing property of WA prompted us to compare the load of mutant huntingtin aggregates in the brain sample of vehicle and WA treated HD mice. Coronal brain sections obtained from both vehicle and WA treated mice (84 days old) were processed for immunohistochemical staining using huntingtin antibody and nuclear aggregates were counted in different brain regions. As shown in the Figure 6, treatment of WA reduced the number of nuclear aggregates in the striatal, cortical and hippocampal areas of HD mice brain compared to saline treated HD group. There was about 40-50% reduction in nuclear aggregates in different brain regions of WA treated HD mice when matched with saline treated HD group (Fig.6B). To further evaluate the level of insoluble mutant huntingtin, cortical samples were analyzed using dot blot assay and results were very similar to aggregate counting data. WA treated HD cortical sample showed nearly 40% reduction in insoluble mutant huntingtin when compared with vehicle treated HD samples (Fig.6C). Next we analyzed the level of soluble transgenic mutant huntingtin in the saline and WA treated cortical samples in immunoblot study using 1C2 antibody that specifically recognize expanded polyQ epitope. As shown in Figure 7, WA treatment significantly reduced the steady state level of soluble transgenic mutant huntingtin. Huntingtin antibody was unable to detect the soluble pool of transgenic mutant huntingtin. Immunoblot analysis confirmed that the level of HSP70 was significantly reduced in the HD mice brain and that was partially restored upon WA treatment indicating the induction of HSR in WA treated animals (Fig.8). However, increase of HSP70 in WA-treated wild type group was not significant. It is possible that the blood brain barrier permeability of WA might be poor. Because of the disruption of blood brain barrier permeability in HD mice, WA might me more accessible to the brain[46]. Treatment of WA also partially restored the reduced level of striatal DARPP32 (a dopamine regulated phoshphoprotein 32) in HD mice brain (Fig.8). This finding correlates well with the restoration of striatal volume.

Reduced inflammation in WA treated HD mice brain

WA is shown to suppress the inflammatory process through the inhibition of NF-κB pathway and thereby could prevent neurodegeneration in animal models of ALS[38]. In R6/2 mice, inflammation is observed in the late stage of disease progression particularly around 12-14 weeks of age[47]. We used Iba1 antibody to detect activated microglia in the brain sample of saline and WA treated HD mice. Iba1 is a well-known marker of activated microglia and its level increases during activation. Immunohistochemical staining

detected activated microglia in the striatum of HD mice as evident from their round cell body with short pseudopodia (Fig9). In wild type mice, cell body of the microglia is comparatively large and have extensive branching. Treatment of WA reduced the number of activated microglia. Immunoblot analysis further detected increased level of Iba1 in the striatum of HD mice and treatment of WA brought down the level to nearly normal range (Fig.9B and C). These results indicate treatment of WA significantly reduces the inflammatory process in HD mice.

Discussion

In the present investigation, we report that the treatment of WA delays the disease progression and increase the survival of a model HD mouse and the beneficial effect could be mediated partly through the activation of HSF1 and subsequent induction of HSR. The HSR is an important physiological process involved in preserving cellular protein quality control and this process is progressively compromised in HD and related neurodegenerative diseases[13, 18–20, 23, 22]. Many of the component of HSR particularly molecular chaperones exceeds their capacity to deal with the misfolded mutant huntingtin protein for their proper folding and clearance as expanded polyQ proteins are prone to misfold and difficulty to degrade through proteasome[19, 48–50]. More importantly, the function of HSF1, a master regulator of HSR is also severely compromised in HD brain[27, 30]. How the activity of HSF1 is affected in HD is still unclear. While one report showed transcriptional repression of HSF1 in HD mice brain[27], another report indicated enhanced degradation of HSF1[30]. HSF1 transcriptional activity also could be reduced as the function of Sirt1 (that deacetylates and activates HSF1) is affected due to its aberrant interaction with mutant huntingtin[51, 52].

In any case, decrease in the activity of HSF1 in HD brain indicates that pharmacological activators of HSF1 could be one of the potential therapeutic strategies in reversing HD phenotypes. Indeed, several brain permeable small molecules activator of HSF1 have been demonstrated to rescue the HD phenotypes in multiple model systems. Some of those includes geldanamycin, 17AAG, NVP-HSP990, HSF1A, celastrol, dexamethasone, azadiradione etc.[29, 27, 33, 53, 34, 35]. Most of these molecules are toxic to the cell at higher concentrations and therefore, identification of relatively less toxic HSF1 activator is critical for prolonged treatment particularly in rescuing neuronal dysfunction and neurodegeneration. In this context, HSF1 activator that has additional cellular targets linked with neuroprotection or enhanced clearance of abnormal proteins could be very important. For example, recently we have identified azadiradione that not only activates HSF1, but also induces the expression of Ube3a (an ubiquitin ligase), that is involved in the degradation of expanded polyglutamine proteins[29]. In the similar line, WA not only activates HSF1, but also demonstrated to have potent anti-inflammatory effect[38]. We have confirmed that WA activates HSF1 through thiol oxidation as treatment of NAC prevented HSF1 activation. This has been suggested in an earlier report[43]. At higher doses, WA has been shown to inhibit proteasomal dysfunction and induction of autophagy and these effects could be linked with its effect on thiol modification in the cell[44]. Treatment of WA to HD150Q cells at relatively less toxic doses significantly increased the expression of HSP70 along with reduced level of soluble mutant huntingtin and their aggregation. These results indicate prolonged treatment of low doses of WA

could be effective in suppressing mutant huntingtin aggregation possibly through their refolding and clearance. In HD mice, WA treatment (1mg/kg body weight) was started at early stages of disease progression and when visible aggregates begin to appear and treatment was continued up to 12 weeks. In this treatment regime, WA significantly increased the lifespan of HD mice along with recovery of deteriorated motor functioning. Improvement of progressive worsening of the body weight of WA treated HD mice indicative of minimum drug-associated toxicity. Significant activation of HSF1 along with reduction of aggregate load in the drug treated HD mice clearly indicates that HSF1-mediated restoration of HSR plays an important role in this process. WA might induce cellular stress at early stage of treatment, but eventually could make the cell more resistant with improved proteostasis. A recent study has shown similar outcome, where dietary restriction improves proteostasis through endoplasmic reticulum stress[54]. All these data indicate that treatment of WA at early stages of disease progression seems to be very effective.

Growing body of literature now indicates the neuroprotective role of *Withania somnifera* plant extracts or its active principal component (particularly WA) in various animal models of neurodegenerative disorders[55, 37]. Semi-purified root extract of this plant containing withanolides and withanosides reversed the behavioural deficits and amyloid plaque load in the brain of APPswe/PS1δE9 transgenic mouse model of Alzheimer's disease[42]. Extensive studies in various animal models of ALS, the root extract or WA itself found to be very effective in rescuing behavioural deficits along with reduction in the level of mutant disease proteins (SOD1 and TDP-43)[40, 41, 39, 56, 38]. The molecular mechanism through which WA reverses the ALS pathology is not clearly understood. WA-mediated reduction in neuroinflammation through inhibition of NF-κB activity is suggested to be playing an important role[38, 39]. However, treatment of WA results in significant reduction in the level of mutant SOD1 or TDP-43 along with induction of some chaperones (HSP25, HSP70) in the respective animal model[39, 56]. These results strongly indicate that there is induction of HSR in WA treated animal models of ALS.

Although, our study primarily focused on WA-stimulated HSR in ameliorating HD progression, we cannot rule out the probable anti-inflammatory role of this drug in the rescue process as inflammation occurs in the brain of HD patients and mouse models of HD. In R6/2 HD mouse model (used in this study), inflammation occurs in the late stage of disease progression (from 12–14 weeks age)[47]. In fact, we have detected activated microglia in the striatum of 12 weeks old HD mice (R6/2) and treatment of WA significantly reduced the number of activated microglia. WA could prevent inflammation in HD mice brain from its inception by inhibiting the activation of NF-κB pathway, a pathway that seems to play a very critical role in HD[57, 58]. Previously we have shown that agent EVP4593, an inhibitor of NF-κB signaling pathway acting due to SOC channel blockade, attenuated pathologically enhanced level of huntingtin in patient-specific iPSC-derived HD neurons and had a neuroprotective effect in the cell[59]. Up-regulation of NF-κB activity through aberrant stimulation of IκB kinase was found in astrocytes of patients and mouse with HD[60].

Altogether, our study demonstrate that WA treatment delays the disease progression and increase the lifespan in a mouse model of HD. HSF1 activating property along with anti-inflammatory function could

brand WA as an attractive therapeutic molecule for treatment of HD and other protein misfolding neurodegenerative disorders.

Declarations

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Conflict of interest statements:

None

Compliance with ethical standards:

All experiments were conducted in accordance to the strict guidelines outlined by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forestry, Government of India and were approved by the Institutional Animal Ethics Committee of the National Brain Research Centre (Protocol number NBRC/IAEC/2019/154).

Data availability statement:

Data will be made available by the corresponding author on request.

Consent to participate:

Not applicable.

Consent for publication:

Not applicable.

Author contributions:

All authors contributed study conception and design. Experimentations were performed by Tripti Joshi and Vipendra Kumar; data were analysed by Tripti Joshi and Nihar Ranjan Jana. First draft of the manuscript was written by Nihar Ranjan Jana and all authors read, commented and approved the final manuscript.

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Figures

Fig.1

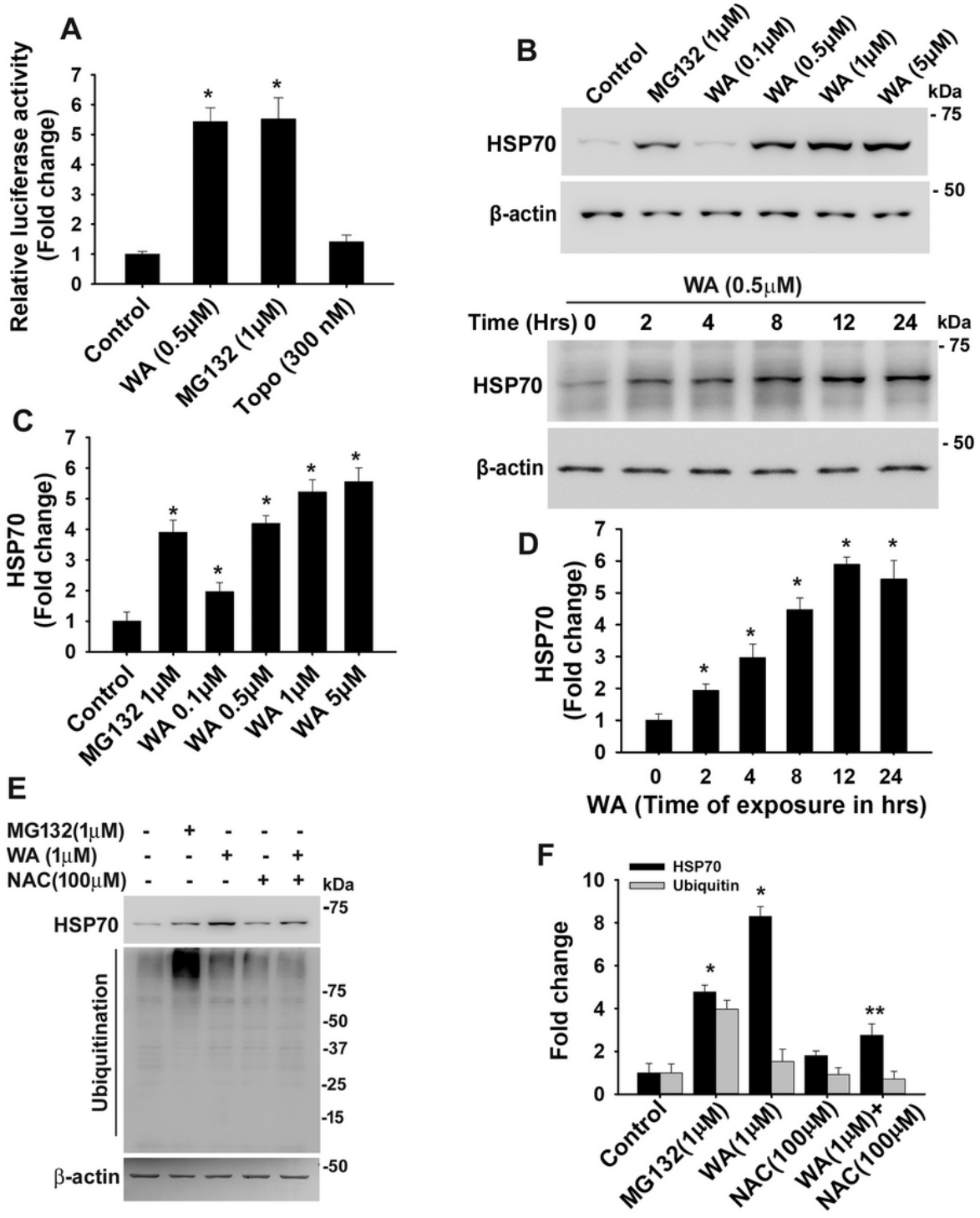

Figure 1

Treatment of WA induces HSR. A) HT22 cells were transiently transfected with a Firefly luciferase plasmid containing HSE along with Renilla normalization plasmid. Twenty four hours of post transfection, cells were treated with various chemicals for 12 h as indicated in the figure. Cells were then

collected and subjected to dual luciferase assay. Values are mean ± SD of three independent experiments each performed triplicate. *P<0.001 as compared to control. MG132 used as positive control. B, C and D) Cells were platted onto 6-well tissue cultured plates and on the following day treated with different doses of WA for 12 h. In some experiment, cells were treated with 0.5μM of WA for different time periods. Collected cells were then processed for immunoblot analysis using HSP70 and β-actin antibodies. Band intensities of HSP70 and β-actin were determined using NIH ImageJ software, level of HSP70 was normalized with β-actin and expressed as fold change (C and D). MG132 used as positive control. E and F) WA-induced HSR is mediated through thiol modification. Cells were left untreated or treated with WA in the absence or presence of NAC for 12 h. Collected cells were then subjected to immunoblot analysis using various antibodies as indicated in the figure. Band intensities of HSP70 and ubiquitin were quantified, normalized and plotted as fold change. In C, D and F, Values are mean ± SD of three independent experiments. *P<0.001 as compared to control and **P<0.001 in comparison with WA treated group.

Fig.2

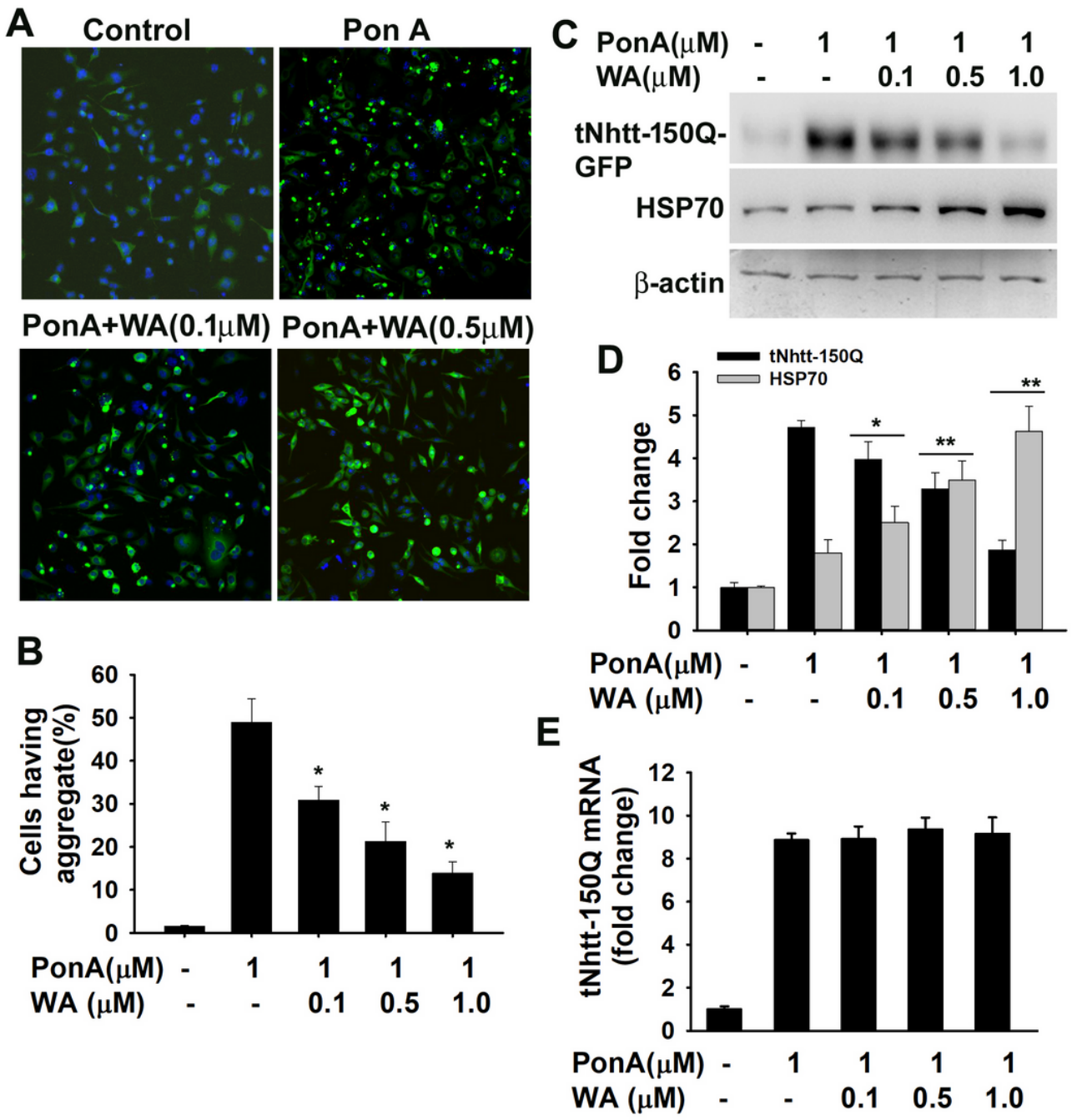

Figure 2

Treatment of WA suppresses mutant huntingtin aggregation in a cellular model of HD. A and B) HD150Q cells were platted onto 2-well chamber slide at low density and on the next day, cells were simultaneously induced with ponasterone A (PonA) and treated with different doses of WA. Forty-eight hours of post treatment, images were taken from multiple fields in each treatment group using fluorescence microscope and number of aggregates were counted and expressed as percentage of cell having aggregates. Multiple aggregates in a single cell were considered single aggregate. Values represented are mean ± SD of three

independent experiments. *P<0.001 as compared to PonA treated group. C, D) HD150Q cells were platted onto 6-well tissue cultured plates and on the subsequent day, cells were induced and treated with WA for 48 h. Collected cells were then processed for immunoblot analysis using GFP (to detect tNhtt-150Q-GFP protein) and HSP70 antibodies. Band intensities of tNhtt-150Q-GFP and HSP70 were measured and normalized as described in Figure 1. E) HD150Q cells were induced and treated with WA as above, total RNA was extracted and subjected to semi-quantitative RT-PCR analysis. Values depicted are mean ± SD of three independent experiments. In D, *P<0.05 and **P<0.001 as compared to respective PonA treated group.

Fig.3

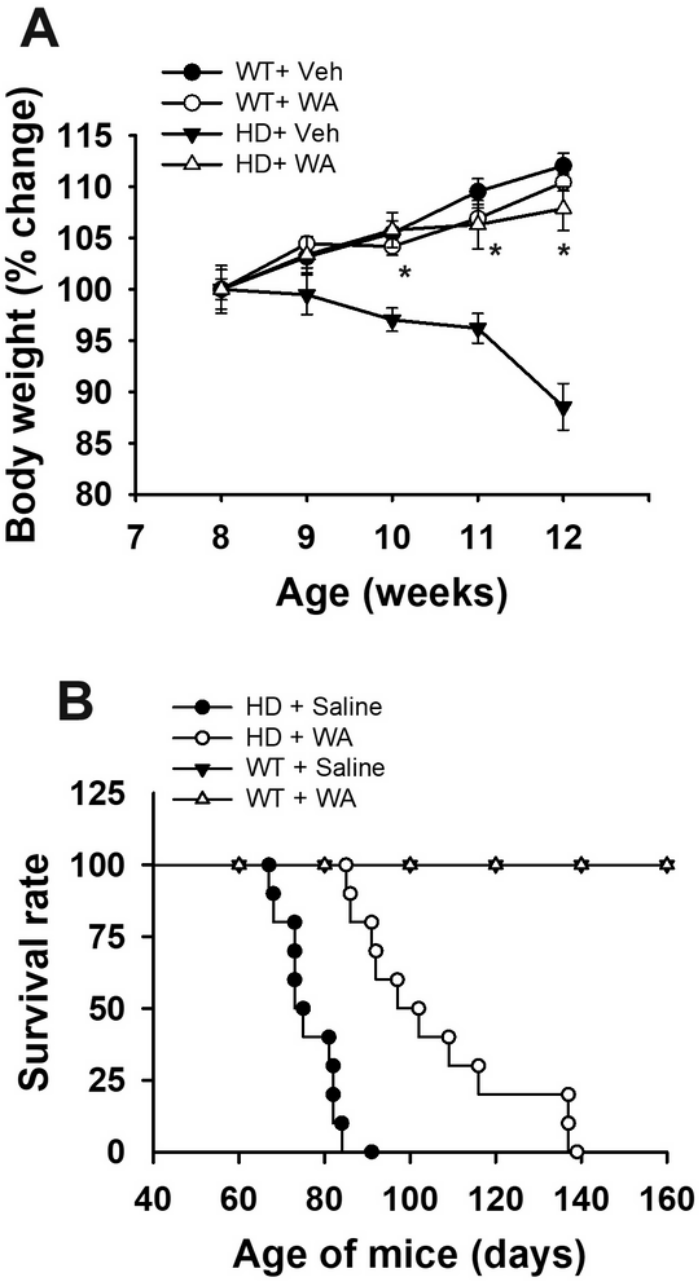

Figure 3

Treatment of WA rescues deteriorated body weight and increases the lifespan of HD mice. Wild type and HD mice were injected with either saline or WA from their age of 56 days and continued up to 84 days. Injections were given every alternate day. Body weight of each mouse was measured every week up to 84 days and then left untouched to monitor their lifespan. A) Percent change of the body weight of saline and drug treated HD and wild type mice groups. B) Comparison of the survival rate of drug and vehicle

treated HD mice. Data was analysed using two-way ANOVA followed by Holm Sidak Post hoc test. Values shown are mean ± SD with 9-12 animals in each experimental group. *P<0.05 as compared to saline treated HD group.

Fig.4

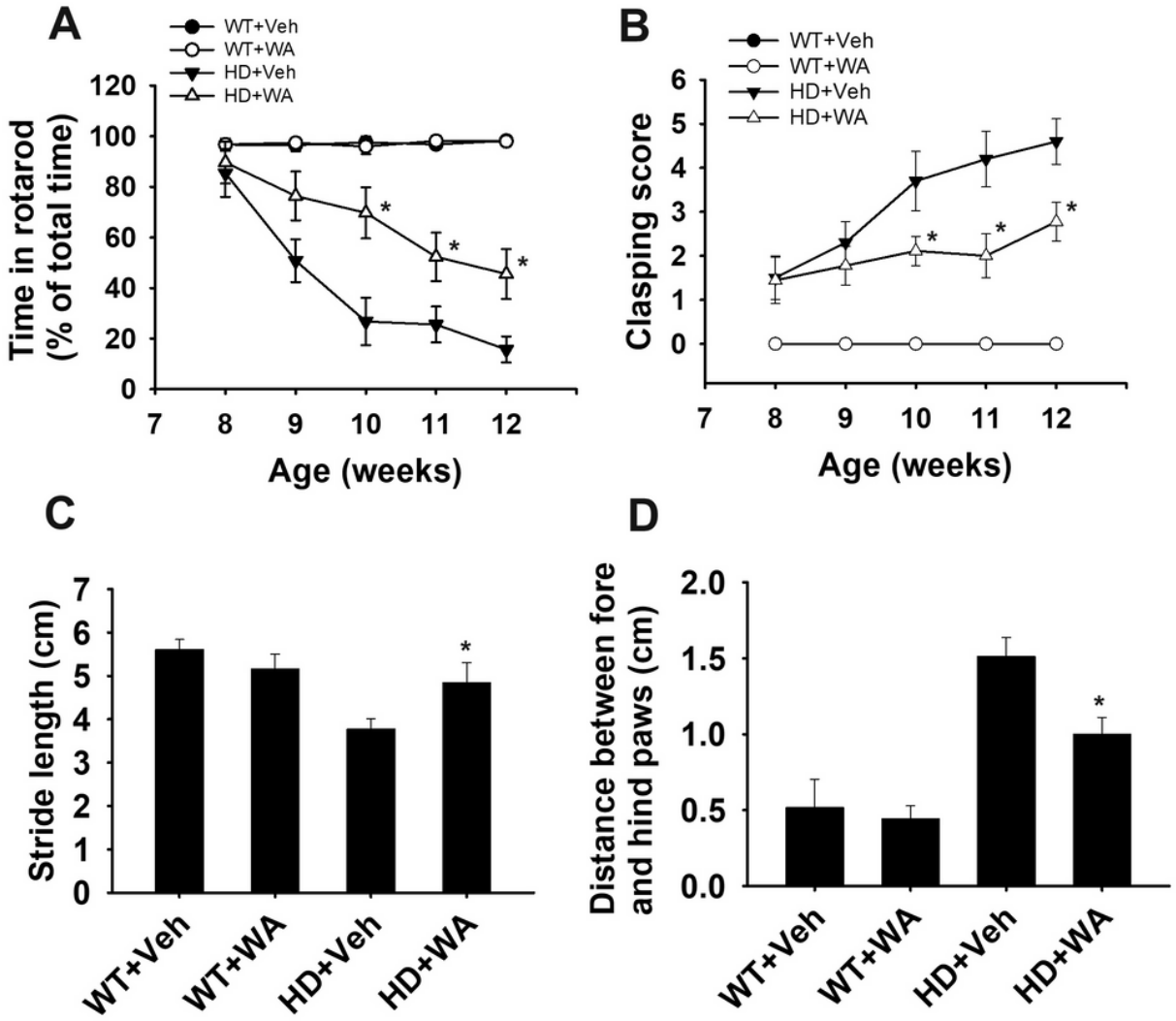

Figure 4

Significant improvement of motor behavioural deficits in WA treated HD mice. Long-term administration of WA improved motor deficits in HD mice. Animals were treated with saline or WA as described above and various tests to assess motor function were conducted at the end of each week. A) Assessment of motor function through rotarod performance test. B) Clasping test. C and D) Foot print gait analysis. Values shown are mean ± SD with 10 animals in each experimental group. *P<0.001 as compared to

vehicle treated HD group. Two-way (in A and B) or one-way (in C and D) ANOVA was used to analyse the data.

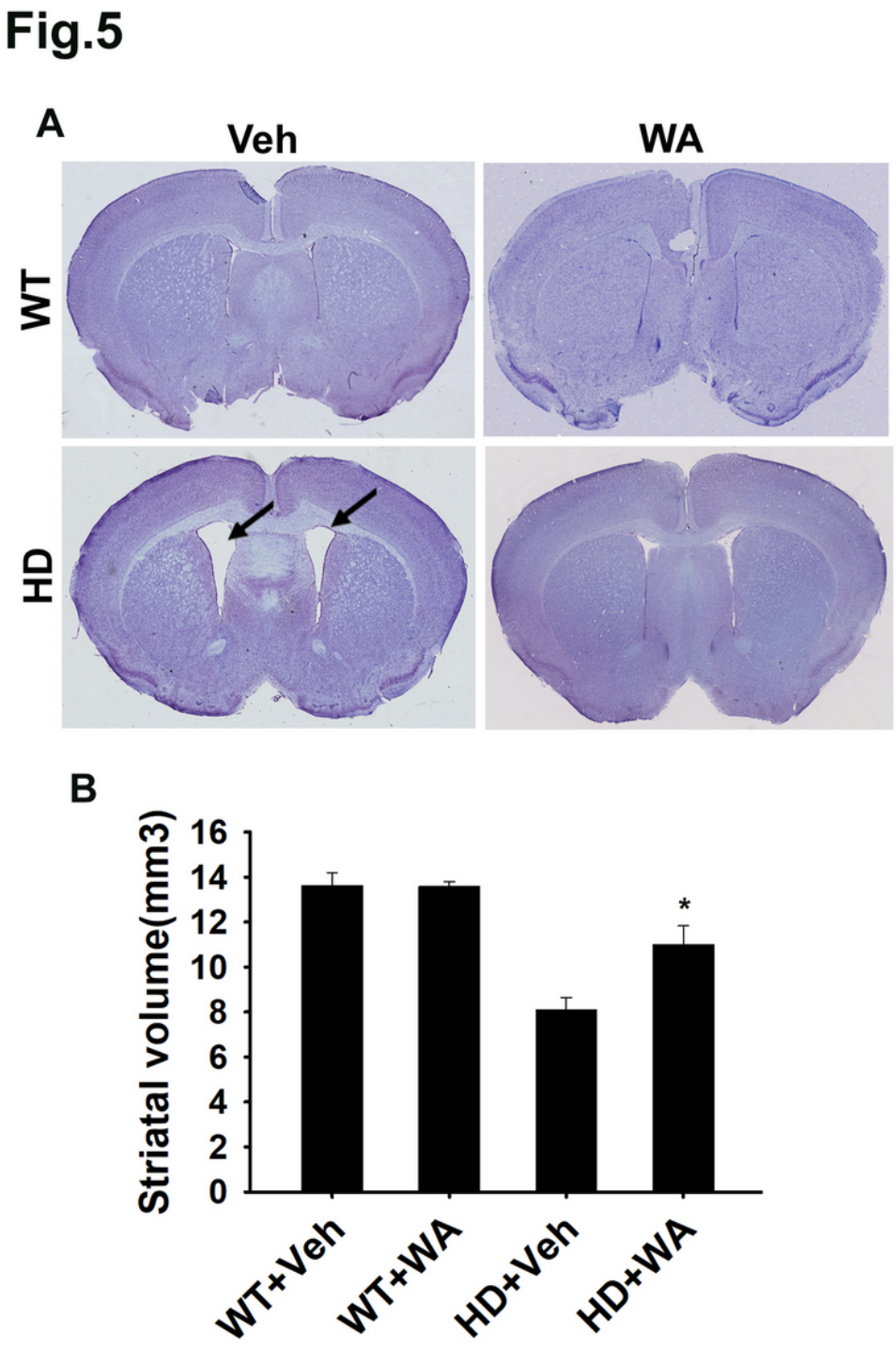

Figure 5

Partial restoration of striatal atrophy in WA treated HD mice. Animals were treated with either vehicle or drug as described in Figure 3 and after last injection (at 84 days), animals were subjected to perfusion under anesthesia, brains were collected and subjected to cryo-sectioning. Serial coronal sections were

placed onto glass slides and processed for Nissl staining followed by stereological evaluation of striatal volume. A) Nissl-stained coronal sections. B) Assessment of striatal volume. Values are mean ± SD with 3 animals in each experimental group. *P<0.01 as compared to vehicle treated HD group (one-way ANOVA followed by post hoc test).

Fig.6

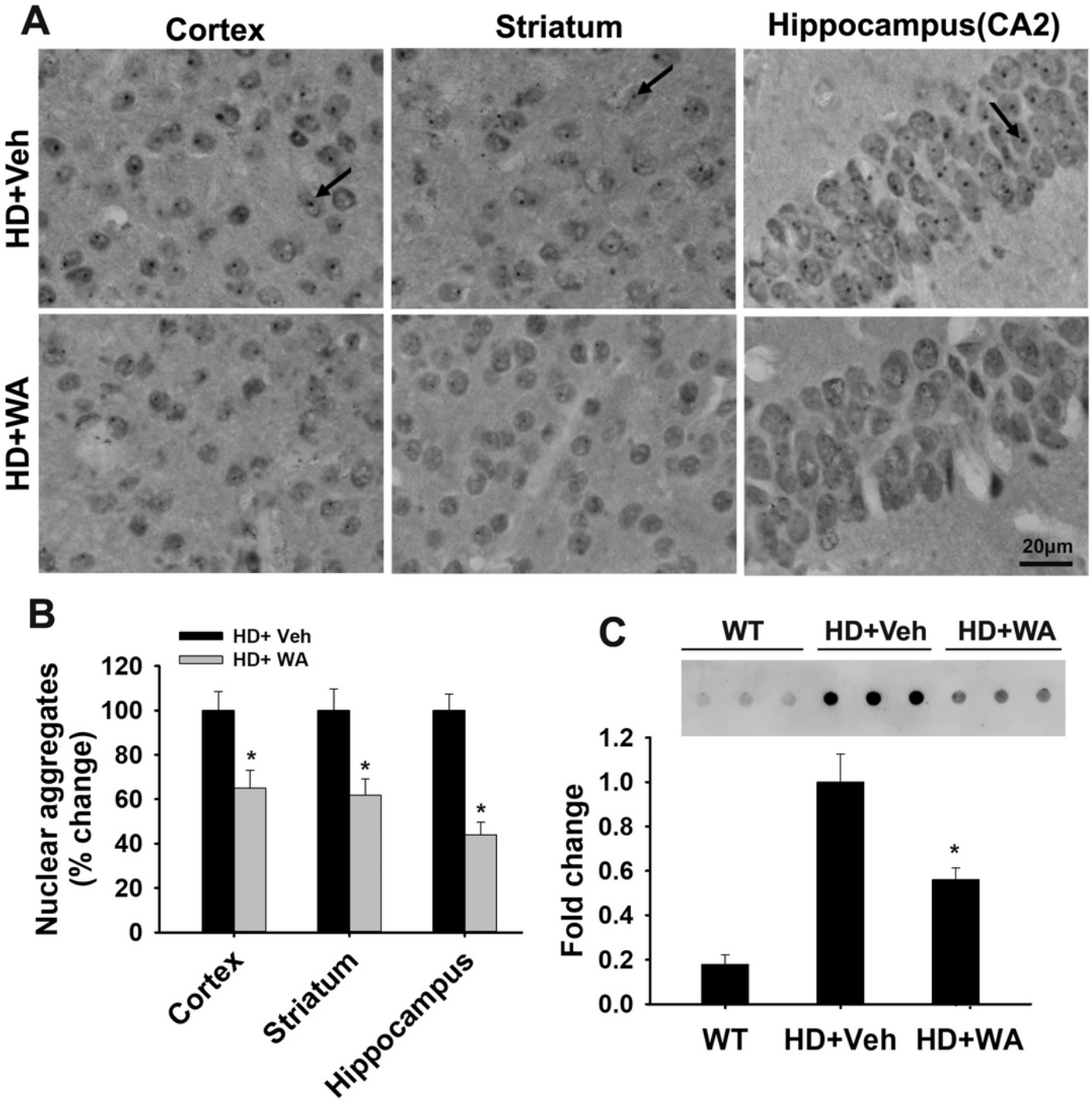

Figure 6

Administration of WA significantly reduces the number of nuclear aggregates in HD mice brain. A) Coronal brain sections acquired from vehicle and WA treated HD mice were processed for IHC staining using huntingtin antibody to detect the aggregates (pointed by arrow). B) Nuclear aggregates were manually counted in 2-3 different field of each brain area. Brain sections from three different animals were evaluated and values were expressed as percent change upon WA treatment. Values are mean ± SD with 3 animals in each experimental group. *P<0.01 as compared to saline treated HD group (one-way ANOVA followed by post hoc test). C) Dot blot analysis of the insoluble fraction prepared from saline and WA treated wild type and HD animals (cortical samples). Blot was detected with IC2 antibody. Intensities of each spot were determined using NIH Image analysis software and expressed as fold change. In each spot, sample from different mouse was used. Values are mean ± SD with 3 animals in each experimental group. *P<0.001 as compared to vehicle treated HD group (one-way ANOVA followed by post hoc test).

Fig.7

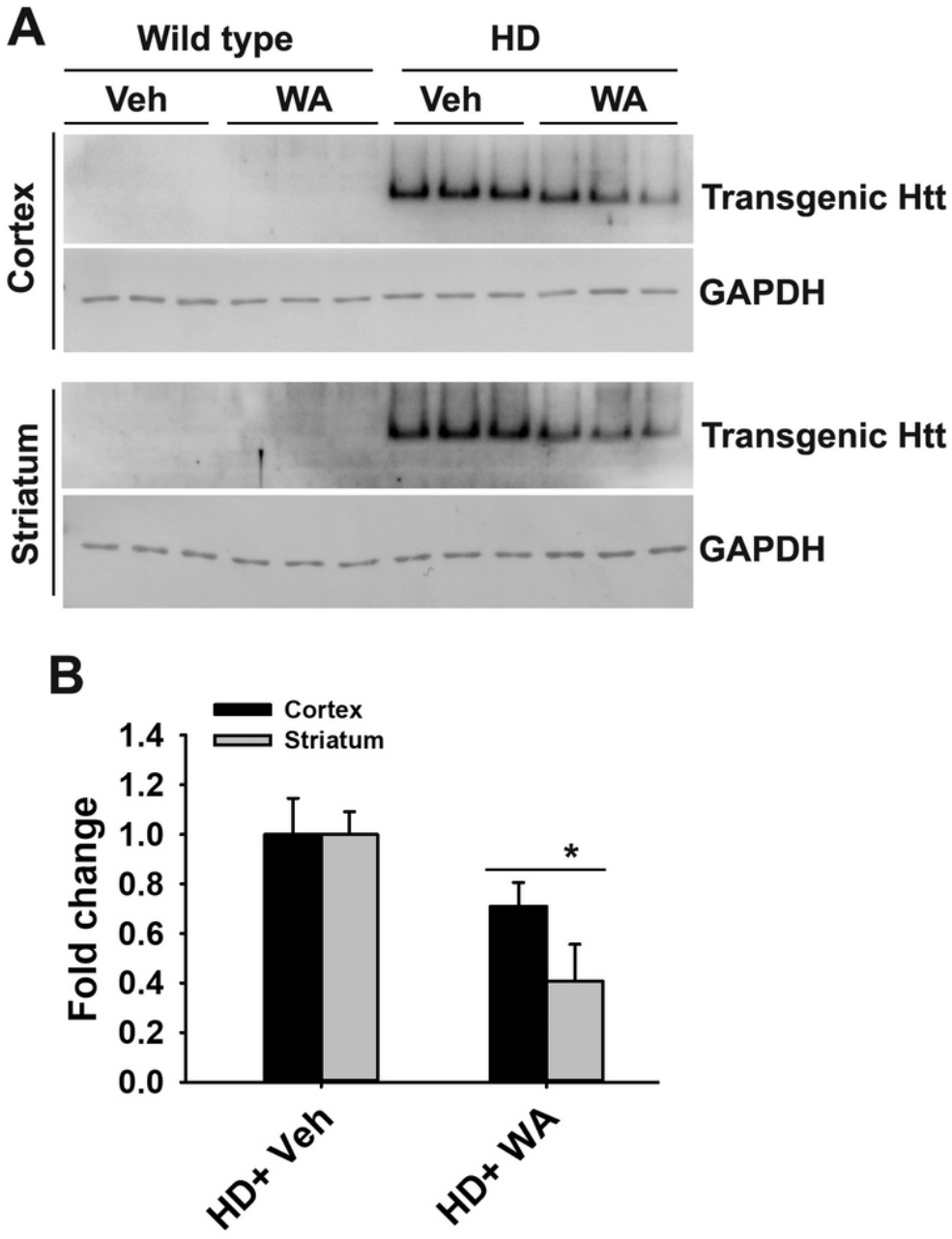

Figure 7

WA treatment significantly reduces the soluble level of transgenic mutant huntingtin. A) Cortical and striatal samples from 3 different mouse in each experimental group were subjected to immunoblot analysis using 1C2 antibody to detect the soluble transgenic mutant huntingtin fragment. B) Band intensity of transgenic mutant huntingtin fragment was analyzed and expressed as fold change. Values

are mean ± SD with 3 animals in each experimental group. *P<0.05 (cortex) and P<0.01 (striatum) as compared to saline treated HD group (Students t-test).

Fig.8

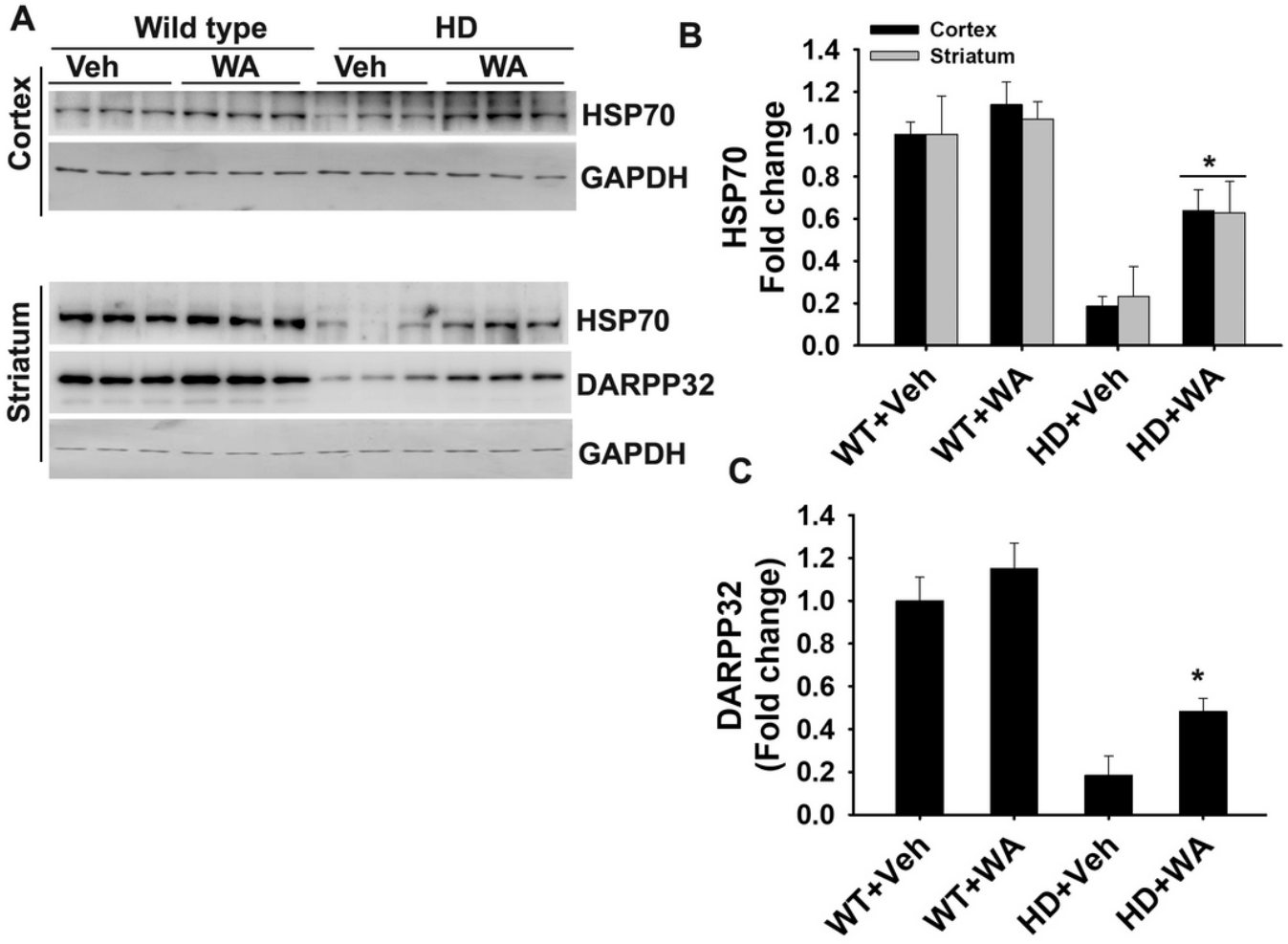

Figure 8

Induction of HSR and improvement of striatal function in WA treated HD mice. A) Cortical and striatal lysates obtained from saline and WA treated wild type and HD mice were subjected to immunoblot analysis using various antibodies as indicated in the figure. Each lane in the blot represent sample from different mice. B) Band intensity of HSP70 from cortical and striatal samples was evaluated and plotted as fold change. C) Quantification of band intensity of DARPP32 in the striatal sample. Data represented as mean ± SD; n=3 animals in each experimental group. *P<0.05 as compared to saline treated HD group (one-way ANOVA followed by post hoc test).

Fig.9

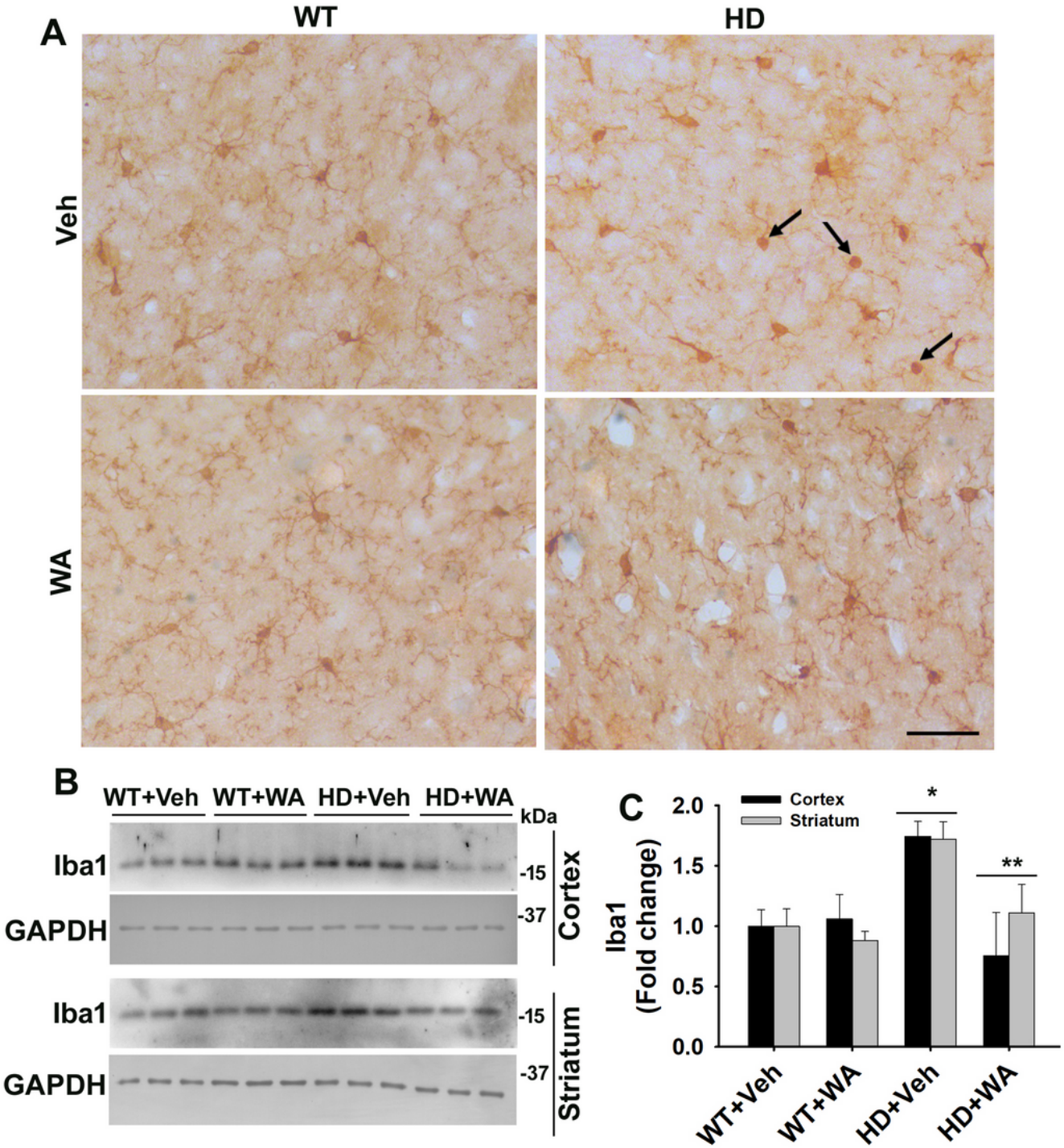

Figure 9

Treatment of WA suppresses the inflammation in HD mice brain. A) Brain sections from wild type and HD mice received either vehicle or WA treatments were processed for immunohistochemical staining using Iba1 antibody. Coronal sections (having striatal and cortical areas) from all four experimental groups were placed on same slide and processed for staining. Arrow indicates the activated microglia. Scale bar, 40μm. B and C) Cortical and striatal lysates were subjected to immunoblot analysis using Iba1 and

GAPDH antibodies. Band intensity of Iba1 was quantified and expressed and fold change. Values are mean ± SD with 3 animals in each experimental group. *P<0.01 in comparison with wild type groups while **P<0.01 with compared to saline treated HD group.