

Efficacy of therapy by MK-28 PERK activation in the Huntington's disease R6/2 mouse model

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Research Article

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Abstract

There is currently no disease-modifying therapy for Huntington's disease (HD) and two recent clinical trials testing antisense oligonucleotides failed. We recently described a small molecule, MK-28, which restored homeostasis in HD models by specifically activating PKR-like ER kinase (PERK) and thus boosting neuroprotection by the unfolded protein response (UPR), and reducing endoplasmic reticulum (ER) stress, a central cytotoxic mechanism in HD and other neurodegenerative diseases. Here we have tested the long-term effects of MK-28 in HD model mice. R6/2 CAG (160) mice were treated by lifetime IP injection, 3 times a week. CatWalk measurements of motor function showed significant improvement after only two weeks of MK-28 treatment and continued with time, most significantly at 1 mg/kg MK-28, approaching WT values. Seven weeks treatment significantly improved paw grip strength. Body weight recovered and glucose levels, which are elevated in HD mice, were significantly lowered. Treatment with another PERK activator, CCT020312, also caused amelioration, although less significant than with MK-28 in some of the parameters. Lifespan, measured in more resilient R6/2 CAG (120) mice with daily IP injection, was significantly extended by 16 days (20%) with 0.3 mg/kg MK-28, and by 38 days (46%) with 1 mg/kg MK-28. No toxicity, measured by weight, blood glucose levels and blood liver function markers, was detectable in WT mice treated for 6 weeks with 6 mg/kg MK-28. Boosting of PERK activity by long-term treatment with MK-28 appears to be a safe and promising therapeutic approach for HD.

Introduction

Aggregation-prone proteins that are the cause of diseases, amongst them many of the neurodegenerative diseases, produce cellular stress, toxicity and death. In HD, the causative agent is a mutant huntingtin gene, containing expansions of CAG repeats encoding for a polyglutamine (polyQ) stretch, which becomes pathogenic when it encompasses over approximately 35 glutamines, leading to the formation of toxic oligomers [1-3]. We had determined that in cells expressing mutant huntingtin, there is a strong induction of endoplasmic reticulum (ER) stress, especially in striatal cells, the cells that first degenerate in HD patients [4]. ER stress induces the unfolded protein response (UPR), which activates several pathways. One of these pathways, the PERK pathway, causes phosphorylation of the translation factor eIF2 α , leading to a general inhibition of protein synthesis in the short term, a cell protective mechanism, because it reduces the ER load [5-7]. We had previously found that striatal cells have a very low level of phosphorylated eIF2 α . This phospho-eIF2 α level was increased by expression of pathogenic huntingtin. We had originally hypothesized that the toxicity of pathogenic huntingtin might be mediated by its effect in increasing the naturally low eIF2 α -P that exists in striatal cells, which can lead to apoptosis in the long term [2]. We synthesized novel compounds based on the structure of A4, which had been reported as a PERK inhibitor *in vitro* [8]. We surprisingly found that the lead compound, MK-28, is a PERK activator, not an inhibitor [9]. In a first evaluation of MK-28 *in vivo*, we delivered the compound transiently, using subcutaneous Alzet pumps for 4 weeks at a dose of 1 mg/kg. This treatment showed encouraging results, with improved motor and executive functions and delayed death onset [9]. Boosting PERK activation would extend the cell-protective inhibition of protein synthesis, reducing ER stress and

cytotoxicity. Long term PERK activation induces the expression of the eIF2 α -P phosphatase (PP1C) regulatory subunit GADD34 (PPP1R15A), tuning the eIF2 α phosphorylation balance to allow protein synthesis recovery and preventing induction of apoptosis [7].

Here, we have extended our evaluation of MK-28, with lifetime IP administration of increasing doses of the compound in the R6/2 HD mouse model. Our results indicate strong neuroprotective effects with dramatic improvement of motor symptoms and unprecedented extension of lifespan.

Materials And Methods

Materials. Thapsigargin (Thap) and common reagents were from Sigma. CCT020312 was from Calbiochem. MK-28 was synthesized as described before [9]. Rabbit anti-PERK was

from Abcam (#ab65142). Goat anti-rabbit IgG conjugated to HRP was from Jackson Labs.

Cells

Mouse embryonic fibroblasts (MEFs) were treated for 3 hours with thapsigargin (2 μ M) as an ER stress inducer. Cells were lysed with PBS containing 1% Triton X-100, 0.5% Sodium deoxycholate (DOC) with phosphatase inhibitors Cocktail 2 and 3 (Sigma). Lysates were incubated on ice for 30 minutes, then centrifuged at 16,000xg for 30 minutes at 4 °C. The supernatants were boiled in SDS loading buffer for 5 min and run on 8% SDS-PAGE. Anti-PERK antibody was used to detect PERK and its phosphorylated forms (1:1000 in blocking solution).

Animals

Female mice model for Huntington's disease B6CBA-R6/2 (CAG 160 +/-) and B6CBA-R6/2 (CAG 120 +/-) (Jackson lab, cat. #002810 and #006494, respectively, USA) were kept on a 12/12 day/night cycle with free access to food and water. Following company instructions, females were bred with non-carrier (wild type) males B6CBAF1/J (cat. 100011).

All procedures were approved by the Tel Aviv university animal care and use committee.

Genotyping

At 3 weeks, mice were genotyped based on mouse-tail DNA extraction. PCR assessment of CAG repeat size was done using the following primers: 5'-ATG AAG GCC TTC GAG TCC CTC AAG TCC TTC-3' and 5'-GGC GGC TGA GGA AGC TGA GGA G-3'.

Animal treatments

Intraperitoneal injection (IP) was given to the mice 3 or 5 times a week, as indicated, with vehicle (50% glycerol and 15% DMSO in PBS) containing different doses of MK-28 or CCT020312.

Toxicity tests

Healthy male C57BL/6 WT 6 weeks old mice were given IP injection every other day (3 times a week) with vehicle containing or not MK-28 (6mg/kg). Blood glucose level measurements were taken from a small cut at the tip of the tail and measured with an Accu-Check glucose monitor (Roche Diagnostics, USA).

Weight was monitored weekly for 6 weeks in total. At the age of 12 weeks, the mice were sacrificed by CO₂, and blood samples were collected and sent to an external company (ALM) for analyzing liver function markers. Brains were taken for future analysis and kept at -80°C

Brain lysate

One hemisphere of each brain was harvested and homogenized in lysis buffer (1% triton, 100mM NaCl, 10% glycerol, 10mM β-Glycerophosphate, 5mM NaF, 1% DOC, 0.5% SDS, 1mM DTT, 1mM sodium vandate, 5μg/ml pepstatin, 5μg/ml leupeptin and phosphatase inhibitors cocktail). Each sample was sonicated three times for 5 seconds. Lysate protein concentrations were quantified using a bicinchoninic acid (BCA) protein assay kit (Thermo scientific, #23225). Equal total protein concentrations were resolved on 8% SDS-polyacrylamide gels.

Grip

Grip strength was measured using a computerized grip strength meter (Model 47200, Ugo-Basile, Varese, Italy) [10] every other week. Each mouse was gently placed in front of a metal net, held by the tail, and allowed to grip the bar with his front paws. Then the mice were pulled back until their grip was lost. The grip meter calculated the amount of force exerted on the grip net. Each mouse went through 5 trails. The three highest scores were used for the statistic.

CatWalk

Mice were placed on a CatWalk path (CatWalk XT - Noldus Information Technology) once a month. The mice were allowed to walk the CatWalk track without involvement until they passed the path without interruptions or hesitation for 5 runs. CatWalk XT software calculated the average speed, paw contact, and paw print area for each mouse.

Statistical analysis

The results are expressed as mean ± standard error except where indicated. Student's

t-test (two-tailed) was used when comparing the means of two groups. P value ≤ 0.05 was considered statistically significant. P ≤ 0.05 (*), P ≤ 0.01 (**), P ≤ 0.001 (***). Statistical analysis was performed using SPSS statistic software 27 and Microsoft Office Excel software.

Results

MK-28 causes PERK activation and no toxicity in WT mice

MK-28 activates PERK when tested with purified components *in vitro* [9]. In cells, this can be observed as an increased in PERK phosphorylation (Fig. 1A). Treatment of WT mice by daily IP injection at 1 mg/kg for 7 days resulted in a significant increase of phosphorylated PERK compared to mice injected with vehicle, when measured in brain lysates (Fig. 1b).

For lifetime evaluation of MK-28 efficacy on R6/2 mice, since daily injections are aversive, especially for this fragile model, and may not be necessary for the beneficial effect of PERK activation, we opted for IP injection 3 times a week and not daily. To ensure that this regime is not toxic, we tested it on WT mice, starting at the age of 6 weeks and for 6 weeks at 6 mg/kg MK-28, which is 6-fold the dose used in our previous study [9] and to observe PERK activation in the brain (Fig. 1b). The treatment caused only a minor but not significant increase in weight (Fig. 1c) and no significant change in blood glucose levels (Fig. 1d). At the end of the 6 week treatment, liver function markers were analyzed in blood samples, showing no significant difference (Fig. 1e).

Intermittent delivery of MK-28 ameliorates physiological parameters in R6/2 HD model mice

B6CBA-R6/2 mice from Jackson laboratories, containing expanded repeats of CAG (160 +/- 5) were treated by IP injection, 3 times a week with different doses of MK28, 0.1, 1, and 10 mg/kg, starting at 3 weeks of age. For comparison, another group was treated with 1 mg/kg of a commercial PERK activator, CCT020312 [11]. As controls, two groups of WT/HD mice were treated with vehicle. R6/2 mice are known for losing weight over time with the progression of the disease [12,9]. It is not clear if the weight loss is a result of difficulty to eat or other reasons, since HD is also characterized with development of diabetes. MK-28 showed a positive influence in the weight, slowing down weight loss (Fig. 2a), significantly at 1 mg/kg. Blood glucose levels are increased in HD mice [13]. Glucose levels were measured at 8 weeks of age, showing significant reduction in HD mice, in mice treated with MK-28 or CCT020312, to normal levels or slightly below (Fig. 2b).

MK-28 significantly improves motor functions

The mice were subjected to a gripping force test every second week. The HD mice showed a significant reduction in the grip strength starting at 10 weeks of age (Fig. 3a). MK-28, as well as CCT020312, increased significantly the grip strength at 10 weeks of age (Fig. 3b). However, the effect was reduced at 12 weeks of age, remaining significant only for the lowest dose of MK-28, 0.1 mg/kg, whereas higher concentrations or 1 mg/kg CCT020312 had no effect.

We used CatWalk tests to test several motor parameters. This is a computerized video-assisted system, which measures automatically and with high sensitivity, gait and motor function in mice while they walk over an illuminated glass walkway, allowing quantitative assessment [14,15]. CatWalk tests were performed once a month, starting at 5 weeks of age. The average speed of the mice, measured as distance over time (cm/s), showed a strong, though variable deficit of the HD mice (Fig. 4a). MK-28 at 1

mg/kg showed a significant improvement in the average speed to near WT levels (Fig. 4a, b), whereas other concentrations or CCT020312 did not have a significant effect. HD mice develop gait problems and difficulty stepping on their paws, leading to more gentle steps and less contact of the paws with the surface. We determined the maximum paw contact intensity, indicating the maximum pressure exerted by each single paw on contact with the walkway, expressed as the average of the 4 paws. We were able to determine maximum contact intensity at 5 and 9 weeks of age, showing a significant reduction in HD mice (Fig. 4c). MK-28 and CCT020312 significantly improved the maximum contact, to normal levels at 5 weeks of age, after only two weeks of treatment (Fig. 4d). It is noteworthy that at 1 mg/kg, MK-28 treated HD mice showed almost the same maximum contact intensity as WT mice over time, which increased from 5 to 9 weeks of age (Fig. 4c). Another parameter that was determined was the print area of each paw upon contact with the walkway. CatWalk tests show a much better use in the paws among treated mice with a better spreading area of the paw's center and fingers, on a larger surface. For the WT mice the print area (average of the 4 paws) increases significantly with age, from 5 to 9 weeks. In contrast, for HD mice there was no increase, consistent with no increase in maximum contact intensity (Fig. 4e, compare with 4c). All treatments with MK-28 and CCT020312 increased the paw print area at 5 weeks of age, above that of the untreated HD and WT mice (Fig. 4e). This effect was reduced with time, except for MK-28 at 1 mg/kg, which caused less increase at 5 weeks and an increment with time, similar to the WT mice. At 9 weeks of age all HD mice treated with MK-28 or CCT020312 showed a significant improvement in paw print area, with measures similar to the WT mice and far larger than the untreated HD mice. This can clearly be seen visually in images of representative left hind paws of each group (Fig. 4f).

Overall, the treatments led to a strong improvement in gait and locomotion, especially with MK-28 at a dose of 1 mg/kg, as can be seen in a CatWalk movie (Suppl. Movie S1 (9 weeks)).

Lifespan extension

R6/2 mice have a significantly reduced lifespan [16]. Mice treated with MK-28 or CCT020312 showed a strong delay in death initiation (Table 1). However, due to the premature death of the untreated R6/2 CAG160 mice, statistical significance could not be achieved in Kaplan-Meier curves. Therefore, we performed an experiment with MK-28, with the more moderate HD model of R6/2 with 120 CAG repeats [17], which were amenable to daily IP injection. In this case, there was significant extension of lifespan with a Kaplan-Meier estimate of 16 days (20%) with 0.3 mg/kg MK-28, and a very substantial extension of 38 days (46%) with 1 mg/kg MK-28 (Fig. 5).

Discussion

There is no disease modifying therapy currently available for HD, although there is an active search for one [18-20]. Current treatments are only for symptom alleviation. There is an urgent need for a novel approach for the development of a viable therapy, which is stressed by the fact that two clinical trials based on antisense oligonucleotides recently failed [21]. A therapy involving a small molecule, such as

the PERK activator which we advance here, would be ideal given the efficiency in delivery to hard to access brain regions.

PERK pathway activation is naturally observed in neurodegenerative diseases, which might suggest a damaging response. However, the fact that in several tauopathies, PERK variants with reduced activity are a genetic risk factor and cause high sensitivity to ER stress [22] suggests that PERK activation in the neurodegenerative diseases is an insufficient physiological attempt to reduce the ER stress [9,23,7].

PERK pathway activation causes transient protein synthesis inhibition, reducing ER protein load, and inducing cell protective pathways through eIF2 α phosphorylation which increases ATF4 expression and by direct phosphorylation and activation of NRF2 [6,7]. PERK activation also stimulates autophagy, which has important neuroprotective effects in HD [24,25].

In our previous work we reported the PERK activator MK-28, its efficient protection of an HD cellular model, a striatal cell line (STHdh^{Q111/111}), its high selectivity for PERK and its neuroprotective properties in short-term treatment of an HD mouse model [9]. PERK pathway activation can also be accomplished by inhibition of eIF2 α -P dephosphorylation by the eIF2 α -P phosphatase regulatory subunit GADD34 restoration of protein synthesis in the long

term by eIF2 α -P dephosphorylation is also repressed, which can lead to activation of pro-apoptotic pathways [26-28]. However, direct PERK activation can boost the protective phases without compromising long-term recovery of eIF2 α function, as GADD34 is still induced. Another direct activator of PERK described so far is the compound CCT020312, which had neuroprotective effects in cellular and mouse models of tauopathies [11]. CCT020312 protected HD cellular models, although with much lower efficacy than MK-28 [9]. Here we found that *in vivo* CCT020312 showed good efficacy, although less than MK-28 in improving weight and CatWalk parameters (Fig. 2 and 4).

The optimal range of MK-28 *in vivo* appears to be ~1 mg/kg, improving HD mice performance to a level close to their WT littermates in weight, blood glucose and CatWalk parameters in the short and long terms (Fig. 2 and 4), and extending significantly lifespan (Fig. 5). A lower dose of 0.1 mg/kg improved significantly CatWalk parameters in the short term and had even better efficacy than 1 mg/kg in the grip strength test (Fig. 3). The effect on grip strength is an important feature, as it was not increased by other small molecules tested, such as the anti-inflammatory drug tolfenamic acid [29] or the mHtt binder and autophagy inducer GLYN122 [30].

The minimal effective doses for MK-28 neuroprotection *in vivo* are noteworthy, if compared to reports with other small molecules (some in clinical trials) on the aggressive R6/2 mouse model, which showed effects in the 5-20 mg/kg range and little or no extension of lifespan [31-34]. This is even more remarkable, considering that we observed the significant improvement in motor function in a regime of IP delivery of only 3 times a week.

Even at a much higher dose than the minimal effective dose, 6 mg/kg, delivery for 5 weeks of MK-28 showed no toxicity in mice, as measured by weight, blood glucose levels and liver function markers (Fig. 1). It did not show any evidence of deleterious effects in the long-term either in HD model mice.

Conclusions

Our study suggests long-term MK-28 treatment as a possible therapeutic avenue for HD. MK-28 is a small molecule with efficient delivery and no observed toxicity. MK-28 shows so far unmatched improvement of motor function, such as significant increase in paw grip, and of lifespan extension. A similar effect, although with less efficacy in some of the tests, of CCT020312, an unrelated compound, asserts direct PERK activation as the protective mechanism, which boosts the unfolded protein response.

Abbreviations

DOC: Deoxycholate

ER: Endoplasmic reticulum

HD: Huntington's disease

IP: Intraperitoneal injection

MEFs: Mouse embryonic fibroblasts

PERK: PKR-like ER kinase

Thap: Thapsigargin

UPR: Unfolded protein response

WT: Wild type

Statements and Declarations

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Competing interests

The authors declare that they have no competing interests.

Author contributions

G.L. conceived and designed the study. D.O. supervised animal experiments. T.S. performed the experiments. T.S. and G.L. wrote the manuscript. All authors read and approved the final manuscript.

Data availability

All data generated or analyzed during this study are included in this article or available upon request.

Ethics approval.

All in vivo experiments followed institutional guidelines and were approved by the Institutional Animal Care and Use Committee of Tel Aviv University.

Consent to participate

Not applicable.

Consent to publish

Not applicable.

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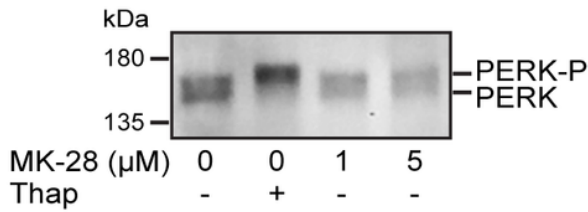
Table

Table 1. Death onset in B6CBA R6/2 HD model mice with expanded CAG repeats (160 +/- 5) in the huntingtin gene treated from the age of 3 weeks by IP injection 3 times a week (n = 9 WT, 5 HD, 8 HD + 0.1mg/kg MK-28, 11 HD + 1mg/kg MK-28, 5 HD + 10mg/kg MK-28, 2 + 1mg/kg CCT020312).

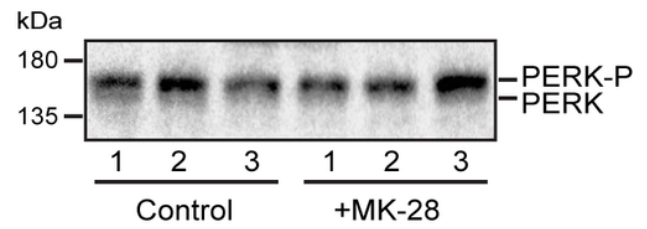
Treatment group	Death onset (weeks)
WT Control	-
HD Control	6
HD+0.1 mg/kg MK-28	9
HD+1 mg/kg MK-28	10
HD+10 mg/kg MK-28	11
HD+1 mg/kg CCT	13

Figures

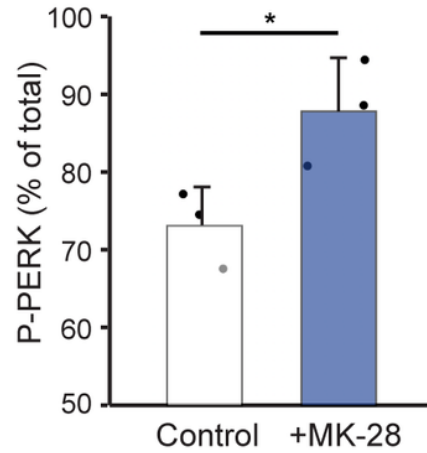
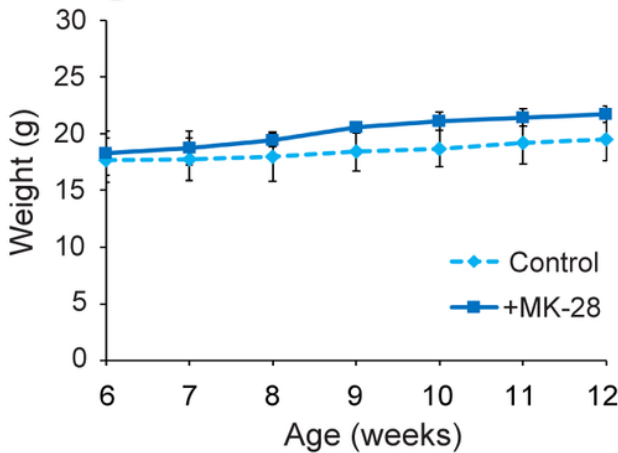
a. MEF cells



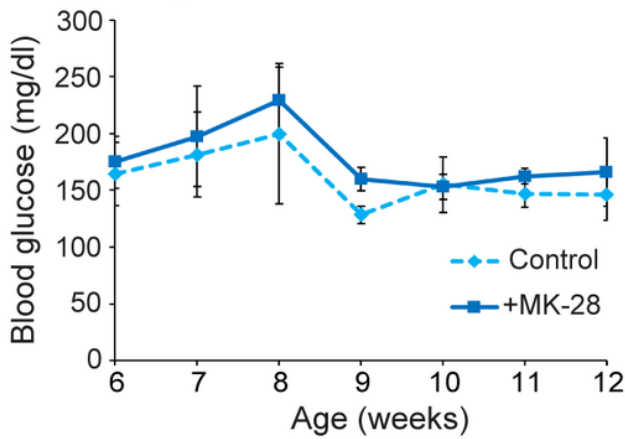
b. Brain lysates



c. Weight



d. Blood glucose



e. Blood liver function markers

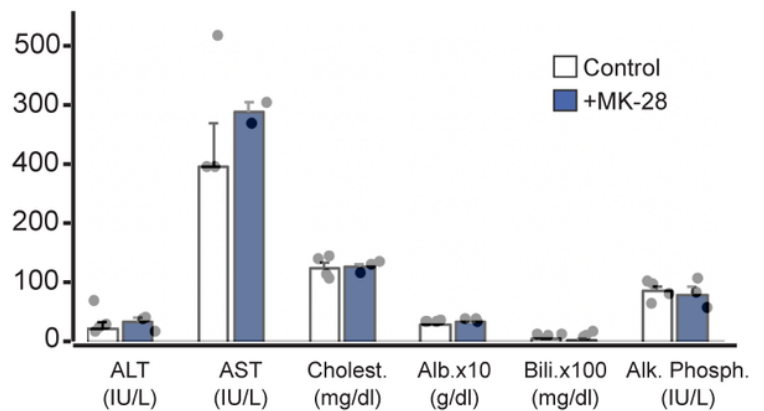


Figure 1

PERK activation by MK-28 causes no toxicity in WT mice. (a) MEF cells were treated for 3 h with the ER stress inducer Thap (2 μM) or with MK-28 at the indicated concentrations. An immunoblot with anti-PERK antibody shows conversion of most molecules to a slower migrating band (PERK-P). MK-28 treatments also show an increase in PERK-P, indicating PERK activation. (b) Six-week old C57BL/6 WT mice were injected (IP) three times a week with 6 mg/kg of MK-28 (n=3) or with vehicle (control, n=3) for 6 weeks. Brain lysates show PERK activation by MK-28 treatment. The graph shows average+SD of P-PERK divided by total PERK. (c) Weight was monitored every week, showing no significant differences between the groups. (d) Blood glucose levels were measured weekly, showing no differences between the groups.

(e) Liver function markers were measured in blood after 6 weeks, showing no significant differences between the groups.

Fig. 2. Shaham et al

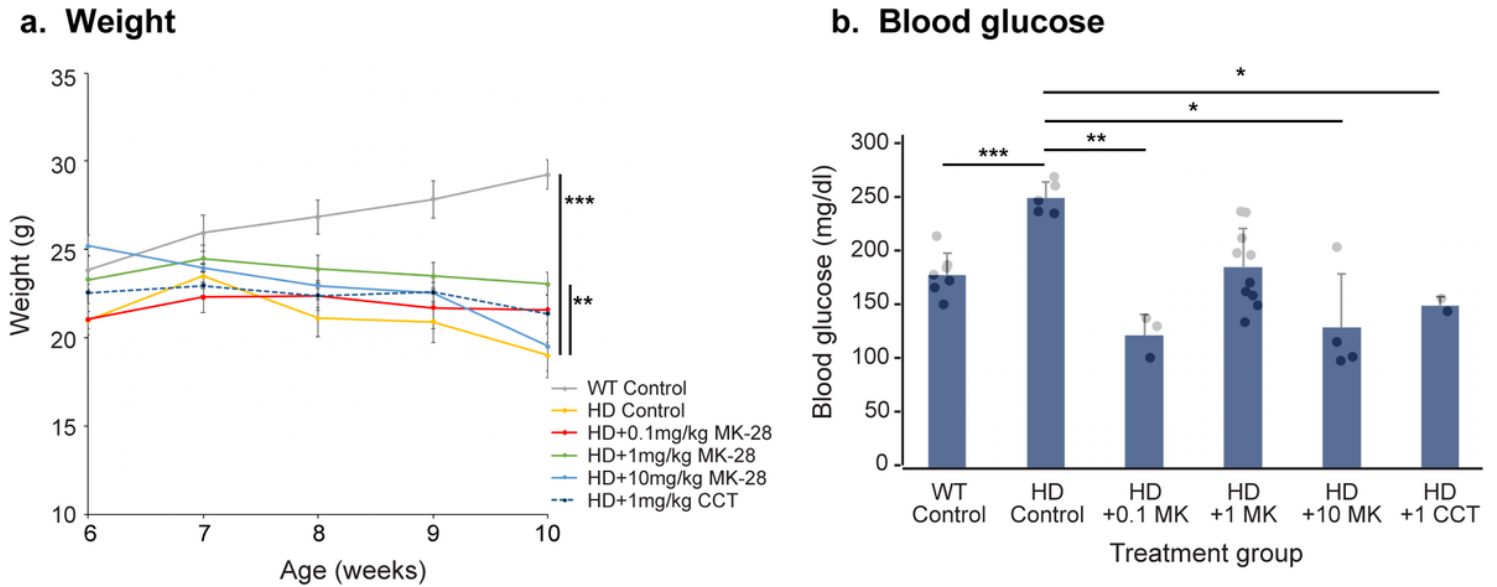


Figure 2

MK-28 improves weight and corrects glucose levels in HD model mice. B6CBA R6/2 HD model mice with expanded CAG repeats (160 +/- 5) in the huntingtin gene were treated from the age of 3 weeks by IP injection 3 times a week with the indicated MK-28 doses compared to CCT020312 (1 mg/kg) or vehicle (HD control). WT littermates injected with vehicle were the control group. (n = 6 WT, 7 HD, 8 HD + 0.1mg/kg MK-28, 11 HD + 1mg/kg MK-28, 4 HD + 10mg/kg MK-28, 2 HD + 1mg/kg CCT020312). (a) The mice were weighed weekly. HD mice lost weight over time, compared to the WT control group. A dose of 1 mg/kg MK-28 treatment reduced the weight loss of HD mice significantly. (b) Blood glucose levels, measured at 8 weeks of age, increased significantly among the HD mice and returned to regular levels, or slightly below, in all the treated groups (doses in mg/kg).

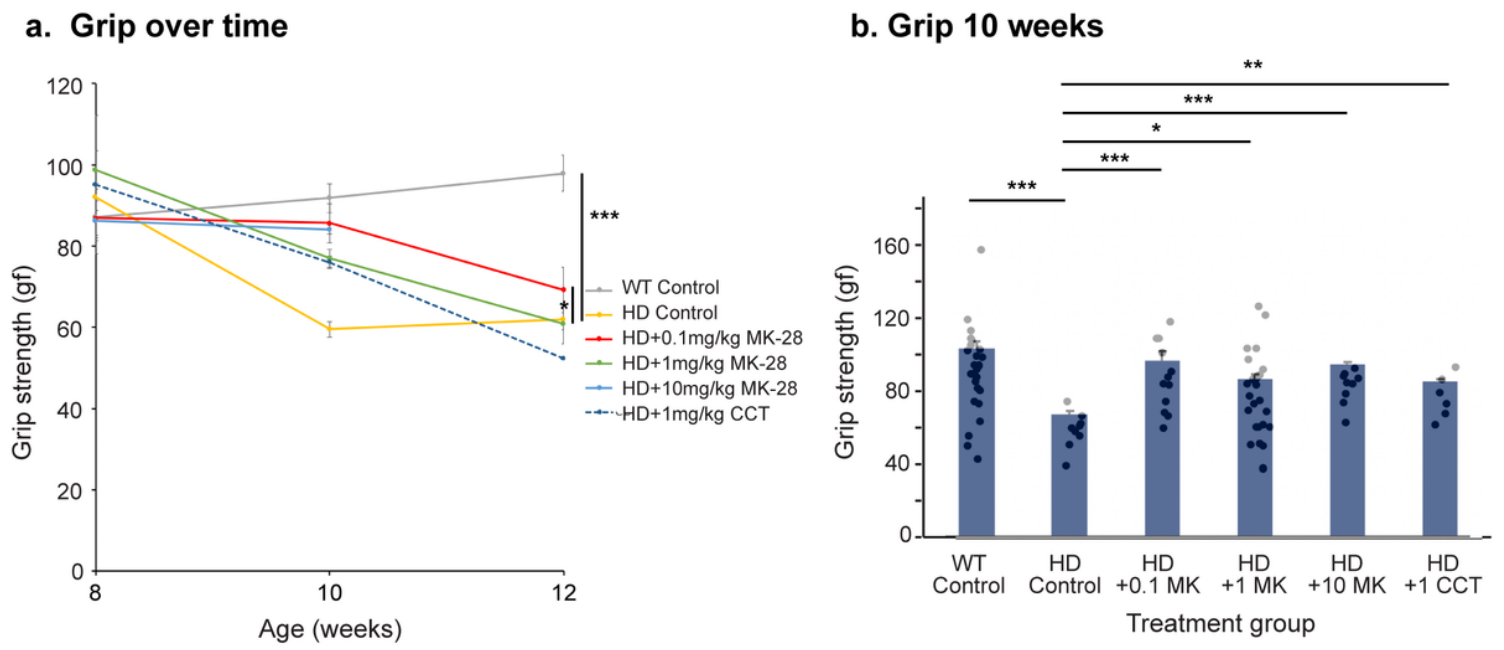


Figure 3

MK-28 improves grip in R6/2 mice. R6/2 mice were treated as in Fig. 2. (a) A grip test was used to test the strength of the front paws. The test showed a significant decrease in paw strength in HD mice compared to controls (best 3 of 5 measurements). Treatments with MK-28 or CCT020312 slowed down the decrease, but at 12 weeks of age the effect remained significant only for MK-28 at a dose of 0.1mg/kg. (n = 9 WT, 5 HD, 8 HD + 0.1mg/kg MK-28, 11 HD + 1mg/kg MK-28, 5 HD + 10mg/kg MK-28, 2 HD + 1mg/kg CCT020312). (b) At the age of 10 weeks, all treated groups showed a significant improvement compared to the untreated HD mice (doses in mg/kg).

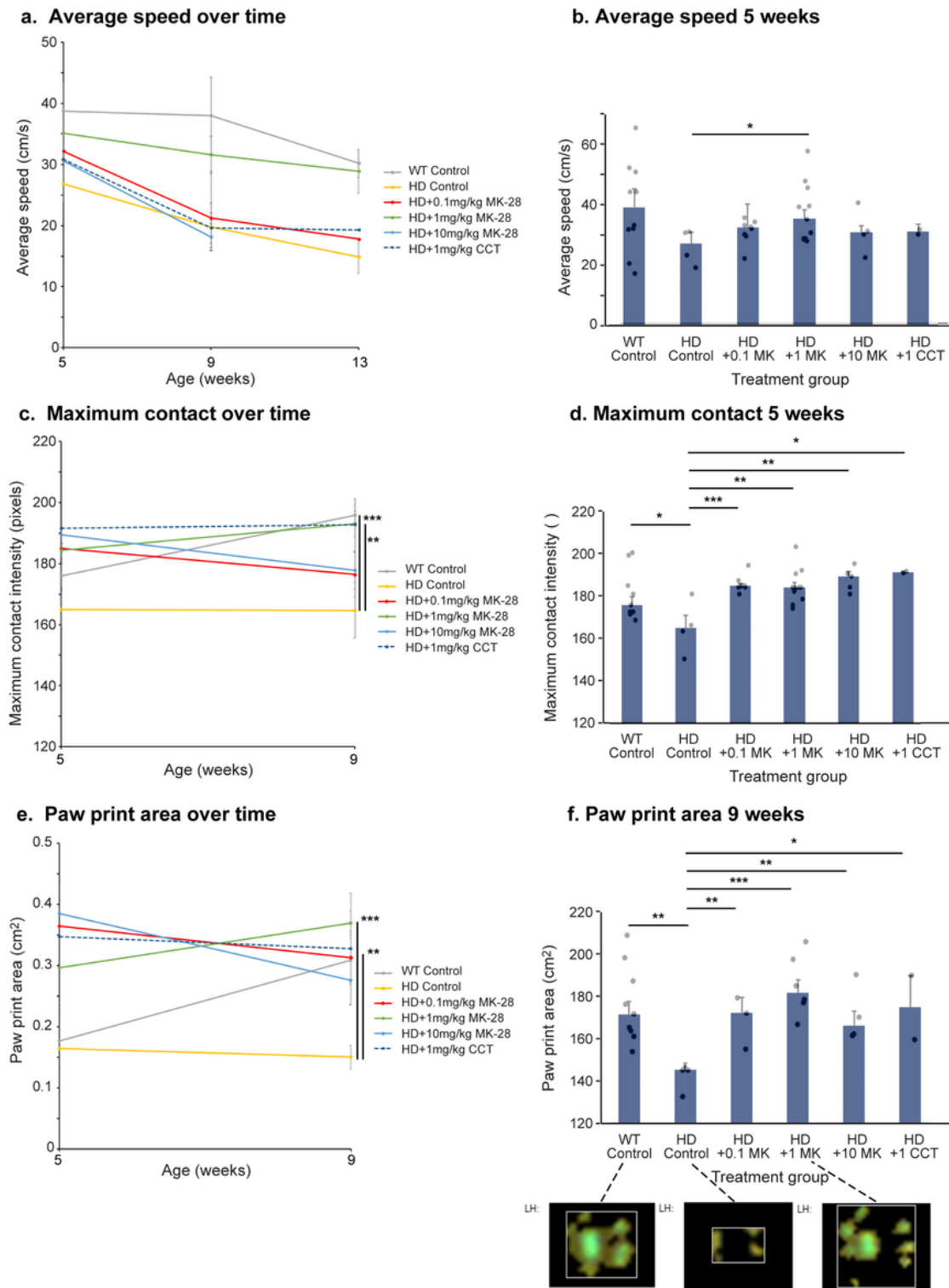


Figure 4

Restoration of motor function by MK-28, as measured by CatWalk parameters in R6/2 mice. R6/2 mice were treated as in Fig. 2 and tested on the CatWalk. (a) Average speed of the mice revealed a strong motor deterioration in HD mice. MK-28 restored the speed considerably at 1mg/kg. (b) The effect of 1mg/kg MK-28 was significant at the age of five weeks, after two weeks of treatment. (c) Maximum paw contact intensity on the walkway, as an average of the 4 paws. HD mice showed a significant reduction

compared to controls. The treatments improved the maximum contact, with 1mg/kg MK-28 being most significant in restoring almost completely to WT levels (d) All treatments caused significant improvement, to levels equal or slightly higher than controls at the age of five weeks. (e) The spreading of the paws on the walkway (paw print area, average of the 4 paws) was strongly reduced in HD mice and did not increase with age. All treatments caused improvement, most significantly with 1mg/kg MK-28, which restored age increase. (f) At the age of 9 weeks all treatment groups showed significant improvement to levels equal or slightly higher than controls. The bottom images illustrate the differences between the left hind paw print areas of WT mice compared to untreated HD mice or treated with 1mg/kg MK-28.

Fig. 5. Shaham et al

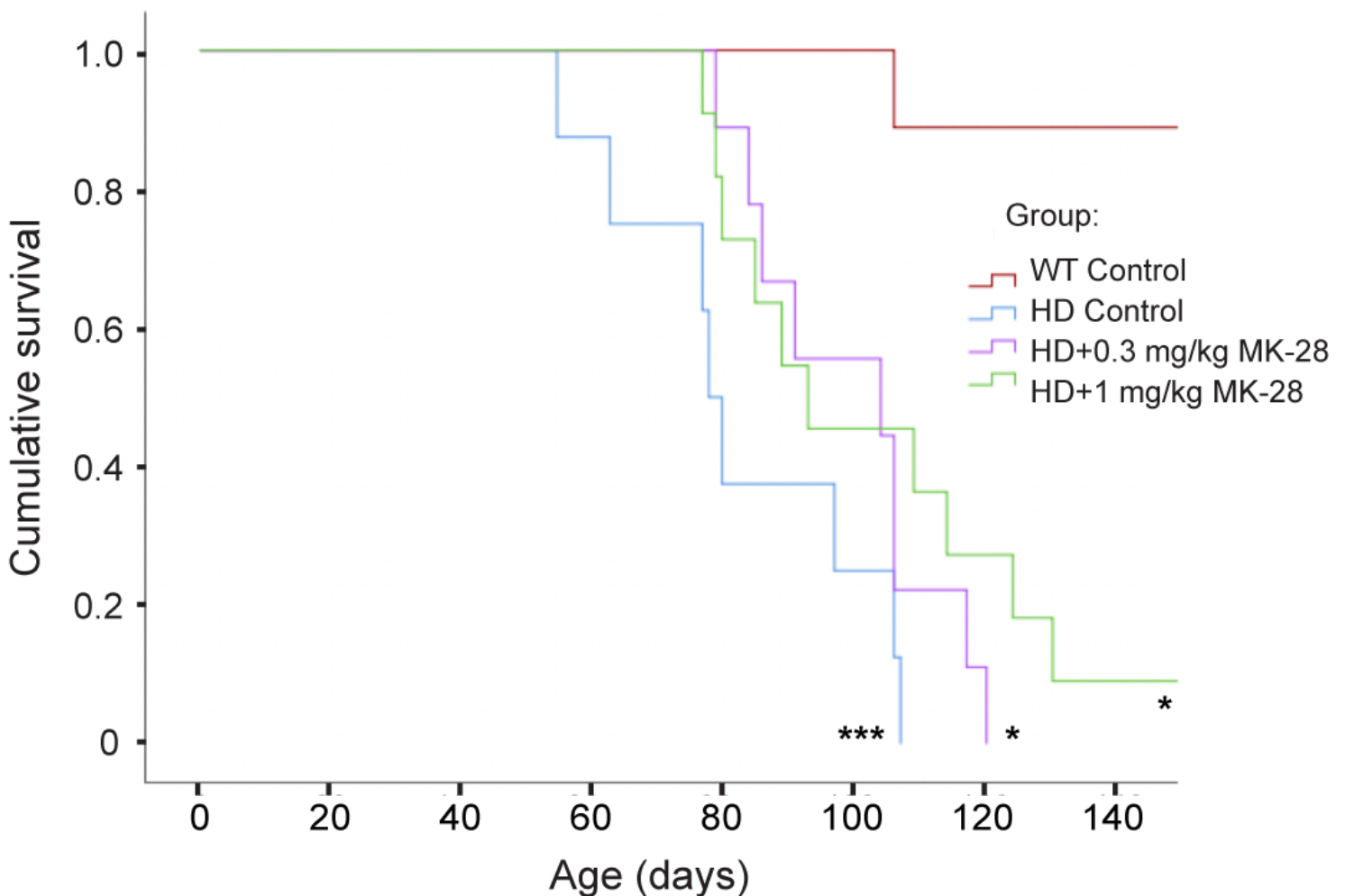


Figure 5

Lifespan extension in MK-28 treated R6/2 mice. B6CBA R6/2 HD model mice with expanded CAG repeats (120 +/- 5) in the huntingtin gene were treated from the age of 3 weeks by IP injection five times a week with 0.3 or 1 mg/kg MK28 or vehicle (HD control). WT littermates injected with vehicle were the WT control. The life expectancy of untreated HD mice is low and increased significantly upon treatment with

MK-28 (n = 9 WT, 8 HD, 9 HD + 0.3mg/kg MK-28, 12 HD + 1mg/kg MK-28). p WT vs HD= 0.00013, HD vs 0.3mg/kg= 0.033, HD vs 1mg/kg= 0.037. (Ttest of ages at death. Breslow-Wilcoxon analysis for comparisons of Kaplan-Meier survival curves also showed significance in Chi-square).

Supplementary Files

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- [VideoS39weeksHDMK1.mp4](#)
- [VideoS413weeksWT.mp4](#)
- [VideoS513weeksHD.mp4](#)
- [VideoS613weeksHDMK1.mp4](#)