

# High-dose 1,25-dihydroxyvitamin D supplementation elongates the lifespan of Huntington's disease transgenic mice

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Huntington's disease is an autosomal dominant progressive neurodegenerative disease, which results in a decreased quality of life and an early death. A high prevalence of vitamin D deficiency was first described in a 2013 study in patients with manifest Huntington's disease, where serum vitamin D level was found to be associated with motor capabilities of the patients. Objectives: Our objective was to investigate the effect of a high-dose vitamin D<sub>3</sub> supplementation on a transgenic mouse model of Huntington's disease. Methods: Our study was performed on N171-82Q Huntington's disease transgenic mice in age- and gender-matched groups. We collected data on the motor state and survival of the mice. Results: The results demonstrate that though vitamin D<sub>3</sub> had no effect on the motor performance of transgenic mice, but significantly increased the lifespan of transgenic animals (Kaplan-Meier survival curves: vehicle-supplemented group: 73 (67–94) days vs. vitamin D<sub>3</sub>-supplemented group: 101 (74–109) days,  $p=0.048$  Mantel-Cox log rank test). Conclusions: Further investigations are needed to determine whether a neuroprotective or a general corroborative effect of vitamin D leads to the measured effect. Our findings support the potential influence of vitamin D deficiency on the disease course and propose that vitamin D may be an effective supplementary treatment to beneficially influence clinical features of Huntington's disease.

Key words: Huntington's disease, vitamin D, N171-82Q transgenic mice

## INTRODUCTION

Huntington's disease (HD) is an autosomal dominantly inherited, progressive neurodegenerative disease, with its initial symptoms presenting mainly during early adulthood. Involuntary choreiform motor symptoms are typical presentations of the disease, accompanied by cognitive and psychiatric symptoms. The primary symptoms are attributable to neuronal dysfunction and death evoked by mutant huntingtin protein (mHtt). The most affected brain region which is responsible for the characteristic movement disorder is the striatum, in particular the medium-sized GABAergic spiny neurons. Disturbances in transcription, translation, protein folding (Trześniewska et al. 2004), energy metabolism, antioxidant defense, and the maintenance of cellular homeostasis cause impaired neuronal function and finally lead to cell death (Szalárdy et al. 2012). Although, the underlying genetic cause and some aspects of the progression are well-known, an effective treatment for HD is still pending (Frank and Jankovic

2010). Symptomatic medications are available for the management of various motor and psychiatric symptoms. Today, decades after the introduction of animal models for HD, a huge database of preclinical studies is available. During this time, numerous drugs have been reported to be beneficial in rodents; however, only tetrabenazine has been concluded to demonstrate consistently positive outcome, though only on chorea (Killoran and Biglan 2014).

In the past years, 1,25-dihydroxyvitamin D<sub>3</sub> (VD<sub>3</sub>) has been shown to be present in the blood in a diminished level in certain neurodegenerative diseases, including Alzheimer's dementia (AD) (Littlejohns et al. 2014) and Parkinson's disease (PD) (Mehanna et al. 2014), often describing decreased bone mass as well in these patients. In addition, the development of these diseases have also been associated with polymorphisms and certain haplotypes of vitamin D receptor (VDR) (Łaczmański et al. 2015, Torok et al. 2013). Furthermore, a negative correlation between serum VD<sub>3</sub> levels and disease severity has repeatedly been reported in PD (Meamar et al. 2015). All these suggest that

VD<sub>3</sub>-deficiency might contribute to the neurodegenerative process in these diseases. Corresponding with findings in AD and PD, a decrease in bone density was described in patients with premanifest HD, suggestive of VD<sub>3</sub> deficiency (Goodman and Barker 2011). More recently, a notably reduced serum level of VD<sub>3</sub> has been reported in advanced HD as a first direct evidence of VD<sub>3</sub> deficiency in the disease, with serum VD<sub>3</sub> levels found to be in positive association with motor capabilities of the patients with HD (Chel et al. 2013). These findings altogether suggest the contribution of VD<sub>3</sub> deficiency/insufficiency in the pathogenesis and/or severity of these neurodegenerative diseases, and propose a potential neuroprotective role of VD<sub>3</sub> supplementation.

VD<sub>3</sub> is discussed to exert neuroprotective effects via VDR, with a number of different mechanisms of action implicated. These proposed mechanisms include the elevation of the antioxidant glutathione levels in neural cells by the upregulation of expression of  $\gamma$ -glutamyl transpeptidase in astroglia (Garcion et al. 1998, 2002); the attenuation of nitric oxide synthesis via the downregulation of inducible nitric oxide synthase (iNOS) (Durzun et al. 2013, Chen et al. 2000, Ciesielska et al. 2003); the upregulation of genes involved in processes counteracting neuroinflammation (Nissou et al. 2014), a feature also characteristic of neurodegenerative disorders; the initiation of histone acetylation and demethylation (Fetahu et al. 2014, Pickholtz et al. 2014), epigenetic modifications widely linked to neuroprotective actions (Ziemka-Nalecz and Zalewska 2014, Zadori et al. 2009, Gardian et al. 2004); as well as the upregulation of neurotrophic factors (Garcion et al. 2002).

These mechanisms of VD<sub>3</sub> may protract the development of symptoms and may take positive effect on the lifespan of patients with neurodegenerative disease, including HD. In line with these proposed protective effects, an increasing number of preclinical evidence suggest the neuroprotective effects of VD<sub>3</sub> supplementation in rodent models of AD (Durk et al. 2014) and PD (Kim et al. 2006, Wang et al. 2001), and promising clinical trials have also been published (Chaves et al. 2014). However, there is a paucity of experimental data on the potential therapeutic effect of VD<sub>3</sub> in HD. Based on the positive results of studies with VD<sub>3</sub> in models of other neurodegenerative diseases, we report the first study assessing the therapeutic potential of VD<sub>3</sub> in an animal model of HD.

## METHODS

### Experimental animals and drug administration

In our experiment, we used N171-82Q, also known as B6C3-Tg(HD82Gln)81Gschi/J HD transgenic mice. This transgenic strain is characterized by a selective neuronal expression of an N-terminal fragment of mutant huntingtin

gene containing exon 1 and 2, including a 82 amino acid-long polyglutamine region. These mice develop a movement disorder, including tremor and hypokinesia, together with intranuclear inclusions, neuritic aggregates, and striatal atrophy at the morphological level (Schilling et al. 1999, Zadori et al. 2011). The decreased life expectancy makes this strain a suitable animal model of the disease. N171-82Q transgenic HD (n=24) and wild-type (WT) (mixed background containing C57BL/6J and C3H/HeJ, referred to as B6C3F1) (n=16) mice were examined longitudinally from 7<sup>th</sup> to 16<sup>th</sup> weeks of age. Groups were matched in age and gender.

The animals were housed in cages under standard conditions with a 12–12-h light–dark cycle and free access to food and water. The experiment was carried out in accordance with the European Communities Council Directive (86/609/EEC) and was approved by the local animal care committee.

Animals of N171-82Q and WT strains were divided into control (WT-vehicle and HD-vehicle) and VD<sub>3</sub>-supplemented (WT-VD<sub>3</sub> and HD-VD<sub>3</sub>) groups (12-12 in the transgenic and 8-8 in the WT group). VD<sub>3</sub>-supplemented mice subcutaneously received cholecalciferol (Vigantol® 50000, Merck KgaA, Darmstadt, Germany) in a dose of 12000 IU/kg (emulsification: 0.5 g Tween 80+8975  $\mu$ l 0.1 M phosphate-buffered saline (PBS)+1025  $\mu$ l Vigantol). Mice in the control groups received vehicle (0.5 g Tween 80+10000  $\mu$ l 0.1 M PBS). Animals were injected 5 times a week after behavioral tests with an average of 120  $\mu$ l of the emulsion.

### Experimental design

Experimental animals were first measured for the native (treatment-free) parameters of their motor capabilities in the rotarod test and open-field test in their age of 7<sup>th</sup> weeks to rule out inherited differences between the randomly generated groups. Treatment was started in the 8<sup>th</sup> week of age of the animals. Motor status of the mice was monitored with rotarod test on a weekly basis, and with open-field test in every second week of the study. Behavior assessments were performed till there was a minimum satisfactory sample size of 5 per group. The treatment was continued until the death of the last mouse from the N171-82Q strain.

### Behavior testing

#### *Open-field test*

Spontaneous locomotor and exploratory activity was investigated with a computer-assisted open-field apparatus (Conducta v2.0, Experimetria Ltd., Budapest, Hungary).

Mice were put into the measure box for 15-min tracking sessions. We used a light intensity of a standard 2 lux for the tracking sessions, measured inside the apparatus. Motimetry tests were repeated on every 14<sup>th</sup> day.

#### Rotarod tests

Motor coordination ability of the animals was examined on a computer-assisted rotarod apparatus (TSE RotaRod Advanced, TSE Systems GmbH, Bad Homburg, Germany) on every 7<sup>th</sup> day. The apparatus is supplied with rods of 30 mm in diameter. Mice were trained on the rotarod for 2 days before the first examination day. Each training day consisted of 3 sessions of a 5-min train. Rotation speeds of 5 rpm (rotation per minute) and 10 rpm were applied on training day #1 and #2, respectively. The examination was performed with an accelerating speed profile gradually increasing from 1 rpm to 30 rpm during the 5-min period. Each animal was examined 3 times on the days of examination with a 30 min resting interval. The latency to fall values were registered and their averages were used for subsequent analysis. Repeated examinations on the subsequent weeks were carried out with the same accelerating speed profile as detailed before. On the days preceding each examination day, reminder trainings were applied, consisting of two 5-min sessions with a speed set to a constant 10 rpm.

#### Collection of data of survival

The mice were euthanized (using Forane, 5%) when severe hypokinetic state was established. Animals were

considered to attain this state when they did not manifest motor reaction when towed by their tails.

#### Statistical analysis

Datasets showing Gaussian distribution (tested with Shapiro-Wilk test) were compared with one-way ANOVA. For the assessment of between-group difference, parametric (Bonferroni *post hoc* test) or non-parametric (Mann-Whitney U test) tests were applied according to the distributions of the datasets. (Variances were equal in all cases, as tested with Levene's test.) Analysis of survival was performed with Mantel-Cox test for the comparison of Kaplan-Meier survival curves. Data are presented in mean  $\pm$  standard deviation and median (interquartile range) in case of normally and non-normally distributed parameters, respectively. A p value <0.5 was regarded as statistically significant.

## RESULTS

#### Open-field tests

The pre-treatment native analysis of ambulatory parameters revealed no significant differences between the *a priori* randomized groups in any of the examined parameters, enabling valid further investigations (data not shown). The waning capability for spontaneous locomotion in the N171-82Q strain was detectable as early as week 11 for both the distance travelled (WT-vehicle: 3721.9 $\pm$ 849.8 cm vs. HD-vehicle: 2099.8 $\pm$ 1249.3 cm,  $p=0.025$  Bonferroni *post*

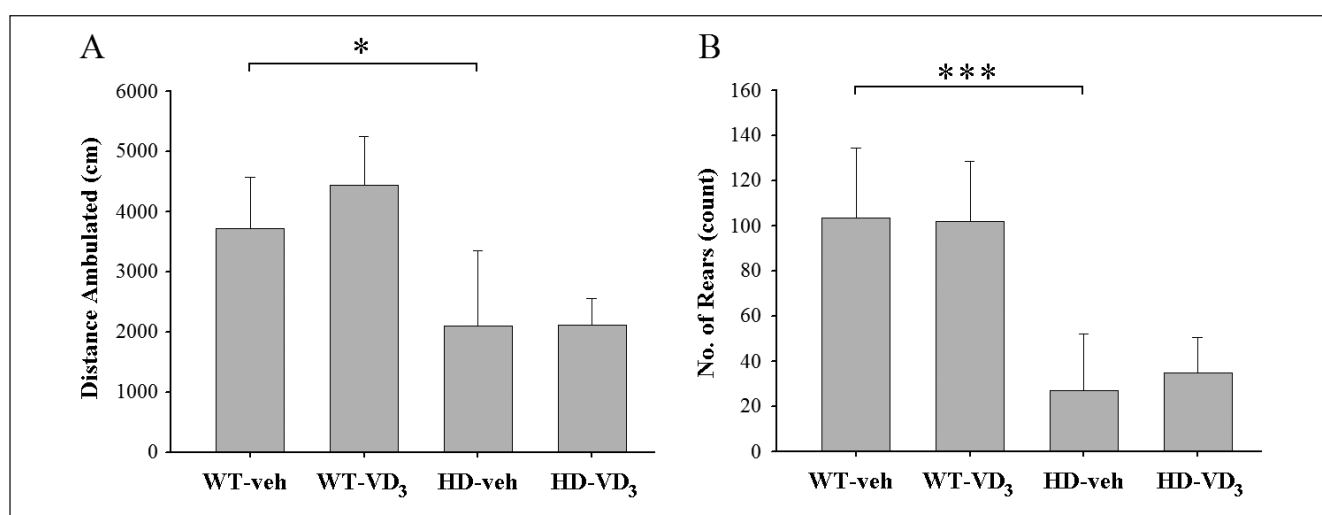


Fig. 1. Representative image of the A total distance ambulated in the open-field and the total number of the rears B at week 13. A significant decrease in the ambulated distance was shown in vehicle-treated HD mice. A robust difference was measured in the number of the rears among WT and HD mice treated with vehicle. HD mice treated with VD<sub>3</sub> did not show alteration in ambulated distance or in the number of rears compared to vehicle-treated HD group. VD<sub>3</sub> treatment had no adverse effect observable in WT mice.

*hoc* test) and the number of rears (WT-vehicle: 103.2±31.1 vs. HD-vehicle: 27.0±25.1,  $p < 0.001$  Bonferroni *post hoc* test), corresponding with prior observations (Chiu et al. 2011). However, there were no statistically significant improvements found in either the spontaneous locomotor activity (HD-vehicle: 2099.8±1249.3 cm vs. HD-VD<sub>3</sub>: 2111.2±449.0 cm,  $p > 0.05$  Bonferroni *post hoc* test) or the total number of rears (HD-vehicle: 27.0±25.1 vs. HD-VD<sub>3</sub>: 34.7±15.8,  $p > 0.05$  Bonferroni *post hoc* test) of the mice supplemented with VD<sub>3</sub> compared to untreated animals throughout the study (Fig. 1).

### Rotarod tests

A trend towards a decreased performance on the rotarod became apparent in HD mice in accordance with, but earlier than the development of the decreased locomotion and exploration (as measured on week 11; WT-vehicle: 149.1±51.9 sec vs. HD-vehicle: 130.2±42.0 sec); however, this did not reach statistical significance in the analyses of variance at any timepoints thereafter ( $p > 0.05$  at each time point; Bonferroni *post hoc* test). No tendentious difference was detected between VD<sub>3</sub>-supplemented and vehicle-treated groups in either of the strains ( $p > 0.05$  at each time point; Bonferroni *post hoc* test).

### Analysis of survival

VD<sub>3</sub> treatment remarkably increased the median lifespan within the HD transgenic group supplemented with VD<sub>3</sub> compared to the vehicle-treated HD group [HD-veh: 73 (67–94) days vs. HD-VD<sub>3</sub>: 101 (74–109) days], which was in accordance with a significant difference in the Kaplan-Meier survival analysis ( $p = 0.048$  Mantel-Cox log rank test; Fig. 2).

## DISCUSSION

In this study, we investigated the effects of high-dose systemic VD<sub>3</sub> supplementation on life expectancy and motor performance of N171-82Q transgenic HD mice. The analysis of survival found a statistically significant 38% increase of the median lifespan of VD<sub>3</sub>-treated HD mice compared to vehicle-treated HD controls. This is a similar magnitude of effect on life expectancy as reported by prior studies on HD transgenic mice with certain potentially neuroprotective agents or combinations, such as valproate (31.4%) (Zadori et al. 2009), the combination of lithium and valproate (31%) (Chiu et al. 2011), the combination of remacemide and coenzyme Q10 (31.8% in R62 HD transgenic strain and 17% in N171-82Q strain)

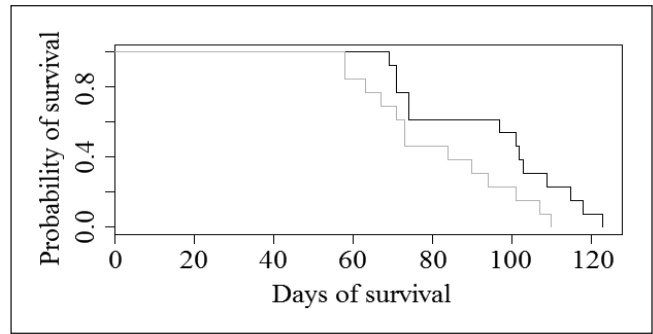


Fig. 2. The Kaplan-Meier survival curves of HD mice treated with VD<sub>3</sub> and vehicle. A significant increase in the days of survival was shown in the VD<sub>3</sub>-treated group compared to the control HD mice [HD-vehicle: 73 (67–94) days vs. HD-VD<sub>3</sub>: 101 (74–109) days,  $p = 0.048$  Mantel-Cox log rank test].

(Ferrante et al. 2002), and a kynurenic acid analogue (30.7%) (Zadori et al. 2011). Being the first in this field, the authors suggest the potential neuroprotective effect of VD<sub>3</sub> in an HD model. This concept seems feasible based on the previously reported proposed protective actions of VD<sub>3</sub> against oxidative injury and neuroinflammation, in part via inhibiting the synthesis of iNOS, though the contribution of VDR-linked neurotrophic effects as well as epigenetic modifications cannot be excluded. As regards the potential role of iNOS downregulation, although the role of iNOS-mediated oxidative injury has been implicated in the pathogenesis of a number of neurodegenerative disorders (Durzun et al. 2013, Chen et al. 2000, Ciesielska et al. 2003) and its activity has been found markedly increased in neurotoxin models of HD (Schmidt et al. 1995, Pérez-De La Cruz et al. 2005, Schulz et al. 1995, Milstien et al. 1994, DiCiero Miranda et al. 2000), its level has been consistently found unaltered or even slightly decreased in the striatum of transgenic HD mice by independent groups (Deckel et al. 2001, 2002, Pérez-Severiano et al. 2002), leaving this issue open for further discussion.

In contrast with such a marked beneficial effect of VD<sub>3</sub> on survival, the treatment did not significantly improve the locomotor performance of HD mice in the open-field. Though by the time rotarod performance started to markedly decrease the serial occurrence of death events among transgenic animals resulted in a gradual loss of statistical power to show significant difference, the apparent trend to exert a decreased performance did not seem to be affected by VD<sub>3</sub> treatment either. This conflict may arise from an impaired physiological profile of the skeletal muscle of N171-82Q mice exhibiting a decreased ability to form type-I (fatigue resistant) muscle fibers, most probably due to a diminished expression of peroxisome proliferator-activated receptor  $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ) protein, a key player in mitochondrial biogenesis and energy metabolism (Chaturvedi et al. 2009). Notably, agents providing similar improvement in survival but no overt effect on

motor performance in HD transgenic mice has been described earlier in the literature (Gardian et al. 2005). Nevertheless, the findings that long-term high-dose VD<sub>3</sub> treatment did not induce motor deficits in the mice confirms the safety of this therapy, with most probably no physiologically significant effect on serum calcium levels, similarly to that had been previously described in mice (Dusad et al. 2015). On interpreting the results, while it is tempting speculate a neuroprotective effect underlying the observed increase in survival, we could not exclude a general corroborative effect of VD<sub>3</sub> supplementation underlying this increase, especially in light of the reports of decreased VD<sub>3</sub> serum levels in HD patients. Further investigations are needed to permit conclusions on this issue.

## CONCLUSIONS

Long-term systemic VD<sub>3</sub> supplementation lead to a remarkable increase in the survival of N171-82Q transgenic HD mice. This is in line with prior reports on beneficial effects of VD<sub>3</sub> treatment in experimental models of other neurodegenerative diseases, as well as with the insufficient levels of VD<sub>3</sub> in HD patients showing a negative correlation with disease severity. While further investigations are needed to determine whether neuroprotective or a general corroborative effect of VD<sub>3</sub> leads to such an elongation of the lifespan of HD transgenic mice, the results supports the safety and the therapeutic potential of VD<sub>3</sub> as a supplementary treatment for HD patients, especially for those with established VD<sub>3</sub> deficiency.

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