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Different Forms of Huntingtin in the Most Affected Organs; Brain and Testes of Transgenic Minipigs

Různé formy huntingtinu v nejvíce postižených orgánech; mozku a varlatech transgenních miniprasat

Abstract

Huntington's disease (HD) is a neurodegenerative disorder caused by the the elongation of CAG triplet repeat in the gene encoding the huntingtin protein (Htt). In patients, in addition to the monomeric form of huntingtin, N-terminal fragments, oligomers, and polymers are present mostly in the affected tissues, even though the mutated huntingtin (mtHtt) is expressed basically in all cells. The most affected tissues are basal ganglia and cerebral cortex. In this study we analyzed the presence of N-terminal fragments and oligomers of Htt in different tissues of 24 and 36 months old experimental animals. This was done in our large animal model of HD, which uses transgenic (TgHD) minipigs expressing N-terminal part of human mtHtt. Among all the tissues tested, we found cortex and testes to contain N-terminal fragments as well as oligomeric smears in TgHD minipigs compared to wild type siblings. On the other hand, we did not detect any fragments or oligomers in muscle and heart of TgHD minipigs, only starting fragmentation in muscles of 36 months old animals. These findings mimic the early progression of the disease in humans, hence presents minipig as a promising model for therapeutic testing of HD.

Souhrn

Huntingtonova nemoc (HD) je neurodegenerativní porucha způsobená elongací CAG repetic v genu kódující protein huntingtin (Htt). U pacientů jsou v postižených tkáních přítomny vedle monomerní formy hlavně N-koncové fragmenty, oligomery a polymery mutovaného huntingtinu (mtHtt), oproti tomu samotná monomerní forma mtHtt je exprimována v podstatě ve všech buňkách. Nejvíce postižené tkáně jsou bazální ganglia a mozková kůra. V této studii jsme analyzovali přítomnost N-koncových fragmentů a oligomerů Htt v různých tkáních 24- a 36měsíčních transgenních (TgHD) miniprasat exprimujících N-koncovou část lidského mutovaného huntingtinu a jejich zdravých sourozenců. Zjistili jsme, že mozková kůra a varlata na rozdíl od svalu a srdce TgHD miniprasat obsahují kromě monomerní formy i N-koncové fragmenty a oligomerní smíry. Ve svalech z 36 měsíčních TgHD miniprasat však již začíná mírná fragmentace. Tato zjištění napodobují časnou progresi onemocnění u lidí, a proto miniprase poskytuje slibný model pro terapeutické testování HD.

The authors declare they have no potential conflicts of interest concerning drugs, products, or services used in the study.
Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.
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Key words

Huntington´s disease – transgenic minipig model – mutant huntingtin – protein fragments – oligomeric structures

Klíčová slova

Huntingtonova nemoc – transgenní miniprasečí model – mutovaný huntingtin – proteinové fragmenty – oligomerní struktury

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Aim of the study

Huntington's disease (HD) is a fatal neurodegenerative disorder caused by the elongation of polyglutamine stretch (lenght > 40) encoded by CAG triplets in the huntingtin protein (Htt). Although the mutant huntingtin (mtHtt) is expressed in virtually every cell type, the neurons of cortex and basal ganglia are most affected [1]. Even though the pathogenesis of the disease is not fully understood, the presence of large inclusion bodies, or aggregates, is tightly correlated with the progression of HD [2]. Nevertheless, the precise role of the aggregates in pathogenesis of HD is still not clear. While aggregates have been linked to cell death [2,3], other studies find cells die without ever forming aggregates, and suggest their protective role against the effects of mtHtt [4]. On the contrary, smaller soluble forms of mtHtt and huntingtin oligomers were described to be toxic to the cells and to be the key factors of cellular dysfunction [5–7].

In order to facilitate the studies of pathogenesis and therapy of HD, we have generated a unique transgenic (TgHD) minipig model using microinjection of a lentiviral vector encoding N-terminal (548 amino acids) part of human huntingtin containing 126 CAG/CAA repeats under the control of the human Htt promoter [8]. The first TgHD minipig was born in July 2009. Afterwards, four successive generations of TgHD minipigs were born up to now.

The aim of our study is to follow the disease development in transgenic minipigs by comparing WT and TgHD siblings. Here we focused on the formation of fragments and oligomers of Htt in different tissues of 24 and 36 months old TgHD minipigs.

Methods

Animals

All experimental procedures were carried out in strict accordance with the Czech legislation and approved by the Animal Ethics Committee (#003/2012) in Prague, Czech Republic. We utilized a novel TgHD minipig model developed in our institute as described by Baxa et al. [8]. The TgHD minipigs bear one copy of N-terminal 548 amino acid sequence of human mtHtt with expanded tract of 124 glutamines and as a large animal disease model are suitable for studies of HD. R6/2 HD mice model carrying N-terminal region of the human mutant Htt with 150 CAG repeats were used for comparison. Tissues from dead animals were put into eppendorf tubes, snap frozen in liquid nitrogen, and stored at –80°C.

SDS-PAGE and Western blot

Tissues were homogenized in liquid nitrogen using a mortar. The prepared tissue samples were lysed in RIPA buffer (Radio Immuno Precipitation Assay Buffer; 150 mm NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mm Tris-HCl pH 8, inhibitors of phosphatases and proteases), vortexed for at least 30 min at 4°C, then sonicated for 10 min and centrifuged at 10,000 g for 10 min at 4°C. Samples were loaded onto 3–8% Tris-Acetate gel (Thermo Fisher Scientific Inc., #EA03755) and run at 150 V. Gel was transferred onto nitrocellulose membrane (Thermo Fisher Scientific

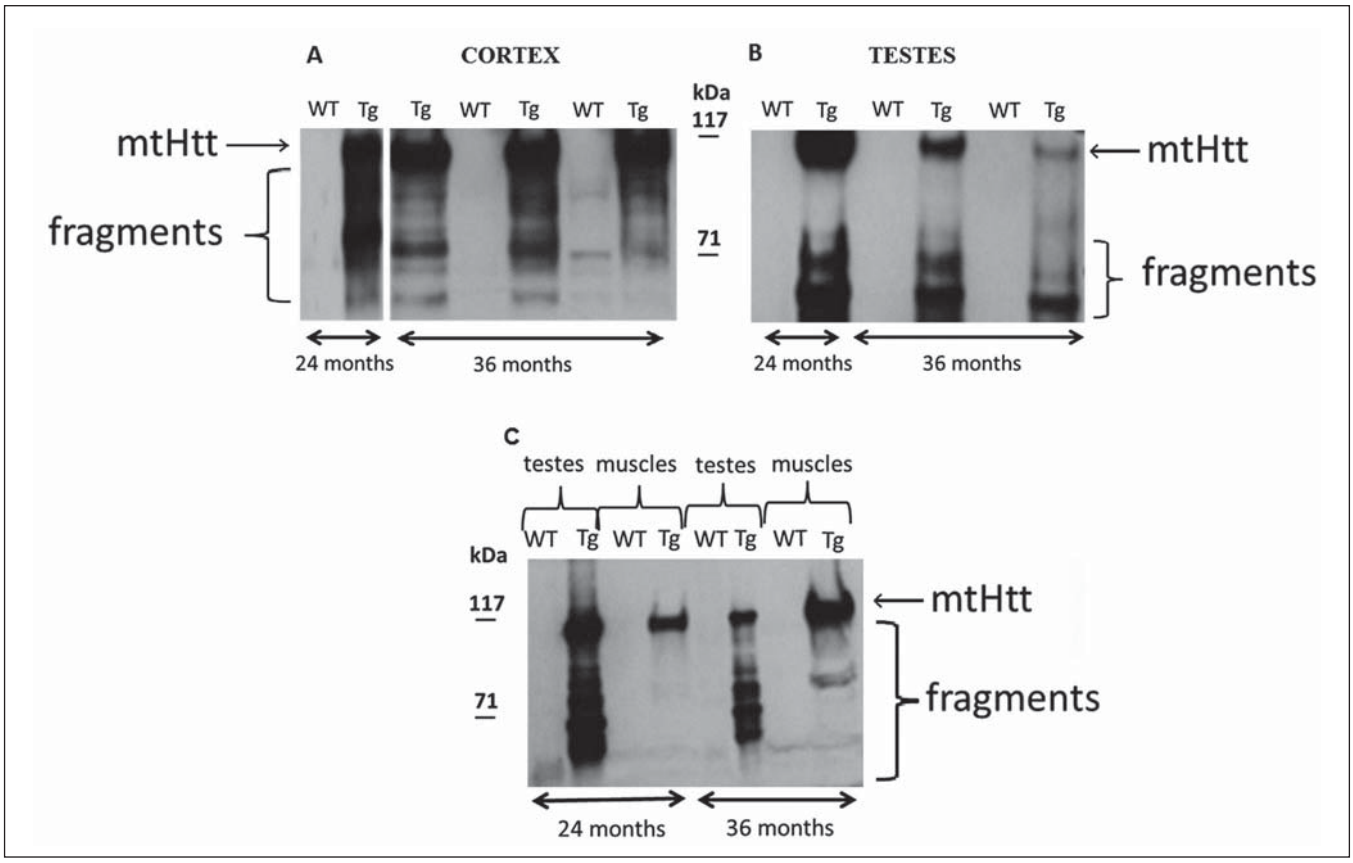

Fig. 1. Fragmented mtHtt detected in 24 and 36 months old TgHD minipigs compared to their WT siblings in different tissues by polyQ 3B5H10 antibody.

Western blot analysis shows fragmented forms of mtHtt (A), cortex (B), testes of TgHD minipigs. Comparing to muscles of TgHD minipigs, testes show increased amount of fragmented mtHtt of 24 as well as 36 months old minipigs (C).

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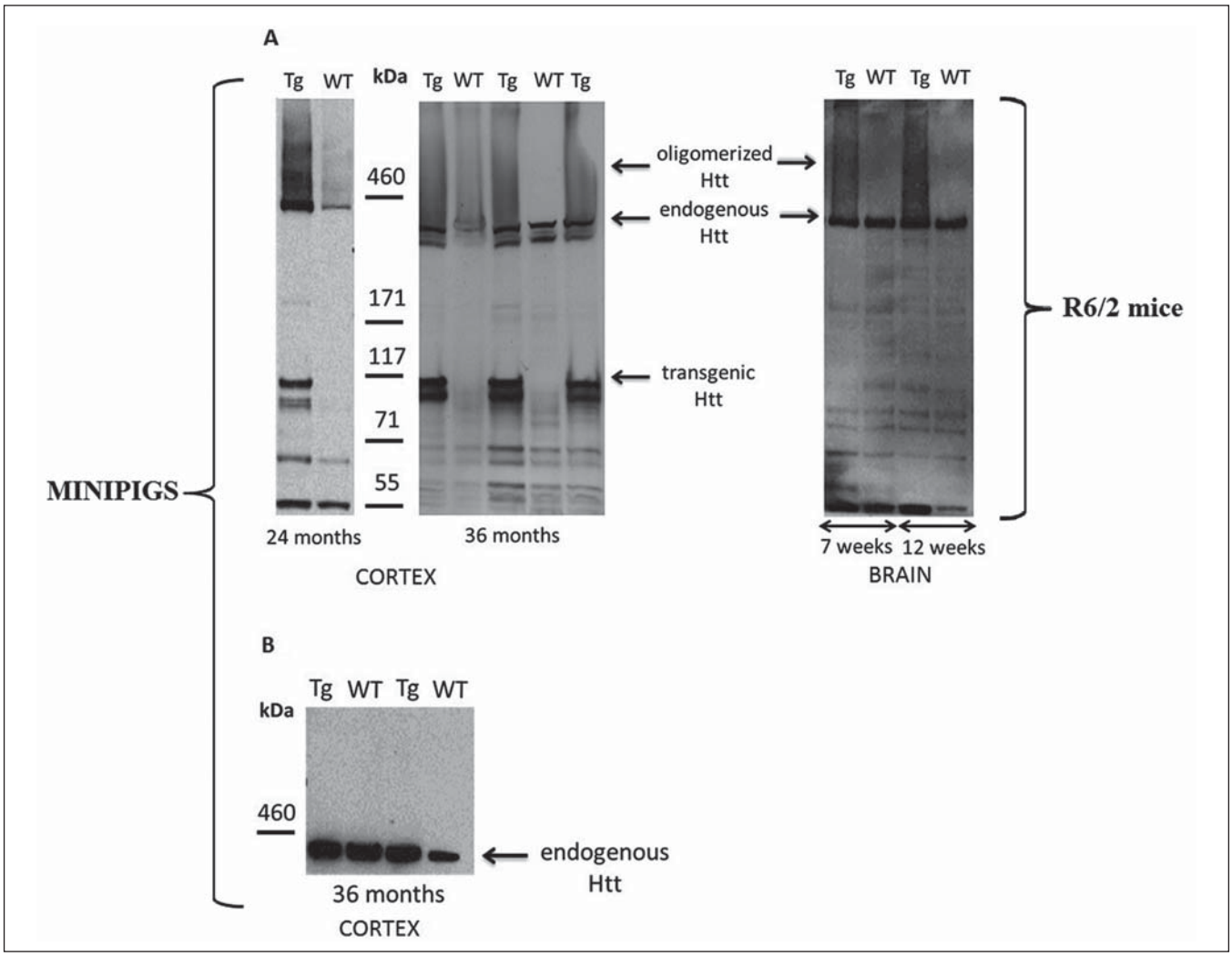

Fig. 2. The presence of oligomeric structures in cortex of 24 and 36 months old TgHD minipigs (A).

Oligomeric structures in TgHD minipigs cortex samples detected by EPR5526 anti-N-terminal Htt antibody by smears in higher molecular weights and comparison with R6/2 mice brain samples. Cortex of 36 months old minipigs reveals no smears after using (H-300) C-terminal Htt antibody (B).

Inc., #IB301001) at 250 mA for 45 min. Membranes were blocked in 5% skimmed milk for 1 hour, and probed overnight with appropriate antibody. For our experiments we used anti-HTT antibody (EPR5526, Abcam, 1 : 3,000), polyQ-antibody (3B5H10, Sigma Aldrich, 1 : 3,000), ((H-300) antibody, Santa Cruz Biotechnologies, 1 : 200) and 1C2 antibody. Secondary antibody conjugated with HRP (anti-mouse, Jackson ImmunoResearch #711–035–152, 1 : 10,000 or anti-rabbit, Jackson ImmunoResearch #711–035–152, 1 : 10,000) was used. Light reaction was induced by ECL (GE Healthcare #RPN2232) and signal was captured on CL-Xposure films (Thermo Scientific P43#34091). The exposed CL-XPosure films (Thermo Scientific, Rockford, IL, USA) were scanned using a calibrated densitometer GS-800 (Bio-Rad, Hercules, CA, USA) and bands were quantified using Quantity One software (Bio-Rad, Hercules, CA) measuring trace quantity.

Results

Previously we showed sperm and testicular degeneration as a result of the presence of mtHtt protein in the testes of TgHD minipig boars [9]. Here we focused on the presence of mtHtt fragments and oligomeric structures in 24 and 36 months old TgHD minipigs, not just in testes, but also in others affected tissues, such as brain and muscles.

Using antibody (3B5H10) directed against N-terminal fragment of human Htt (171 amino acids containing 65 glutamins), we found fragmented forms of mtHtt, which were reported to cause cellular toxicity, in cortex and testes of TgHD minipigs (Fig. 1) [10]. Light bands in WT sample might be due to a cross reactivity of the antibody with other polyglutamine proteins. Interestingly, muscles from 24 months old animals did not show any fragmented mtHtt, and muscles form 36 months old animals showed just minor fragmentation compared to the mtHtt fragments in testes (Fig. 1C).

Moreover, anti-Htt antibody (EPR5526), detected next to the endogenous and mtHtt also smears in higher molecular weights in cortexes of TgHD, but not in WT minipigs (Fig. 2). We detected the same smears in the brains of R6/2 (N-terminal HD transgenic mice), but not in their wild type siblings (Fig. 2A). In some WT samples we detected

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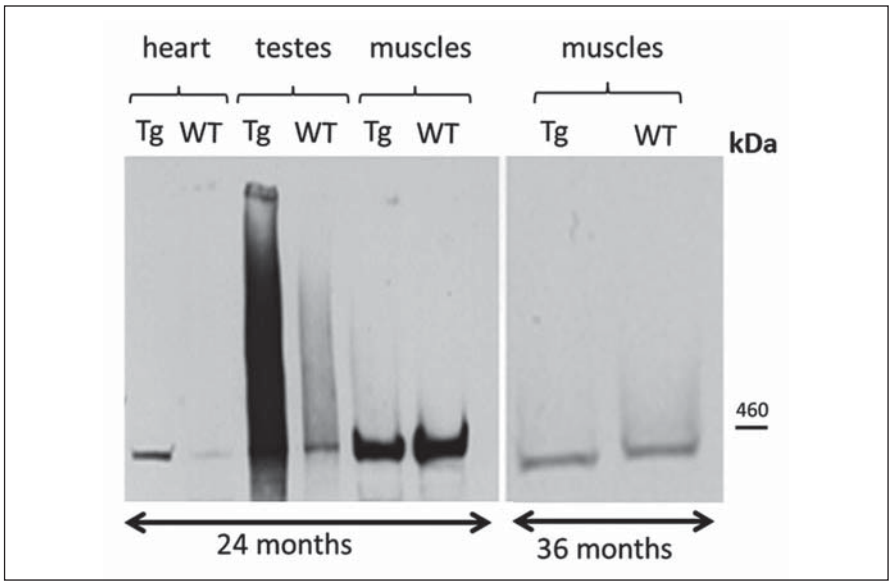

Fig. 3. Detection of oligomeric structures in TgHD minipigs compared to WT in additional tissues.

Anti-N-terminal Htt antibody EPR5526 shows oligomeric structures by smear in higher molecular weights also in testes of TgHD minipigs, but not in heart or muscles.

also a light smear, but not comparable to the smear of TgHD animals. This might be due to the fact that non mutated Htt containing around 14 CAG can also form some oligomers in specific tissues, but not in such extend. Since the C-terminal Htt antibody revealed no smears (Fig. 2B), we suppose the smear represents N-terminal oligomeric structures of mtHtt.

Anti-N-terminal Htt antibody EPR5526 was also used for detection of oligomeric structures in additional tissues, such as heart, testes, and muscle. Surprisingly, aside from cortex, the oligomeric mtHtt structures were found also in testes, but not in heart or muscle (Fig. 3). This finding is in accordance with the presence of fragmented mtHtt, which we found also in cortex and testes, but not in other tissues analyzed. However, we detected endogenous Htt as well as mtHtt in all tested tissues (Fig. 3).

Conclusion

In this study, we show the presence of fragmented and oligomerized forms of mtHtt, in addition to the intact monomeric form, in cortex and testes of TgHD minipigs at the age of two and three years. Furthermore, at this age, we did not detect these forms in other tissues tested. These findings are consistent with the progression of HD in human patients, where the most affected tissues are brain, but also testes [11]. The pathological findings in testes received much less attention, since the clinical manifestation of HD is after the reproductive period, mostly in the mid-thirties. Interestingly, among all organs, the testes display the most comparable gene expression pattern to the brain [12]. In patients, the expanded Htt is also found rather in the form of N-terminal fragments, oligomers and polymers then in monomeric form in the affected areas of the brain [13]. Therefore our finding in TgHD minipigs recapitulates the progression of HD in humans, and thus gives a stronger argument for using the minipig model for preclinical testing of HD therapeutics.

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