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ADDENDUM

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The calpain-suppressing effects of olesoxime in Huntington's disease

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ABSTRACT

Olesoxime, a small molecule drug candidate, has recently attracted attention due to its significant beneficial effects in models of several neurodegenerative disorders including Huntington's disease. Olesoxime's neuroprotective effects have been assumed to be conveyed through a direct, positive influence on mitochondrial function. In a long-term treatment study in BACHD rats, the latest rat model of Huntington's disease, olesoxime revealed a positive influence on mitochondrial function and improved specific behavioral and neuropathological phenotypes. Moreover, a novel target of the compound was discovered, as olesoxime was found to suppress the activation of the calpain proteolytic system, a major contributor to the cleavage of the disease-causing mutant huntingtin protein into toxic fragments, and key player in degenerative processes in general. Results from a second model of Huntington's disease, the *Hdh*^{Q111} knock-in mouse, confirm olesoxime's calpain-suppressing effects and support the therapeutic value of olesoxime for Huntington's disease and other disorders involving calpain overactivation.

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The therapeutic compound olesoxime

Olesoxime is a cholesterol derivative, which was discovered in a small-molecule screen for neuroprotective compounds at Trophos SA, Marseille.[1] In the initial screening, primary motor neurons were deprived of serum, incubated in the presence or absence of olesoxime, and survival was monitored.[1] The screen revealed highly beneficial effects of the compound. Further assays on neurons subjected to other pathological stimuli all showed increased survival.[2]

Binding and localization analysis revealed that the compound concentrates at the site of mitochondria[2] and interacts with the voltage-dependent anion channel (VDAC)[3] as well as the translocator protein (TSPO)[4] on the outer mitochondrial membrane.[1] As these proteins are considered to play a role in mitochondrial permeability transition (mPT),[5] it was proposed that olesoxime's mechanism of neuroprotection was the inhibition of mPT and subsequent cell death.[1] In accordance with this idea, olesoxime was found to prevent cell death induced by various toxins[1,2,6] to a similar magnitude as cyclosporine A, a potent mPT inhibitor. Further studies discovered a variety of interesting aspects around olesoxime's molecular effects, but the exact mechanism of cytoprotection remained elusive. Olesoxime was reported to prevent cytochrome *c* release from mitochondria and the subsequent activation of caspases in the cytosol.[7] These effects did not seem to derive from improved mitochondrial Ca^{2+} handling or inhibition of Ca^{2+} release.[2] Olesoxime further did not directly inhibit caspases, activate prosurvival kinases or modulate the transcription of proapoptotic genes via the transcriptional regulator p53.[7] Besides the direct effect on the initiation of mitochondria-mediated apoptosis, olesoxime was found to promote microtubule-based neurite outgrowth and microtubule-dependent mitochondrial transport, specifically in differentiated neuronal cells,[8] and to improve oligodendrocyte-mediated remyelination.[9]

In vivo studies demonstrated consistently beneficial outcomes regarding neuroprotection and regeneration in animal models of motor neuron diseases[1,2,10] as well as neurodegenerative disorders,[2,9,11] and neuroprotective

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and antinociceptive effects in models of peripheral neuropathies.[8,12-14]

The therapeutic potential of olesoxime was further investigated in clinical trials on motor neuron diseases. Olesoxime showed excellent safety and tolerability in the patients.[2] Moreover, the treatment was easy to apply, as olesoxime reaches the brain in sufficient amount upon oral administration of one daily dose,[15] and therefore medication does not involve invasive or time-consuming procedures. Two trials, one on survival in end-stage amyotrophic lateral sclerosis (ALS) patients[15] and one on motor symptom onset in early stage spinal muscular atrophy (SMA) patients[16] were conducted. The ALS trial revealed a negative outcome, while the trial on SMA patients showed striking beneficial effects on the clinical end point, suggesting better efficacy of olesoxime in early phases of disease.

As *in vitro* data pointed to positive effects of cholesterol-oximes in Huntington's disease (HD),[17] and as mitochondrial dysfunction is considered to contribute substantially to the HD pathology,[18] it was considered to be of significant interest to investigate the potential therapeutic effects of olesoxime in an *in vivo* model of this disorder.

Main effects of olesoxime in the BACHD rat model of HD

BACHD rats were treated with olesoxime, supplied via the food, from the age of 5 weeks until the age of 13 months. The rats' behavior as well as their neuropathological phenotypes were investigated.[19]

Olesoxime-treated BACHD rats showed specific improvements in behavior and neuropathology compared to placebo-treated BACHD rats. These concerned the selective amelioration of cognitive and psychiatric abnormalities, increased cortical thickness and a significant reduction in the aggregation and nuclear accumulation of the disease-causing mutant huntingtin (HTT) protein throughout the brain. The drug effect appeared to be the result of reduced calpain-mediated proteolysis, which was found to be the main source of mutant and wild type HTT fragments in the BACHD rat. Furthermore, olesoxime seemed to have influenced mitochondria in several ways: Olesoxime treatment led to a restoration of respiratory function, reduced the association of HTT fragments with mitochondria and modulated the levels of several mitochondrial proteins involved in mitochondrial cholesterol import, metabolite exchange, respiratory chain function, Ca^{2+} homeostasis, calpain activation and mitochondrial dynamics. Thus, it was hypothesized that the compound had exerted its beneficial effects by improving mitochondrial function, thereby stabilizing Ca^{2+} homeostasis, decreasing Ca^{2+}-related calpain activation and aborting the generation of toxic HTT fragments, as well as their exaggerated negative influence on mitochondria.

Validation of olesoxime's effects in other models of HD

The beneficial effects of olesoxime in HD are not restricted to the BACHD rat model. Several analyses have been performed, or are ongoing, in the Hdh^{Q111} knock-in mouse[20] as well as in STHdh^{Q111} cells, a stable cell line derived from striatal progenitor cells of this mouse model[21] (Fig. 1). Evaluating olesoxime's effects in these models represents a relevant extension of the BACHD rat study in terms of validating the initial results and determining their general importance for HD. Hdh^{Q111} knock-in mice represent a particularly valuable model, as they provide a more precise reproduction of the human genetic conditions due to the polyQ mutation being inserted into the mouse huntingtin gene (*Hdh*). The intact genetic context as well expression of the mHTT protein on an endogenous level, result in a slow progressing phenotype, which might better resemble the symptoms observed in HD patients.[23]

Olesoxime has been found to restore abnormally high mitochondrial membrane fluidity not only in the BACHD rat, but also in Hdh^{Q111} knock-in mice and STHdh^{Q111} cells.[22] Moreover, olesoxime's prominent effects on calpain activation and HTT fragmentation reported for the BACHD rat could be reproduced in the $Hdh^{Q111/Q111}$ knock-in mouse (Fig. 2 and 3). Compared to wild type mice, 3 months old knock-in mice showed significantly increased levels of large and small fragments containing the N-terminal (Fig. 2A, *4C8 detection*) and/or middle (Fig. 2B, *D7F7 detection*) part of HTT. In addition, HTT aggregation was already moderately increased at this young age (Fig. 2C, *4C8 and 1C2 detections*), all of which is indicative of enhanced HTT cleavage. In accordance with this, as well as in line with the results obtained from BACHD rats,[19] calpain-1 was found to be

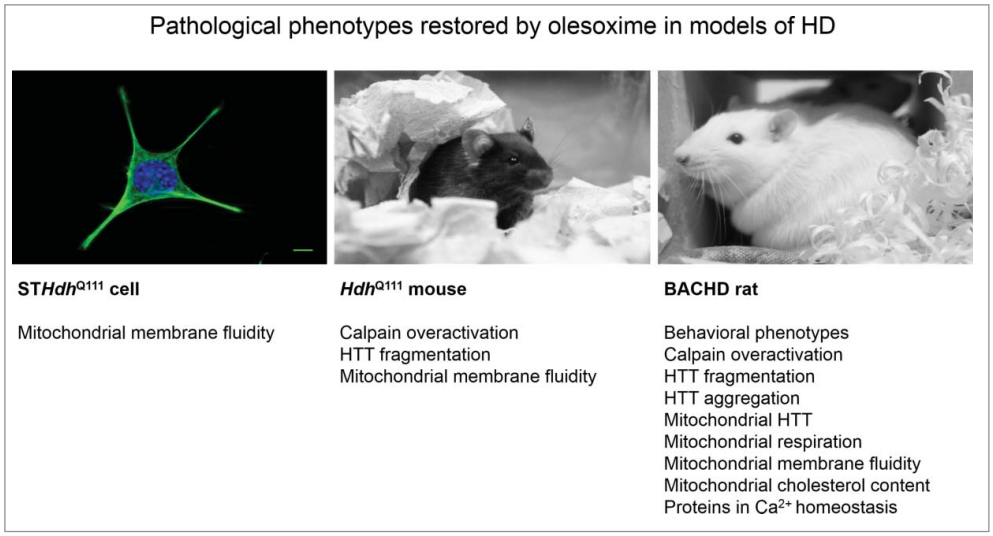

Figure 1. Overview of pathological phenotypes restored by olesoxime in models of HD. The effect of olesoxime on pathological phenotypes has been studied in 3 models of HD: the ST*Hdh*^{Q111} cell line, the *Hdh*^{Q111} knock-in mouse and the BACHD rat.

overactivated in the knock-in mice, as demonstrated by an increased ratio of fully processed to processed calpain-1, increased levels of the cleaved form of the calpain substrate α (alpha)-spectrin, see labeling in Fig. 3A as well as reduced levels of its endogenous inhibitor calpastatin (Fig. 3A). A possible reason for this overactivation could be intracellular Ca^{2+} derangements, as elevated protein levels of IP3R 1 were detected as well (Fig. 3B).

Olesoxime treatment, which had been started prenatally, via feeding the dams with olesoxime-loaded diet, significantly reduced both HTT fragmentation (Fig. 2A and B) and calpain overactivation (Fig. 3A), similar as in the BACHD rat.[19] Other effects found in the rats, such as reduced HTT aggregation, as well as reduced IP3R 1 and increased VDAC 1 and TOM20 levels,[19] did not reach statistical significance in the knock-in mice, although trends were apparent (Fig. 3B). This might be ascribed to the fact that these phenotypes were mostly not very strong from the start.

Additional insight into the causal relationship between olesoxime's effects on mitochondrial function, Ca^{2+} homeostasis, calpain activation, HTT fragmentation and HTT aggregation might be gained by studying the compound's therapeutic effects in a fragment model of HD. Due to the constitutive expression of mHTT fragments through the transgene in such a model, the effects on HTT fragment generation can be assumed to be marginal, potentially enabling the dissection of direct effects of olesoxime on mitochondrial function from indirect treatment effects via reduced fragment generation.

Olesoxime's mechanism of action

Although Ca^{2+} levels or Ca^{2+} handling were not directly measured in our studies, the overactivation of the Ca^{2+}-dependent protease calpain-1 (Fig. 2) as well as the increased protein levels of the endoplasmic reticulum Ca^{2+} exporter IP3R 1 in BACHD rats[19] and *Hdh*^{Q111} knock-in mice (Fig. 3) suggest a baseline disturbance of Ca^{2+} homeostasis in these models. The ameliorating effects of olesoxime on these parameters (although only in trend for IP3R 1 levels in *Hdh*^{Q111} knock-in mice) further support the idea that Ca^{2+} homeostasis is somehow involved in olesoxime's mechanism of action. The direct effects of olesoxime on Ca^{2+} balance are currently under investigation in several HD cell models including the ST*Hdh*$^{Q111/Q111}$ cell line.

Brain region-selective effects of olesoxime

Olesoxime was found to ameliorate deficits, which were restricted to the cortex and not present in the striatum of the BACHD rat.[19] The absence of increased IP3R 1 levels, calpain-1 overactivation and extensive HTT fragmentation in the striatum were interpreted as an indicator of a compensatory reduction in Ca^{2+} sensitivity in this brain region, since this had been reported earlier for HD mouse models including the *Hdh*^{Q111} knock-in mouse. [24]

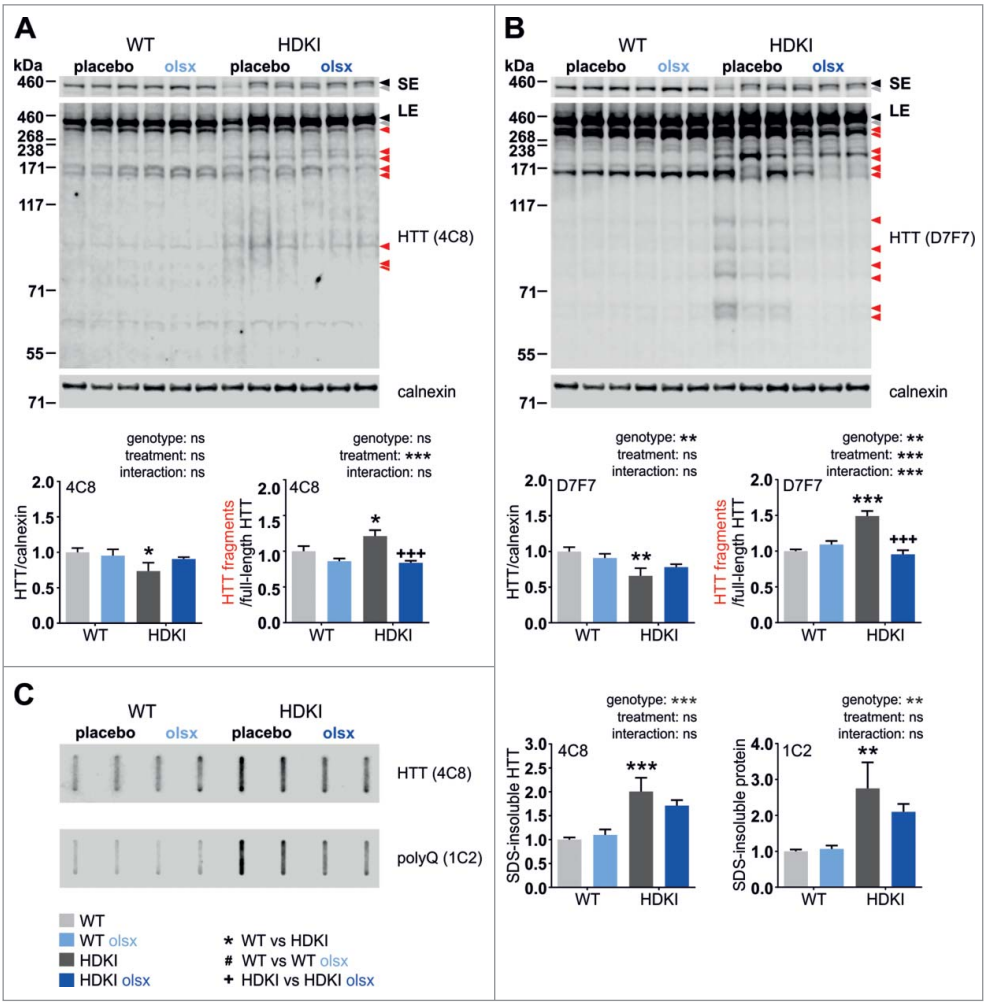

Figure 2. Olesoxime treatment reduces mHTT fragmentation in *Hdh*^{Q111} knock-in mice. Effects of olesoxime on HTT fragmentation were analyzed by western blotting of half brain lysates from 3 months old wild type (WT) and heterozygous *Hdh*^{Q111} knock-in mice (HDKI) receiving placebo or olesoxime-loaded diet (olsx). Full-length and fragment forms of HTT were assessed using the HTT-specific antibodies 4C8 (A) and D7F7 (B). Black arrowheads: full-length mHTT, gray arrowhead: full-length HTT, red arrowheads: HTT fragments, calnexin: loading control. SE: short exposure; LE: long exposure. (**C**) SDS-insoluble proteins were trapped on a nitrocellulose membrane and probed with the HTT-specific antibody 4C8 or the polyQ-specific antibody 1C2 to quantify the amount of aggregated HTT. Data were analyzed using 2-way ANOVA and Fisher LSD posttest; $^{*}/^{\#}/^{+}$: $P < 0.05$; $^{**}/^{\#\#}/^{++}$: $P \leq 0.01$ and $^{***}/^{\#\#\#}/^{+++}$: $P \leq 0.001$.

Data from 6 months old knock-in mice show that calpain overactivation is specifically found in the striatum and not in the cortex of these animals, as an increased ratio of fully processed to processed calpain-1 as well as increased levels of the cleaved form of alpha-spectrin (see comment above or Fig. 3A) were found to be restricted to the striatum (Fig. 4). Further analysis of samples from 12 months old mice is still outstanding and will reveal, if the phenotype is reversed to cortical instead of striatal calpain overactivation at this older age, and then resembles the results obtained from BACHD rats. In line with the increased striatal calpain activation, mutant HTT aggregates are also predominantly detected in the striatum and not in the cortex at earlier ages in the *Hdh*^{Q111} knock-in mice (unpublished data).

Importantly, the fact that olesoxime treatment was effective in restoring calpain overactivation in the striatum of *Hdh*^{Q111} knock-in mice, suggests that the compound is capable of affecting this pathology independent of the brain region it is found in at a given time or in a given animal model.

Phenotype-Specific effects of olesoxime

Olesoxime had failed to ameliorate some pathological phenotypes in the BACHD rat. Pathologies with particularly early onset, such as Rotarod performance[25]

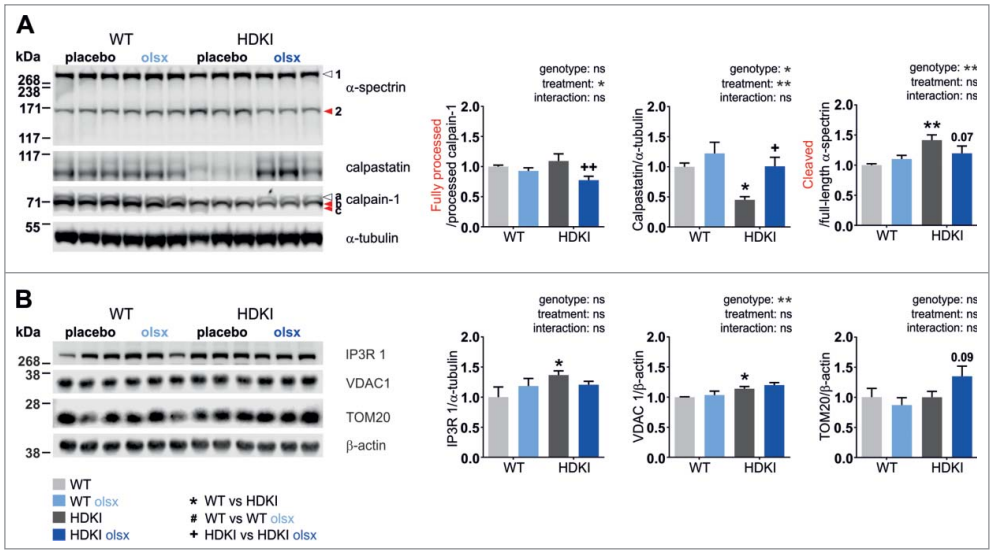

Figure 3. The calpain-suppressing effects of olesoxime are replicated in *Hdh*^{Q111} knock-in mice. The calpain-suppressing effects of olesoxime were investigated by protein gel blot analysis of half brain lysate from 3 months old wild type (WT) and heterozygous *Hdh*^{Q111} knock-in mice (HDKI) receiving placebo or olesoxime-loaded diet (olsx). (A) Calpain activation was assessed based on the processing of calpain-1, protein levels of the endogenous calpain inhibitor calpastatin and the cleavage of the calpain substrate α-spectrin. Arrowhead 1: full-length α-spectrin, arrowhead 2: α-spectrin fragment, arrowhead a: full-length calpain-1, arrowhead b: processed calpain-1, arrowhead c: fully processed calpain-1 (active calpain-1 refers to the ratio c/b), α-tubulin: loading control. (B) Expression levels of the endoplasmic reticulum Ca^{2+} transporter IP3R 1, outer mitochondrial membrane channel VDAC 1 and mitochondrial import receptor subunit TOM20 were assayed. β-actin: loading control. Data were analyzed using 2-way ANOVA and Fisher LSD posttest; $^{*}/^{\#}/^{+}$: $P < 0.05$; $^{**}/^{\#\#}/^{++}$: $P \leq 0.01$ and $^{***}/^{\#\#\#}/^{+++}$: $P \leq 0.001$.

or whole brain size (which correlates with reduced body length due to a developmental deficit[26]), did not show improvement upon olesoxime treatment. However, it is possible that the compound is not *per se* incapable of restoring such phenotypes, but that the treatment might have simply started too late to reverse

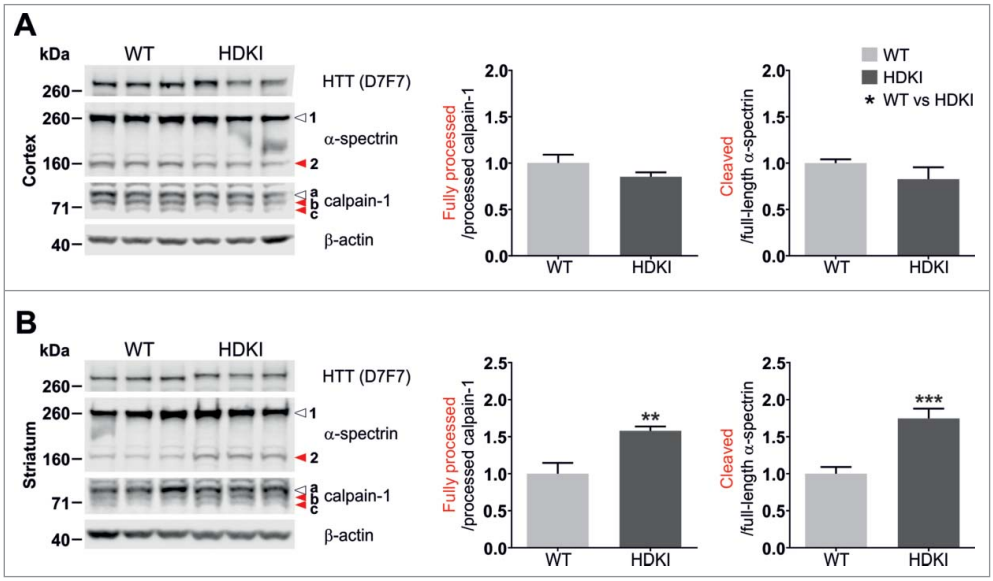

Figure 4. Calpain overactivation is restricted to the striatum in 6 months old *Hdh*^{Q111} knock-in mice. Calpain activation was assessed by western blot analysis of cortical (A) and striatal (B) lysates from 6 months old wild type (WT) and homozygous *Hdh*^{Q111} knock-in mice (HDKI), based on the processing of calpain-1 and the cleavage of its substrate α-spectrin. Arrowhead 1: full-length α-spectrin, arrowhead 2: α-spectrin fragment, arrowhead a: full-length calpain-1, arrowhead b: processed calpain-1, arrowhead c: fully processed calpain-1 (active calpain-1 refers to the ratio c/b), β-actin: loading control. Data were analyzed using unpaired Student's *t*-test; $^{*}/^{\#}/^{+}$: $P < 0.05$; **: $P \leq 0.01$ and ***: $P \leq 0.001$.

pathologies that were already manifest. In this regard, olesoxime treatment of late stage ALS patients did not yield significant improvements,[15] while treatment of children and young patients with SMA had significant, beneficial effects.[16] Also, the compound was highly effective in preclinical studies on ALS models, in which treatment had started early.[10] These findings might indicate that olesoxime is most effective when applied at earliest stages of disease, although it is clear that the direct comparison of preclinical and clinical outcomes from two different diseases is difficult. Therefore, it would be of particular interest to investigate the specific effects of an earlier start of olesoxime treatment on phenotypes that were not ameliorated by the later onset of treatment in the BACHD rat.

Earliest start of treatment was achieved in the *Hdh*^{Q111} knock-in mouse by feeding olesoxime already to the parental animals during breeding, as the drug crosses the placental barrier and is secreted into the dam's milk (Rebecca Pruss, personal communication). The data further demonstrate that calpain overactivation is an early phenotype, and that olesoxime effectively suppresses this pathology also after shorter treatment duration.

Summary and conclusion

The new data confirm that olesoxime suppresses calpain activation and HTT fragmentation *in vivo*, and strengthen its potential as a treatment for HD. Olesoxime had been presumed to improve mitochondrial function and, through this, restore Ca^{2+} balance as well as downstream pathological events. Deciphering olesoxime's exact mechanism of action might be of great value for the understanding of neurodegenerative processes and the development of effective treatments, also, but not only, for HD.

Methods

Ethical statement

All experiments described herein involving animals were carried out by persons with appropriate training and expertise. Operating procedures were approved by the local ethics committees and carried out in accordance with the guidelines of the Federation of European Laboratory Animal Science Associations and the German Animal Welfare Act, based on European Union legislation (Directive 2010/63/EU).

Animals

Hdh^{Q111} knock-in mice were kindly provided by Dr. Marcy MacDonald (Harvard Medical School, Boston, USA) and bred on C57BL/6 background for more than 10 generations in our laboratory.

The mice were kept under controlled environmental conditions of 21–23°C ambient temperature, 55 +/− 10 % humidity and a 12/12 h light/dark cycle, in groups of same sex in standard type III autoclavable plastic cages with wooden bedding and nesting material. Food and tap water were delivered *ad libitum*.

In order to investigate the effect of olesoxime on calpain activation in *Hdh*^{Q111} knock-in mice, breeding pairs of wild type and heterozygous *Hdh*^{Q111} knock-in mice received either placebo or olesoxime-loaded food pellets (Altromin C1000, 0.6 g/kg olesoxime). Offspring from these breeding pairs was weaned and genotyped at 21 days of age, and maintained on the respective diet. Five mice per genotype and treatment, of mixed sex, were sacrificed at 3 months of age for *ex vivo* analyses.

To study brain region-specific calpain activation in *Hdh*^{Q111} knock-in mice, 6 homozygous mice were sacrificed at the age of 6 months.

Brain tissue sampling

The animals were sacrificed by CO_2 inhalation. Brains were immediately dissected on ice and whole brain, or brain regions, were sampled. Tissue was shock-frozen in liquid nitrogen and stored at −80°C. Tissue sampling was performed within 2 hours during the late light phase to minimize variation based on any circadian phase-related changes.

Immunoblotting

Immunoblotting was performed with half brain lysates from 3 months old mice (olesoxime effect on calpain activation) or striatal and cortical lysates from 6 months old mice (region-specific calpain activation). For the former, mouse brains previously stored at −80°C were first placed on dry ice and cut in half along the medial longitudinal fissure. Half brains were then thawed and homogenized on ice in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, pH 7.5) containing protease inhibitors (cOmplete® Protease Inhibitor

Cocktail, Roche). Homogenates were diluted 1:10 in RIPA detergent (5% sodium deoxycholate and 1% sodium dodecyl sulfate in RIPA buffer, pH 7.5) and incubated for 25 min at 4°C. For preparation of the half brain lysates, homogenates were centrifuged for 15 min at 16,100 *g*. The supernatant was collected and stored at −80°C containing 10% glycerol. Striatal and cortical lysates were generated by homogenizing the respective tissue on ice in TES buffer (50 mM Tris, 2 mM EDTA, 100 mM NaCl, pH 7.5) containing protease inhibitors (cOmplete® Protease Inhibitor Cocktail, Roche). Homogenates were diluted 1:10 in TNES buffer (90% TES buffer, 10% Igepal CA630), incubated for 1 h at 4°C and centrifuged twice for 30 min at 16,100 *g*. The supernatant was collected and stored at −80°C containing 10% glycerol.

At the time of immunoblotting, all samples were thawed on ice and the protein concentration was measured spectrophotometrically using Bradford reagent. Western blot analysis was performed according to standard procedures. Briefly, 30 μg of lysate protein were assayed by SDS-PAGE using homemade Bis-Tris polyacrylamide gels or purchased Bolt® Bis-Tris or NuPAGE® Tris-Acetate gels (Life Technologies). Proteins were transferred on nitrocellulose membranes and probed overnight at 4°C with the respective primary antibody: mouse anti-β-actin (1:10,000; clone AC-15, A5441, Sigma Aldrich), rabbit anti-calnexin (1:1000; C4731, Sigma Aldrich) rabbit anti-calpain-1 (1:1000; ab39170, Abcam), rabbit anti-calpastatin (1:1000; 4146, Cell Signaling), rabbit anti-huntingtin (1:1000; clone D7F7, #5656, Cell Signaling), mouse anti-huntingtin (1:500; clone 4C8, MAB2166, EMD Millipore), rabbit anti-IP3R 1 (1:1000; ab5804; Abcam), mouse anti-polyglutamine-expansion diseases marker (1:1000; clone 5TF1-1C, MAB1574, EMD Millipore), mouse anti-α-spectrin (1:1000; clone AA6, EMD Millipore), rabbit anti-TOM20 (1:500; sc-11415, Santa Cruz), mouse anti-α-tubulin (1:5000; clone DM1A, CP06, EMD Millipore), and rabbit anti-VDAC1 (1:10,000; AB10527, EMD Millipore). One hour of incubation with a respective HRP-conjugated secondary (sheep anti-mouse or donkey anti-rabbit 1:2500, GE Healthcare) or IRDye® antibody (goat anti-mouse, donkey anti-mouse or goat anti-rabbit, 1:10,000; LI-COR Biosciences) at room temperature followed. Chemiluminescence and fluorescence signals were detected with the LI-COR ODYSSEY FC Imaging system (LI-COR Biosciences). Protein levels were quantified by densitometry using Image Studio™ Lite software version. 4.0 (LI-COR Biosciences) or ImageJ[27].

Filter retardation assay

For the detection of SDS-insoluble HTT species, 25 μg homogenate protein were diluted in 100 μl DPBS (Life Technologies) with 2% SDS and 50 mM DTT, and incubated for 5 min at 95°C. A nitrocellulose membrane (0.45 μm; Bio-Rad) was equilibrated in 0.1% SDS in DPBS and samples were filtered through this membrane using a Minifold II Slot Blot System (Schleicher & Schuell). The membrane was then washed twice with TBS and blocked with 5% SlimFast (Unilever) in TBS for 1 h at room temperature. Retained SDS-insoluble HTT was detected using the primary antibodies mouse anti-HTT (1:1000; clone 1HU-4C8, MAB2166, EMD Millipore) and mouse anti-polyglutamine-expansion diseases marker (1:1000; clone 5TF1-1C, MAB1574, EMD Millipore) as well as respective anti-mouse IRDye® 800CW secondary antibodies (1:10,000; LI-COR Biosciences). Fluorescence signals were detected with the LI-COR ODYSSEY FC Imaging system and quantified using the ODYSSEY® Server software version 4.1 (both LI-COR Biosciences).

Abbreviations

BACHD rat — Huntington's disease rat model expressing full-length mutant huntingtin from a bacterial artificial chromosome

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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