Peripheral huntingtin silencing does not ameliorate central signs of disease in the B6.*Htt*$^{Q111/+}$ mouse model of Huntington's disease

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21 # Results

22 ## Dose-response and washout studies

23 We piloted four different doses of *Htt* ASO and qualitatively assessed HTT
24 knockdown by western blotting. At the two highest doses, no HTT could be detected,
25 despite robust HTT signal in protein lysates extracted from off target ASO and
26 uninjected mice (Figure in S1 Figure). Therefore we selected 50 mg per kg body mass
27 (mpk) per week as the dose for the efficacy study; this was the minimal dose at which
28 complete HTT knockdown was observed. β-Actin was used as a loading control and
29 was successfully detected in all samples regardless of treatment.

30 To determine how frequently *Htt* ASO must be administered in order to maintain
31 constant HTT knockdown, we conducted a washout study. Wild type mice were given
32 weekly injections of *Htt* ASO or off target ASO for three weeks and sacrificed every
33 other day for 24 days. During this time, we observed complete HTT silencing for 14
34 days after cessation of treatment (Figure in S2 Figure), and even 24 days after the final
35 injection, HTT did not recover to physiological levels. For the efficacy trial, we chose to
36 conservatively administer *Htt* ASO via weekly injections.

HTT knockdown in interim silencing cohorts

At two points midway through the efficacy trial, we confirmed HTT knockdown in three peripheral tissues of interest: liver, perigonadal white adipose tissue, and interscapular brown adipose tissue. HTT levels were quantified via mesoscale discovery (MSD) assays, as described in the primary manuscript. No differences in mutant or total HTT suppression patterns were observed between timepoints, therefore data were grouped for statistical analysis. *Htt* ASO treatment had a significant effect on total HTT levels (Figure in S3 Figure) in all three tissues (effect of treatment in the liver: $F_{(2, 18)}$ = 9.8, p = 1.8 x 10^{-3}, white adipose tissue: $F_{(2, 18)}$ = 15.7, p = 1.2 x 10^{-4}, and brown adipose tissue: $F_{(2, 18)}$ = 11.3, p = 6.5 x 10^{-4}). As expected, Tukey's HSD *post hoc* comparisons revealed *Htt* ASO treatment suppressed HTT compared to both control ASO (liver: p = 8.8 x 10^{-3}, white adipose tissue: p = 3.1 x 10^{-3}, and brown adipose tissue: p = 2.2 x 10^{-3}) and saline treatment (liver: p = 2.6 x 10^{-3}, white adipose tissue: p = 1 x 10^{-4}, and brown adipose tissue: p = 1.4 x 10^{-3}). Similarly, mHTT was significantly reduced by *Htt* ASO treatment in all tissues quantified (effect of treatment in the liver: $F_{(2, 18)}$ = 36.8, p = 4.4 x 10^{-7}, white adipose tissue: $F_{(2, 18)}$ = 9.6, p = 1.4 x 10^{-3}, and brown adipose tissue: $F_{(2, 18)}$ = 5.6, p = 0.01). Tukey's HSD *post hoc* tests verified reduction of mHTT following *Htt* ASO treatment compared to saline treatment (liver: p = 1.2 x 10^{-6}, white adipose tissue: p = 1.0 x 10^{-3}, and brown adipose tissue: p = 0.01). Interestingly, no difference in mHTT levels was observed by Tukey's HSD *post hoc* comparisons between *Htt* ASO and control ASO treatment in either of the adipose tissues (white adipose tissue: p = 0.16, and brown adipose tissue: p = 0.11), despite *Htt* ASO treatment reducing mHTT levels relative to control treatment in the liver (p = 3.5 x 10^{-6}).

60 # Methods

61 ## Mice

62 In order to determine an appropriate ASO dose and frequency of administration,
63 we conducted dose-response (n = 5 per arm, total N = 30) and washout studies (n = 1
64 per arm, total N = 24) in male, wild-type C57BL/6J mice (stock no. 000664) acquired
65 from the Jackson Laboratories (Bar Harbor, ME). Mice were housed under standard
66 vivarium conditions with *ad libitum* access to food and water. After shipment, mice
67 habituated to local vivarium conditions for at least two weeks prior to every study. For
68 both the washout and dose-response studies, treatment began at three months of age
69 and continued for three weeks. For the dose-response study, mice were sacrificed two
70 days after the final injection as described in the primary manuscript. For the washout
71 study, mice were sacrificed every other day after cessation of treatment.
72 ASO uptake in peripheral tissues was qualitatively assessed in a small cohort
73 made up of female, C57BL/6J mice bred locally (n = 2 per arm, total N = 4). Mice in the
74 tissue distribution cohort were maintained with *ad libitum* access to food and water.
75 Treatment began at 4 months (121 ± 1 days) of age and continued for four weeks. At 5
76 months (147 ± 4 days) of age, or two days after cessation of treatment, mice were
77 sacrificed via intraperitoneal injection of at least 250 mpk sodium pentobarbital and
78 transcardially perfused with phosphate buffered saline (PBS). Liver, perigonadal white
79 adipose tissue (WAT), interscapular brown adipose tissue (BAT), kidney, spleen, heart,
80 and gastrocnemius were dissected and fixed overnight in 10% neutral buffered formalin.

81 All procedures were reviewed and approved by the animal care and use committee at
82 Western Washington University (protocol 14-006).

83 Antisense Oligonucleotide Administration

84 In an initial dose-response study, we administered a pan-*Htt* targeted ASO (Ionis
85 419637, *Htt* ASO) via weekly IP injection at four different doses: 17.9, 35.8, 50, or 100
86 mpk. As a control, an off target ASO was administered weekly at 35.8 mpk. The off
87 target ASO was selected as a control because it shares a similar chemical structure
88 with *Htt* ASO, both of which are 20 nucleotide 5-10-5 2'-methoxyethyl gapmers. In
89 addition, we compared both *Htt* ASO and off target ASO treated mice to uninjected
90 mice. Based on this initial dose-response study, we selected a dose of 50 mpk per week
91 for all subsequent studies.

92 To roughly characterize *Htt* ASO duration of action, we conducted a washout
93 study in which mice were treated with *Htt* ASO or off target ASO at 35.8 mpk per week
94 for three weeks. After cessation of treatment, mice were sacrificed every other day for
95 24 days and HTT levels quantified by western blot.

96 For the tissue distribution study, mice received weekly injections of *Htt* ASO at 50
97 mpk for four weeks.

98 Immunoblotting

99 Tissue (half of dissected brown adipose tissue and 35 - 55 mg of liver tissue) was
100 transferred to BeadBug tubes prefilled with 1.5-mm zirconium oxide beads (Benchmark)
101 and homogenized for 2 minutes at 4,000 rpm using the BeadBug Microtube

102 Homogenizer (Benchmark). Protein was extracted using 450 µL of RIPA buffer (150 mM
103 NaCl, 25 mM Tris-HCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing
104 protease and phosphatase inhibitors (Thermo Fisher Scientific). Concentration of
105 protein lysates was determined by Pierce BCA assay (Thermo Fisher Scientific)
106 according to the manufacturer's protocol.
107 Total HTT levels were quantified via western blotting. Equal amounts of protein
108 were loaded into 3-8% Tris-Acetate Mini Gels (Thermo Fisher Scientific) and separated
109 electrophoretically by molecular weight. Protein was transferred at 4℃ for 17 – 20 hours
110 to a PVDF membrane.
111 Western blot assay membranes were blocked for 1 hour at room temperature
112 using Odyssey Blocking Buffer (LiCor) before incubating for 1 hour at room temperature
113 or overnight at 4℃ in primary antibodies. Monoclonal primary antibodies were diluted
114 1:1,000 in Odyssey blocking buffer plus 0.2% tween and consisted of mouse anti-HTT
115 (EMD Millipore: MAB2166; Ab_2123255) and rabbit anti-β-Actin (Cell Signalling:
116 mAB#8457, Ab_10950489). Membranes were rinsed three times in PBS plus 0.05%
117 tween (PBST), then incubated in secondary antibodies for 1 hour at room temperature.
118 Polyclonal secondary antibodies were IRDye 800CW conjugated goat anti-mouse IgG
119 (LiCor: 926-32210; Ab_621842) and IRDye 680RD conjugated goat anti-rabbit IgG
120 (LiCor: 926-68071; Ab_10956166) diluted 1:15,000 in Odyssey Blocking Buffer plus
121 0.2% tween and 0.01% SDS. After rinsing three times in PBST, membranes were
122 washed in PBS before imaging for 2 minutes each at 700 and 800 nm.

123 ## Immunohistochemistry and Imaging

124 As part of the tissue distribution study, formalin-fixed liver, WAT, BAT,
125 gastrocnemius, heart, spleen, and kidney tissues were paraffin embedded, cut into 5-
126 µm sections, and mounted on glass slides at Histology Consultation Services (Everson,
127 WA). Sections were deparaffinized and heat mediated antigen retrieval was conducted
128 in Tris-EDTA buffer before staining overnight in anti-ASO primary antibody (1:1,000) at
129 4℃. Sections were stained in goat anti-rabbit secondary antibody conjugated to
130 Alexa568 (1:1,000) for one hour at room temperature and mounted with DAPI
131 Fluoromount-G (Southern Biotech). All confocal images were acquired with an IX-81
132 laser-scanning confocal microscope using Fluoview 1000 software (Olympus). Image
133 processing and analysis was performed as described [1].

134 # References

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