Genetic knockdown of HDAC4, or sub-chronic treatment with a novel selective Class IIa HDAC inhibitor, reverses elevated membrane excitability in striatal medium spiny neurons from R6/2 and zQ175 Huntington's disease model mice

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Introduction

Huntington's disease (HD) is a lethal autosomal dominant neurodegenerative disorder that leads to deficits in motor control widely believed to reflect structural and/or functional changes in neurons of the basal ganglia. *In-vitro* brain slice recordings of both the R6/2 and Q175 mouse models of HD have revealed a paucity of glutamatergic innervation of the striatum as well as alterations in intrinsic membrane properties of striatal medium spiny neurons (MSNs, exemplified by a large increase in membrane resistance (Rm) and a reduced rheobasic current (Rh). Such changes are reflective of corticostriatal pathway degeneration and MSN hyper-excitability and are likely to result in aberrant striatal output. A 50 % reduction in HDAC4 restored these and other electrophysiological changes in both the R6/2 model, a transgenic over-expresser of Exon 1 HTT with an expanded polyglutamine repeat, and heterozygous Q175 knock-in mice (Q175 +/-), which carry one normal and one mutant HTT allele with an expanded repeat of ~190 polyglutamines, in addition to reversing behavioral alterations in R6/2 mice (Mielcarek et al, 2013; PLOS Biology, in press).

We examined MSN properties in R6/2 mice and Q175 heterozygous knock-in mice (Q175 +/-) after sub-chronic *in vivo* exposure (4 weeks or 4 months respectively) to a novel selective Class IIa HDAC inhibitor, CHDI-00390576, to evaluate whether this could mimic the genetic HDAC4 knockdown data. MSNs in R6/2, Q175 and WT mice had nearly identical resting membrane potentials, but R6/2 and Q175 MSNs exhibited significantly elevated Rm and lower Rh. Rm in both models was partially reversed by CHDI-00390576, while Rh was partially reversed in R6/2 but not significantly in Q175. Neither Rm nor Rh was affected in drug-treated WT mice. Action potential amplitude and threshold in the HD models were decreased and increased, respectively, relative to WT controls. These changes, which were reversed in HDAC4 knockdown Q175 mice, were unaffected in drug-treated R62 mice. In R62 and Q175 (+/-) mEPSC frequency was significantly lower compared to WT MSNs, but no drug-related rescue was seen for either measure. In contrast, reduced mEPSC frequency was reversed by HDAC4 knockdown in both R62 and Q175.

Our findings indicate that disease-specific HD phenotypes in MSNs can be partially reversed by manipulating HDAC4 activity/expression in R6/2 and Q175 mice, suggesting a therapeutic potential for Class IIa HDAC inhibitors in HD.

Methods

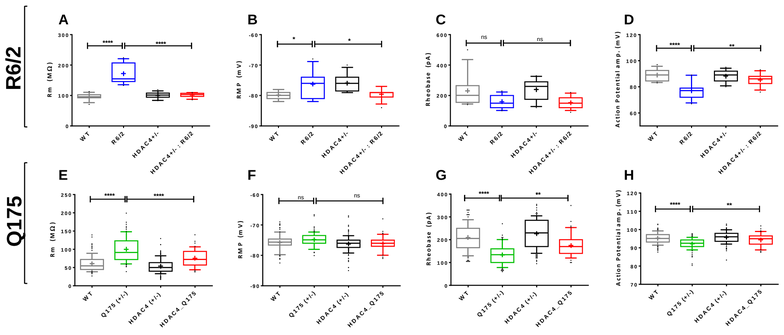
Animal Dosing: A suspension of CHDI-00390576 was prepared in 10% (w/v) HP-β-CD in 50mM Citrate Buffer (pH 5.5) to a concentration of 100 mg/ml and serially diluted to desired doses. R6/2 transgenic mice and WT littermates (C57/B6 R6/2 strain) or Q175 het and WT mice were dosed beginning at 4 weeks old. Doses (P.O.) were administered at 10mL/kg, twice per day (BID) for 4 weeks (R6/2, performed at Psychogenics)) and 5 months (Q175; performed at Charles River Finland). The last dose was administered no sooner than 24 h and up to 72 hours (R6/2) or 2 weeks (Q175) prior to electrophysiology assessment. Mice were housed and maintained on a 12/12 light/dark cycle. Food and water were provided *ad libitum*. All researchers were blinded to drug treatments and genotypes throughout the length of the study.

Electrophysiology: Brain slices were prepared from 8-9 week old R6/2 cohort mice, or ~ 6 month Q175het cohort mice (gender-balanced). The brain was rapidly removed and cooled in ice-cold oxygenated sucrose-ACSF (in mM): Sucrose 220; KCl 2.5; CaCl2 0.5, MgSO4 3, NaH2PO4 1.2, NaHCO3 26, glucose 5. Parahorizontal slices (300 micron) were prepared in ice-cold oxygenated ACSF then warmed to 36ºC for 30min, allowed to cool to room temperature, and transferred as needed to a submerged slice chamber mounted on the stage of an upright microscope, perfused at 2 ml/min with oxygenated normal ACSF (in mM): NaCl 124; KCl 3.5, $CaCl_2$ 2.5, MgSO4 1.2, NaH_2PO4 1.2, NaHCO3 26, glucose 11, 40uM picrotoxin. Whole-cell patch clamp recordings were made under IR/DIC optics from visually identified MSNs with pipettes (4-6MΩ) containing (in mM): K-gluconate 105, KCl 30, EGTA 0.3, HEPES10, $MgCl_2$ 4, Na_2ATP 4, Na_3GTP 0.3, Tris-phosphocreatine 10, pH adjusted to 7.2. For mEPSC recording, the pipette solution was (in mM): Cs-methansulfonate 110, EGTA 10, HEPES 10, TEA-Cl 10, NaCl 10, $CaCl_2$ 1, Mg-ATP 5, Na_2GTP 0.5, Qx314-Cl 5, pH 7.2. Active and passive membrane properties were measured either in current clamp at RMP or in voltage clamp at a holding potential of -80mV using PClamp 10 software. Rheobase was determined by applying 300ms depolarizing current pulses of increasing amplitude. Rm values were determined with +5mV pulses using the automated "Membrane Test" function built into the Clampex software. Miniature EPSCs (mEPSCs) were isolated by including 0.5uM TTX in the bath solution, recordings were filtered at 1KHz and collected continuously for 5 minutes from each cell at a holding potential of -80mV. Inwardly rectifying (Kir) currents were evoked by a series of 500ms hyperpolarizing steps from a holding potential of -70mV.

Data Analysis: Data were analyzed by either a 1-way or a 2-way repeated measures ANOVA, followed by a Tukey's or Bonferroni post-hoc test, respectively, mEPSC analyses were performed using Minianalysis software; all events were visually verified following automated detection. Kir currents were leak-subtracted off-line using Clampfit.

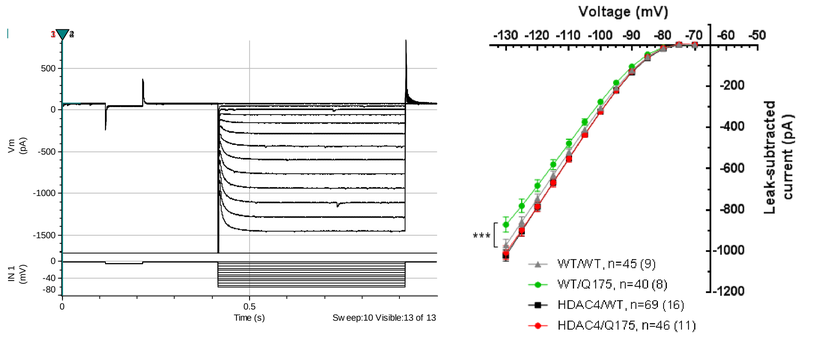
Reversal of HD model neurophysiological phenotypes on reduction of HDAC4

MSN Membrane properties of Q175het KI and R6/2 HD mice



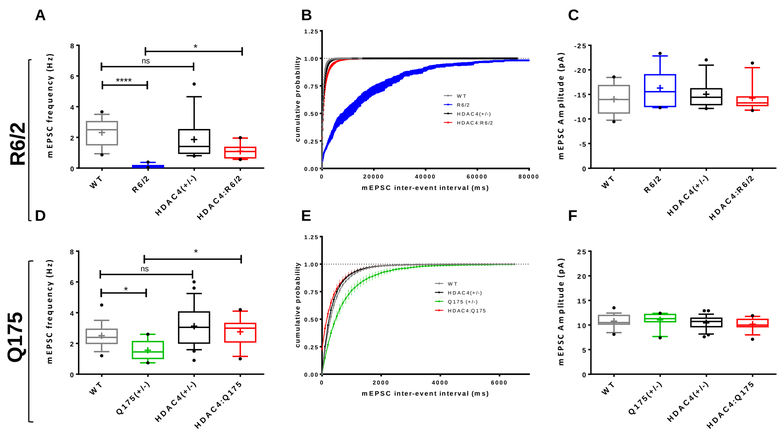
Medium spiny neurons display a hyper-excitable phenotype in both 8 week old R6/2 **(A-D)** and 6 month old Q175 (+/-) mice **(E-H)** compared to WT or HDAC4 (+/-) mice. This is characterized by elevated membrane resistance (**A and E**), modest changes in resting membrane potential (**B and F**), reduced rheobasic current (**C and G**) as well as reduction in action potential amplitude (**D and H**). The HD models crossed with HDAC4 (+/-) mice show significant rescue of all phenotypes. Membrane properties of WT MSNs are unaffected by reduction of HDAC4 levels. Data is displayed as box and whisker plots (box indicated upper and lower 25 percentiles, line indicates median values, + indicates mean values, error bars indicate 10-90% levels, and outlier values are indicated by black dots). Significance is determined by One-Way ANOVA with Tukey's post-hoc analysis. * p<0.05, ** p < 0.01, *** p <0.001.

Decreased K_{ir} channel function in Q175 het KI MSNs – rescue by genetic reduction of HDAC4



K_{ir} currents evoked from MSNs are reduced in 6 month old Q175 het mice. HDAC4 knockdown reversed this shift in Q175 het mice but had little or no effect on currents evoked from WT controls. Cells were held at -70mV. Number of mice for each group are given in parentheses. ***P<.001, 2-way ANOVA.

Rescue of impaired striatal glutamatergic innervation in HD models by reduction of HDAC4



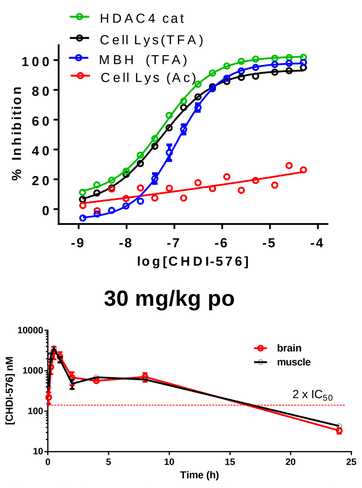
Mean miniature excitatory post-synaptic current (mEPSC) frequency (**A and D**) and inter-event interval cumulative probability (**B and E**) are significantly decreased in both 8 week old R6/2 **(A-B)** and 6 month old Q175 (+/-) mice **(D-E)** compared to WT or HDAC4 (+/-) mice. The HD models crossed with HDAC4 (+/-) mice show significant rescue of this phenotype, suggesting a preservation of striatal glutamatergic innervation on genetic reduction of HDAC4. mEPSC amplitude is unchanged in either R6/2 **(C)** or Q715 (+/-) **(F)** compared to WT, and unaffected by reduction of HDAC4 levels.

HDAC Class IIa inhibition

CHDI-00390576 is a selective HDAC Class IIa inhibitor

Biochemical & Cellular assays	
HDAC4 IC_{50} (μM)	0.05
Cellular Class IIa IC_{50} (μM)	0.07
Mouse brain homoganate (MBH) Class IIa C_{50} (μM)	0.14
Cellular Class I IC_{50} (μM)	>50
Selectivity over Class IIa HDACs	none
Selectivity over Class IIb HDACs	400 x
Selectivity over Class 1 HDACs	230 - 478 x

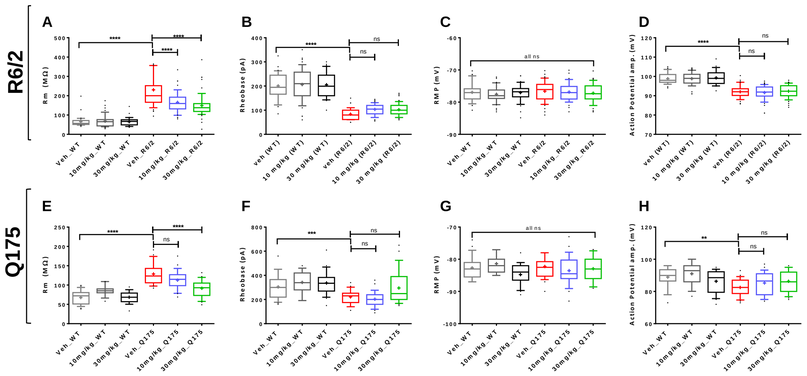
ADMET/PK	
Human liver microsomes Cl_{int} ml/min/kg	<28
Mouse liver microsomes Cl_{int} ml/min/kg	<76
Cerep Profile @ 10 μM	Clean
ALL CYP (μM)	>35
EER	1.2
Papp nm/sec	300
% inhibition hERG [μM]	>10
PK: F%	44
PK: Clp L/h/kg	3.1
PK Vdss L/kg	7.3



Biochemical, cellular and ADMET/PK properties of CHDI-00390576, a novel, orally bioavailable, brain penetrant, selective inhibitor of Class IIa HDACs. A *po bid* chronic dosing schedule starting from four weeks of age in wild-type, R6/2 and Q175 heterozygous mice was used to assess efficacy in rescuing the HD electrophysiological deficits shown to be responsive to HDAC4 genetic knock-down. R6/2 mice were dosed for 4 weeks, and Q175 het mice for 5 months, followed by *ex vivo* brain slice electrophysiology performed 3 days (R6/2) or up to 2 weeks (Q175) post last dose . Further details of the Med Chem program and assays employed can be found in SFN 2013 poster: Aziz *et al*; Class IIa HDAC inhibitors as a therapy for Huntington's disease. Session C.05.a. Huntington's disease: Mechanisms.

Effect of chronic dosing of CHDI-00390576

Restoration of elevated Rm in HD model MSNs, but not other aberrant membrane properties, following CHDI-00390576 dosing



A partial, highly significant, dose-dependent restoration of the elevated Rm in MSNs from 8 week old R6/2 (**A**) and 6 month old Q175 het mice (**E**) was seen following subchronic dosing with CHDI-00390576 (10 and 30 mg/kg po bid, for 4 weeks in R6/2, and 5 months in Q175 het mice). There was no significant improvement in the other parameters studied which showed robust HD dependent phenotypes; i.e no improvement in rheobasic current (**B and F**), or action potential amplitude (**D and H**), in contrast to the improvement in these properties following the genetic reduction of HDAC4. CHDI-00390576 was without effect on any parameter measured in WT MSNs (**A-H**), and did not affect resting membrane potential in either WT or HD MSNs (**C and G**). Significance is determined by One-Way ANOVA with Tukey's post-hoc analysis. ns = not significant * p<0.05, ** p < 0.01, *** p <0.001, **** p <0.0001.

An additional cohort of R6/2 and WT mice were dosed po bid with 100 mg/kg CHDI-00390576 (data not shown). This increased dose did not offer any further improvement over the 30 mg/kg effect size.

No rescue of impaired striatal glutamatergic innervation in HD models following CHDI-00390576 dosing.

Mean miniature excitatory post-synaptic current (mEPSC) frequency (**A and D**) and inter-event interval cumulative probability (**B and E**) following dosing of R6/2 and WT **(A-B)** and 6 month old Q175 (+/-) and WT mice with 10 and 30 mg/kg po bid CHDI-00390576 **(D-E)**. There was no significant improvement of mEPSC frequency in either HD model, although a slight trend to improvement was noted in the 30 mg/kg dosed Q175 mice compared to vehicle treated Q175. mEPSC amplitude is unchanged in either R6/2 **(C)** or Q715 (+/-) **(F)** compared to WT, and unaffected by CHDI-00390576 treatment.

Conclusions

1. MSNs in both the R6/2 and Q175 models of HD exhibit electrophysiological shifts in cell properties consistent with a hyper-excitable phenotype, each of which was either partially or completely reversed by genetic HDAC4 knockdown.

2. Decreases in glutamatergic innervation of the striatum in both HD models were also reversed by HDAC4 genetic knockdown.

3. Chronic exposure to a novel, selective HDAC4 class IIa inhibitor, CHDI-00390576, selectively reduced the large increase in membrane resistance (Rm) in HD model MSNs without significantly affecting other disease-specific properties.

4. These findings demonstrate that drugs which target the active/catalytic site of HDAC4 may not fully emulate reduced HDAC4 enzyme levels. This dissociation suggests there are HD-relevant biological activities of HDAC4 that may be unrelated to the 'catalytic' or Ac-Lys binding activity of HDAC4 *per se*.

Acknowledgments and disclosures

CHDI is a private, not for profit biomedical research organization exclusively dedicated to the development of therapies for Huntington's Disease. CHDI-00390576 was synthesized at BioFocus UK. The R6/2 and Q175 mice crossed to HDAC4 (+/-) mice were generated at King's College London with funding from CHDI. CHDI-00390576-treated R6/2 and WT mice and Q175 HDAC4 knockdown cohort mice were assessed at Psychogenics; CHDI-00390576 treated Q175 mice and HDAC4-knockdown R6/2 cohort mice were assessed at Neuroservice. R6/2 mice were dosed at Psychogenics, Q175 mice were dosed at Charles River Finland and shipped to Neuroservice for evaluation. BioFocus, Psychogenics, Charles River Finland and Neuroservice are CROs that conducted the work on a purely fee-for service basis for CHDI.