Restoring GABAergic inhibition rescues memory deficits in a Huntington’s disease mouse model

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Edited by Mu-ming Poo, Chinese Academy of Sciences, Shanghai, China, and approved January 5, 2018 (received for review September 25, 2017)

Huntington’s disease (HD) is classically characterized as a movement disorder, however cognitive impairments precede the motor symptoms by ~15 y. Based on proteomic and bioinformatic data linking the Huntington protein (Htt) and KCC2, which is required for hyperpolarizing GABAergic inhibition, and the important role of inhibition in learning and memory, we hypothesized that aberrant KCC2 function contributes to the hippocampal-associated learning and memory deficits in HD. We discovered that Htt and KCC2 interact in the hippocampi of wild-type and R6/2-HD mice, with a decrease in KCC2 expression in the hippocampus of R6/2 and YAC128 mice. The reduced expression of the Cl−-extruding cotransporter KCC2 is accompanied by an increase in the Cl−-importing cotransporter NKCC1, which together result in excitationary GABA in the hippocampi of HD mice. NKCC1 inhibition by the FDA-approved NKCC1 inhibitor bumetanide abolished the excitatory action of GABA and rescued the performance of R6/2 mice on hippocampal-associated behavioral tests.

Significance

Huntington’s disease (HD) is a fatal neurodegenerative disorder that currently has no cure. Although HD is classically considered a motor disorder, HD patients experience learning and memory deficits years before the onset of motor symptoms, and these deficits resemble those observed in HD mouse models. In this work, using transgenic mouse models of HD, we demonstrate that the action of the neurotransmitter GABA has switched from inhibitory to excitatory. By treating HD mice with a clinically used diuretic (bumetanide), which restores inhibitory GABA, we rescued the learning and memory deficits. Our data suggest a potential therapeutic approach for the treatment of the cognitive deficits in early HD that can improve patient quality of life and reduce caregiver burden.

Author contributions: Z.D., J.C.K., and M.A.W. designed research; Z.D., J.Y.B., V.M., C.S.K., S.B., and G.M.P. performed research; Z.D., J.Y.B., V.M., and C.S.K. analyzed data; and Z.D. and M.A.W. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1716871115/-/DCSupplemental.
We determined that Htt and KCC2 interact in the hippocampus of both wild-type (WT) and the R6/2 transgenic mouse model of HD. We also discovered that decreased KCC2 and increased NKCC1 expression in the hippocampi HD mice result in a depolarizing shift in the reversal potential for GABAA receptor-mediated Cl− currents (E_{GABA}), which renders GABAA receptor signaling excitatory in R6/2 mice. The treatment of R6/2 mice with the FDA-approved NKCC1 inhibitor bumetanide restored E_{GABA} to the value seen in WT mice, abolished the excitatory action of GABA, and rescued the hippocampal-associated memory deficits in R6/2 mice.

Results

Htt Interacts with KCC2 in the Hippocampus of WT and R6/2 Mice.

Based on the bioinformatic and proteomic studies linking KCC2 and Htt (25, 26), we first determined biochemically whether KCC2 and Htt interact. We performed coimmunoprecipitation (co-IP) assays from hippocampal brain lysates prepared from WT and the R6/2 mouse model of HD. R6/2 mice express the exon 1 human Htt with 120 CAG repeats (31) and develop a relatively fast progressing neurological phenotype similar to HD. At birth, R6/2 mice are indistinguishable from their littermate controls and develop normally until ∼8 wk of age (31). Because we hypothesized that KCC2 dysfunction and weakened synaptic inhibition contribute to the learning and memory deficits in early stages of the disease, we performed the co-IP on hippocampal brain lysates collected from 7-wk-old WT and R6/2 mice. Using anti-Htt antibodies, we found that Htt precipitates KCC2, indicating the existence of a KCC2–Htt complex in vivo (Fig. 1 and SI Appendix, Fig. S1 A–D). KCC2 exists as both monomers (∼140 kDa) and oligomers (>250 kDa), with the oligomeric form believed to be the functional form of the transporter in the mature brain (32, 33). We found that Htt interacts with both monomeric and oligomeric KCC2. As a control, we probed for NKCC1, which is not present in the Htt interactome (26), and found no interaction with Htt (Fig. 1 and SI Appendix, Fig. S1E). To determine whether KCC2 can interact with mHtt, we performed co-IP assays in COS-7 cells transfected with KCC2 and the normal or expanded polyglutamine tract of Htt (Htt-FL-15Q-HA and Htt-FL-128Q-HA, respectively). We found that both normal and mutant forms of Htt could precipitate KCC2 (Fig. 1B and SI Appendix, Fig. S2).

Fig. 1. KCC2 and Htt interact in the hippocampus of WT and R6/2 mice. (A) Native Htt complexes from C12E9-solubilized hippocampal brain lysates immunoprecipitated with anti-Htt antibodies and immunoblotted with the antibodies indicated at right (Htt, KCC2, NKCC1). Shown is a representative example of five independent biological replicates (full blots presented in SI Appendix, Fig. S1). Input, input fraction (2% of IP); IP, immunoprecipitation; M, monomer; O, oligomer. (B) Co-IP experiments performed in COS-7 cells transfected with 15Q-Htt-HA, or 128Q-Htt-HA and KCC2-Myc solubilized in RIPA buffer, immunoprecipitated with anti-HA antibodies, and immunoblotted with the antibodies indicated at right (KCC2, HA). Shown is a representative example of five independent biological replicates (full blots presented in SI Appendix, Fig. S2).

KCC2 and NKCC1 Protein Expression Are Altered in the Hippocampus of HD Mice. mHtt interacts with proteins involved in synaptic transmission and can alter the function, cellular distribution, and total expression of these interacting proteins (34–36). To determine if the KCC2–mHtt interaction alters KCC2 protein expression, we performed Western blot analysis from hippocampal brain lysates and found a significant decrease in total KCC2 protein (oligomer + monomer) at 7 wk in R6/2 mice relative to WT (Fig. 2A and SI Appendix, Fig. S3 A–C; WT, 0.59 ± 0.08; R6/2, 0.33 ± 0.04; P = 0.0104). We also observed a significant decrease in fluorescence intensity of KCC2 at the surface membrane in the hippocampus of R6/2 mice compared with WT controls (Fig. 2E and SI Appendix, Fig. S4; WT, 69.07 ± 2.09; R6/2, 46.27 ± 2.03; P < 0.0001). To determine if the altered KCC2 and NKCC1 expression in the R6/2 hippocampus is common in HD, we repeated our protein expression experiments in the YAC128 mouse model of HD, which expresses full-length human Htt with 128 CAG repeats (37). This model has a later onset of motor symptoms (compared with R6/2) and closely recapitulates the motor dysfunction and neuropathology observed in human HD (37). We performed Western blots on hippocampal brain lysates collected from 9-wk-old WT and YAC128 mice. Consistent with our findings in R6/2, we also observed a significant decrease in KCC2 expression in YAC128 mice (0.80 ± 0.07 compared with WT (1.16 ± 0.12; P = 0.030) (Fig. 2B and SI Appendix, Fig. S3 G and H). We also probed for NKCC1, because in other neurological disorders where KCC2 is decreased, increases in NKCC1 have been reported (38, 39) and are thought to represent a reversion to an immature GABAergic phenotype (39, 40). Interestingly, although NKCC1 is not in the Htt interactome (25, 26) and does not co-IP with Htt (Fig. 1A), we found a significant increase in NKCC1 protein expression at 7 wk in R6/2 mice (Fig. 2C and SI Appendix, Fig. S3 D–F; WT, 0.53 ± 0.09; R6/2, 2.02 ± 0.37; P = 0.0104) but not YAC128 mice (Fig. 2D and SI Appendix, Fig. S5 I and J; WT, 1.30 ± 0.14; YAC128, 1.53 ± 0.21; P = 0.568).

To determine if gene transcription underlies the alterations in protein expression, we performed real-time qPCR on samples from R6/2 and WT mice using isoform-specific amplification of Slc12a5 (KCC2) and Slc12a4 (NKCC1). We used previously identified stable control genes for R6/2 mice (Rpl13a, ATP5b, Ubc, Cnx) (41) and found a significant decrease in KCC2 mRNA from R6/2 mice (7 wk old) compared with WT, consistent with bioinformatic predictions (27) (Fig. 2F and SI Appendix, Fig. S5).
Fig. 2. KCC2 and NKCC1 protein expression is altered in the hippocampi of HD mice. (A, Top) Representative immunoblot images for KCC2 in protein extracts from samples of hippocampus lysate from WT and R6/2. (A, Bottom) Quantification of total KCC2 (oligomer + monomer) in WT (n = 11) and R6/2 (n = 11) normalized to β-Tubulin (P = 0.0104; Student’s unpaired t test). (B) Similar to A but for WT (n = 8) and YAC128 (n = 8) (P = 0.030; Student’s unpaired t test). (C, Top) Representative immunoblot images for NKCC1 in protein extracts from samples of hippocampus lysates from WT and R6/2. (C, Bottom) Quantification of NKCC1 in WT (n = 11) and R6/2 (n = 11) normalized to β-Tubulin (P = 0.0104; Mann–Whitney test). (D) Similar to C but for WT (n = 8) and YAC128 (n = 8) (P = 0.368; Student’s unpaired t test). (Full blots are presented in SI Appendix, Fig. S3). For all panels, circles indicate values from individual animals. (E) Representative confocal images of hippocampal sections from 7-wk-old WT (Left) and R6/2 (Right) mice double labeled for KCC2 (red) and NeuN (green). Scale bars, 100 μm (Left), 25 μm (Right), and 5 μm (Insets). See SI Appendix, Fig. S4 for quantifications. (F) Relative fold difference of pan-KCC2 (P = 0.011), KCC2a (P = 0.033), KCC2b (P = 0.046), and (G) pan-NKCC1 (P = 0.547), NKCC1a (P = 0.822), NKCC1b (P = 0.852) abundance in R6/2 (n = 5) hippocampi normalized to WT (n = 5) using Student’s unpaired t test. See SI Appendix, Fig. S5 for NKCC1 or KCC2 mRNA quantifications normalized to the geometric means of housekeeping genes. All summary figures represent mean ± SEM. *P < 0.05.

A–C). The same set of control genes, however, showed variability between WT and YAC128 hippocampus and showed no statistical differences in the relative expression of KCC2 mRNA expression (SI Appendix, Fig. S5D). Pan-NKCC1 transcript, but not NKCC1a/b, was also significantly increased in R6/2 hippocampal samples but only compared with one of the control genes, Rpl13a (Fig. 2G and SI Appendix, Fig. S5 A–C).

E GABA Is Depolarized in CA1 Neurons from HD Hippocampi. In mature neurons, relatively high expression of KCC2 results in low levels of intracellular CI⁻ and a hyperpolarized reversal potential for GABA (E GABA) (18, 19). When KCC2 is reduced and/or NKCC1 is increased, E GABA depolarizes and can even render GABA excitatory (21, 42, 43). To address whether the altered protein levels of KCC2 and NKCC1 in the hippocampus of R6/2 mice disrupt the polarity of GABA (hyperpolarizing vs. depolarizing), we recorded E GABA from CA1 pyramidal neurons in hippocampal slices using whole-cell patch-clamp recordings. We found that E GABA from R6/2 mice was significantly depolarized (−58.05 ± 1.68 mV) compared with WT mice (−67.45 ± 1.47 mV; P = 0.0009; Fig. 3 A and B), with no significant differences in the resting membrane potential (WT, −65.88 ± 2.12 mV; R6/2, −67.82 ± 1.23 mV; P = 0.41; Fig. 3D) or synaptic conductance (WT, 5.20 ± 0.98 pS; R6/2, 5.23 ± 0.75 pS; P = 0.97; Fig. 3E). During whole-cell patch-clamp recordings, the contents of the intracellular pipette dialyze with cytoplasm, which can alter the CI⁻ gradient and E GABA. To preserve the intracellular CI⁻ concentration, we performed gramicidin-perforated patch-clamp recordings and again found that the E GABA was significantly depolarized in R6/2 hippocampal neurons compared with WT mice (WT, −71.37 ± 1.70 mV; R6/2, −62.07 ± 3.095 mV; P = 0.02; Fig. 3C). KCC2 function is optimally tested in the presence of a CI⁻ load, which simulates the physiological context during inhibition (44). To examine KCC2 function in the presence of a CI⁻ load to drive transporter function, we loaded the intracellular compartment with CI⁻ through the whole-cell patch pipette (44) (30 mM CI⁻). Again, we found a significant depolarizing shift in E GABA in R6/2 hippocampal neurons compared with WT (WT, −50.73 ± 3.59 mV; R6/2, −39.13 ± 2.32 mV; P = 0.01; Fig. 3C). To determine if E GABA was also depolarized in YAC128 hippocampal neurons (9 wk old), we repeated our whole-cell patch-clamp recordings and again found a significant depolarization of E GABA compared with age-matched controls (WT, −70.93 ± 1.61 mV; YAC128, −62.18 ± 1.72 mV; P = 0.004; Fig. 3 F and G) with no significant change in resting membrane potential (WT, −66.71 ± 2.25 mV; YAC128, −66.62 ± 1.79; P = 0.97; Fig. 3H).

Lastly, we investigated whether altered KCC2 and NKCC1 expression and depolarized E GABA also occur in other brain regions. We performed Western blot analysis and whole-cell patch-clamp recording in the cortex of R6/2 and WT mice. We found an increase in the expression of NKCC1 and a decrease in the expression of KCC2 in the cortices of R6/2 mice relative to that in WT mice (SI Appendix, Fig. S6). E GABA was also depolarized in the somatosensory area L4/5 of the cortex in R6/2 mice compared with WT (SI Appendix, Fig. S7).

GABAergic Inhibition Is Converted into Excitation in R6/2 Mice. A depolarization of E GABA can result in loss of inhibitory drive and increase neuronal excitability. To directly test this, we made cell-attached patch-clamp recordings from CA1 pyramidal neurons and recorded the baseline spontaneous spiking activity in WT and
R6/2 mice. Our results showed that the baseline spiking activity was significantly higher in R6/2 (0.25 ± 0.04 Hz) compared with WT mice (0.07 ± 0.01 Hz) (P = 0.001), which was abolished by application of the GABA_A receptor antagonist bicuculline (Fig. 4A and B).

The depolarization of E_GABA also suggests that GABAergic transmission may be excitatory. To determine if GABA is excitatory in the hippocampus of R6/2 mice, we tested the effect of GABA application on spike frequency. Specifically, we made cell-attached patch-clamp recordings from CA1 pyramidal neurons during bath application of increasing concentrations of exogenous GABA. GABA application at increasing concentrations in WT neurons did not result in spiking, consistent with the known inhibitory action of GABA in the adult brain. However, GABA application at the same concentrations elicited a strong increase in spike frequency in neurons from R6/2 mice (Fig. 4 C and D), which was abolished by application of the GABA_A receptor antagonist bicuculline (SI Appendix, Fig. S8).

**NKCC1 Is Responsible for Depolarized E_GABA in R6/2 Hippocampal Neurons**. We next asked whether the depolarizing shift of the GABA reversal potential is due primarily to an increase in NKCC1 and/or a decrease in KCC2 expression. To answer this question, we first determined the effect of the FDA-approved NKCC1 inhibitor bumetanide on E_GABA. In WT CA1 pyramidal neurons, bumetanide did not significantly change E_GABA (Fig. 5 A–C; E_GABA change: −1.34 ± 1.40 mV; P = 0.37), consistent with the known low expression of NKCC1 in mature neurons (45, 46). In contrast, bumetanide produced a significant hyperpolarization of E_GABA in neurons from R6/2 mice (Fig. 5 A–C; E_GABA change: −6.22 ± 0.76 mV; P = 0.01), consistent with the increased expression of NKCC1 we observed in Fig. 1C. To isolate the contribution of KCC2 to E_GABA, we performed an occlusion experiment using furosemide (1 mM), which is an NKCC1 and KCC2 inhibitor, together with the NKCC1 inhibitor bumetanide.

As expected, E_GABA in WT neurons depolarized after KCC2 inhibition with furosemide (in the presence of bumetanide) (Fig. 5 A–C; E_GABA change: 10.46 ± 1.70 mV; P = 0.0009), while E_GABA did not significantly change in R6/2 neurons (Fig. 5 A–C; E_GABA change: 1.10 ± 1.05 mV; P = 0.33). This suggests that relatively high expression of NKCC1 accounts for the relatively depolarized E_GABA in hippocampal pyramidal neurons of R6/2 mice. We also asked whether inhibitory synapses in R6/2 following treatment with bumetanide are functionally similar to WT animals [in artificial cerebrospinal fluid (aCSF)]. We answered this question by comparing inhibitory synaptic conductance between the two groups and found no significant difference in conductance between WT (aCSF) and R6/2 treated with bumetanide (P = 0.70) (Fig. 5D), suggesting that inhibitory synapses are not significantly altered presymptomatically in the R6/2 hippocampus.

Having determined that bumetanide can hyperpolarize E_GABA in CA1 pyramidal neurons from R6/2 mice, we next asked whether bumetanide can abolish the excitatory action of GABA in hippocampal CA1 pyramidal neurons of HD mice. (A and F) Example current–voltage curves of inhibitory postsynaptic current (IPSC) recorded at different holding potentials from −80 to −40 mV in whole-cell patch-clamp configuration in CA1 hippocampal neurons from WT (black) and R6/2 (green) or YAC128 (orange) mice. Insets show sample traces of the corresponding current induced by electrical stimulation in the presence of the glutamate blocker DNQX (20 µM). (Scale bars: 40 pA and 10 ms for A; 100 pA and 10 ms for F.) E_GABA is shown at the arrow. (B) Summary of individual E_GABA recordings obtained from all IV curves in WT (n = 8) and R6/2 (n = 11) (P = 0.0009; Student’s unpaired t test). (C) Similar to B, but for E_GABA using Cl− loading whole-cell patch-clamp recording between WT (n = 9) and R6/2 (n = 13) (P = 0.010; Student’s unpaired t test); gramicidin perforated recording between WT (n = 6) and R6/2 (n = 7) (P = 0.029; Student’s unpaired t test). (D) Similar to B but for resting membrane potential (RMP) from WT (n = 8) and R6/2 (n = 11) (P = 0.412; Student’s unpaired t test). (E) Similar to B but for synaptic conductance from WT (n = 8) and R6/2 (n = 11) (P = 0.977; Student’s unpaired t test). (G) Similar to B but for E_GABA from WT (n = 7) and YAC128 (n = 13) (P = 0.004; Student’s unpaired t test). (H) Similar to B but for RMP in WT (n = 7) and YAC128 (n = 13) (P = 0.973; Student’s unpaired t test). For all panels, circles indicate values from single recordings across a minimum of three mice, and all summary figures represent mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

**Fig. 3.** E_GABA is depolarized in hippocampal CA1 pyramidal neurons of HD mice. (A and F) Example current–voltage curves of inhibitory postsynaptic current (IPSC) recorded at different holding potentials from −80 to −40 mV in whole-cell patch-clamp configuration in CA1 hippocampal neurons from WT (black) and R6/2 (green) or YAC128 (orange) mice. Insets show sample traces of the corresponding current induced by electrical stimulation in the presence of the glutamate blocker DNQX (20 µM). (Scale bars: 40 pA and 10 ms for A; 100 pA and 10 ms for F.) E_GABA is shown at the arrow. (B) Summary of individual E_GABA recordings obtained from all IV curves in WT (n = 8) and R6/2 (n = 11) (P = 0.0009; Student’s unpaired t test). (C) Similar to B, but for E_GABA using Cl− loading whole-cell patch-clamp recording between WT (n = 9) and R6/2 (n = 13) (P = 0.010; Student’s unpaired t test); gramicidin perforated recording between WT (n = 6) and R6/2 (n = 7) (P = 0.029; Student’s unpaired t test). (D) Similar to B but for resting membrane potential (RMP) from WT (n = 8) and R6/2 (n = 11) (P = 0.412; Student’s unpaired t test). (E) Similar to B but for synaptic conductance from WT (n = 8) and R6/2 (n = 11) (P = 0.977; Student’s unpaired t test). (G) Similar to B but for E_GABA from WT (n = 7) and YAC128 (n = 13) (P = 0.004; Student’s unpaired t test). (H) Similar to B but for RMP in WT (n = 7) and YAC128 (n = 13) (P = 0.973; Student’s unpaired t test). For all panels, circles indicate values from single recordings across a minimum of three mice, and all summary figures represent mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.
A

WT baseline

bicuculline 20 µM

R6/2

B

WT

R6/2

Spike frequency (Hz)

C

WT baseline

GABA 10 µM

GABA 50 µM

GABA 100 µM

R6/2

D

WT

***

R6/2

Spike frequency (Hz)

Fig. 4. R6/2 mice exhibit excitatory responses to the exogenous application of GABA. (A) Sample traces of spontaneous spiking activity in cell-attached patch-clamp configuration in acute slices derived from R6/2 mice and WT littermates at baseline and after bath application of bicuculline (20 µM). (Scale bars: 50 pA and 10 s.) (B) Quantification of the mean ± SEM before and after application of bicuculline (20 µM) in neurons from R6/2 (n = 9; P = 0.003; Wilcoxon matched-pairs signed-rank test) and WT (n = 6; P = 0.049; Student’s paired t test). The difference between spiking activity in WT and R6/2 mice was determined using Mann–Whitney test (P = 0.001). (C) Example traces of spontaneous spiking in R6/2 mice and WT littermates before (baseline) and during bath application of GABA at different concentrations (10, 50, and 100 µM). (Scale bars: 50 pA and 5 s.) (D) Quantification of the mean ± SEM summarizing the effect of GABA application on spontaneous spiking activity in neurons from R6/2 mice (n = 14 for the baseline, n = 12 for GABA 5 µM, n = 11 for 10 µM, and n = 10 for 50 and 100 µM) and WT mice (n = 8 for the baseline and n = 6 for 5, 10, 50, and 100 GABA application). Significant differences were determined by comparison with baseline frequencies using two-way repeated-measure analysis of variance (ANOVA) (F1, 79 = 28.66; P < 0.0001) followed by Sidak’s multiple comparisons test (P < 0.01). (C) Sample traces of spontaneous spiking activity in neurons from WT and R6/2 mice before and after bath application of bicuculline (20 µM). (Scale bars: 50 pA and 10 s.) *P < 0.05, **P < 0.01, ***P < 0.001.

Bumetanide Treatment Rescues Memory Deficits in R2/2 Mice. Human HD patients display deficits in spatial and recognition learning and memory tasks (3, 4), and these behavioral deficits have been replicated in HD mouse models including R6/2 (5, 6, 10). We hypothesized that the GABA-mediated excitation contributes to the learning and memory deficits in R6/2 mice in the early stages of the disease before prominent motor deficits are observed. To test this hypothesis, we evaluated hippocampal-dependent learning and memory tasks after 1 and 2 wk of systemic bumetanide i.p. injection (0.2 mg·kg−1·i.p. daily; Fig. 64). We subjected the mice to the T-maze spontaneous alternation task, the novel object recognition test (NORT), and novel object location test (NOLT), which enable testing of spatial memory performance and long-term memory. Consistent with a previous study (10), we observed that R6/2 mice with saline injection made significantly fewer alternations in the T-maze, indicating poor spatial memory performance (Fig. 6B). Neither group differed in their mean latency to enter the arms, which indicates that R6/2 mice did not have impairments in locomotor activity (SI Appendix, Fig. S9). R6/2 mice also showed poor novelty-discrimination capability in the NORT compared with WT littermates (Fig. 6C), with no object preference in the NOLT (Fig. 6D). Bumetanide administration by daily i.p. injection for either 1 or 2 wk restored the poor T-maze alternation performance of R6/2 mice (Fig. 6B), indicating a recovery of spatial-memory performance. Consistently, R6/2 mice with bumetanide administration performed as well as WT mice in NOLT (Fig. 6D). In NORT, we found that the poor novelty-discrimination capability of R6/2 mice after 24 h was rescued by bumetanide administration (Fig. 6C).

To determine whether the effect of bumetanide is long-lasting, we performed electrophysiological experiments on mice at the end of behavioral testing, after bumetanide had been washed out. We discovered that in the absence of continual bumetanide, E\textsubscript{GABA} in previously administrated R6/2 mice with bumetanide was depolarized to the point where it was in R6/2 mice with saline injection and not significantly different (P = 0.88) (SI Appendix, Fig. S10).

Previous studies have reported that brain penetration of bumetanide may not be optimal following systemic administration, due to its pharmacokinetic properties (47, 48). To address this, we used micro-osmotic pumps implanted into the lateral ventricle of the brain (Fig. 64; 0.8 mg·kg·h) and repeated the behavioral tests. Consistent with i.p. injection, we found that the spatial memory deficits in R6/2 mice were rescued following 1 or 2 wk of administration of bumetanide (Fig. 6E). We also retested the R6/2 mice on NORT and NOLT and found that the brain infusion of bumetanide rescues the memory deficits in R6/2 mice (Fig. 6 F and G).

Discussion

In the present study, we discovered that aberrant hippocampal GABAergic signaling through GABA\textsubscript{A} receptors contributes to the learning and memory deficits in the R6/2 mouse model of HD. Specifically, we found that cation-chloride cotransporter expression is altered in the hippocampus and results in depolarized E\textsubscript{GABA} in both the R6/2 and YAC128 HD mouse models, which renders GABA an excitatory neurotransmitter. Inhibition of the Cl−-importing transporter NKCC1 with the FDA-approved bumetanide restored hyperpolarizing GABAergic inhibition and rescued hippocampal-dependent learning and memory deficits.
Fig. 5. Inhibition of NKCC1 with bumetanide hyperpolarizes $E_{\text{GABA}}$ and reverses the excitatory action of GABA in R6/2 hippocampal neurons. (A) Example current–voltage curves of inhibitory postsynaptic current (IPSC) recorded in whole-cell patch-clamp recording configuration showing $E_{\text{GABA}}$ at different holding potentials from WT mice before, 20 min after perfusion with the NKCC1 inhibitor bumetanide (10 µM), and 20 min after application of bumetanide and the KCC2 and NKCC1 blocker, furosemide (1 mM). Insets show the sample traces of the corresponding current for WT and R6/2. (Scale bars: 100 pA, and 10 ms.) (B) Summary of $E_{\text{GABA}}$ before and after application of bumetanide and furosemide in WT ($n = 7$; bum, $P = 0.373$; bum + fur, $P = 0.0009$; Student's paired t test) and R6/2 ($n = 7$; bum, $P = 0.015$; bum + fur, $P = 0.468$; Wilcoxon matched-pairs signed rank test). Bum, bumetanide; fur, furosemide. (C) Summary graph showing the changes in $E_{\text{GABA}}$ in the presence of bumetanide alone ($P = 0.009$; Student's unpaired t test) and bumetanide and furosemide together ($P = 0.0005$; Student's unpaired t test). (D) Summary of individual synaptic conductance obtained from the slope of the IV curves in WT–aCSF ($n = 7$) and R6/2 treated with bumetanide ($n = 7$) ($P = 0.7038$; Student's unpaired t test). (E) Sample traces of spontaneous spiking activity in WT (Left) and R6/2 (Right) from CA1 neurons in the presence of GABA 100 µM with and without bumetanide (10 µM). (Scale bars: 50 pA and 20 s.) (F) Quantification of spike frequency before and after application of bumetanide in WT ($n = 5$; $P = 0.625$; Student's paired t test) and R6/2 ($n = 9$; $P = 0.0294$; Student's paired t test). The difference between spiking activity between WT and R6/2 mice was determined using Mann–Whitney test ($P = 0.001$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Taken together, these findings suggest bumetanide as a potential therapy for the treatment of early cognitive deficits in human HD patients.

Recent proteomic and bioinformatic data linked (m)Htt and KCC2 (25, 26), and in the present study, we provide the biochemical validation of this protein interaction. We found that KCC2 interacts with both Htt and mHtt and KCC2 protein expression is decreased in the hippocampus of two predominant mouse models of HD (R6/2 and YAC128), consistent with a recent report of decreased KCC2 protein expression in the cortex and striatum of R6/2 mice (49). While a decrease in $Slc12A5$ mRNA accounts for some of the reduction in KCC2 protein expression, there is still KCC2 protein present in hippocampal neurons of HD mouse models, and thus additional mechanisms likely also regulate KCC2 protein expression. The expanded polyglutamine repeat in mHtt is toxic and can cause aberrant protein–protein interactions, which interfere with the function and expression of diverse cellular proteins (50). mHtt aggregates intracellularly, and thus KCC2 may be sequestered into the protein aggregates. However, it is also possible that the mHtt effect on KCC2 is indirect and mediated by additional protein interactions. For example, Htt interacts with brain-type creatine kinase (CKB), an enzyme involved in energy homeostasis (26, 49, 51). Reduced expression of CKB in neurons expressing mHtt is a key event in the pathogenesis of HD and contributes to the neuronal dysfunction associated with HD (51). CKB also interacts, phosphorylates, and activates KCC2 transporter function (52). Therefore, the decreased KCC2 expression in the R6/2 hippocampus may result from the reduced CKB-mediated phosphorylation and activation of KCC2.

In addition to a decrease in KCC2 expression, we also found a significant increase in NKCC1 protein in R6/2 mice, despite
the fact that NKCC1 does not interact with Htt. Increases in NKCC1 have been previously reported when disruptions in KCC2 are observed and are thought to represent a reversion to an immature GABAergic phenotype (38, 53). However, it is also possible that the increase in NKCC1 results from the secondary effects of toxic mHtt. For example, mHtt impairs brain-derived neurotrophic factor (BDNF) gene transcription and reduces the expression of the BDNF in HD patients and mouse models of HD (54). BDNF has been reported to regulate NKCC1 protein expression. NKCC1 was increased in the rat hippocampus of pilocarpine-induced temporal lobe epilepsy, which was decreased by BDNF application (55). Dysregulation of BDNF in
HD could be a reason for altered NKCC1 protein we observed in this study. While we did not observe an increase in NKCC1 protein in YAC128 mice, this may be due to the delay in onset of behavioral symptoms in full-length transgenic HD models (56). The mechanism underlying the increase in NKCC1 protein expression in the R6/2 hippocampus is not clear. While our real-time qPCR results do not indicate an increase in Slc12a2, subtype gene expression difference is challenging to detect. This is especially true in HD, where transcriptional dysregulation is a central pathogenic mechanism underlying the disease.

Presymptomatic HD patients show deficits in attention, working memory, verbal learning, verbal long-term memory, and learning of random associations, and these deficits are the earliest cognitive manifestations in HD-gene carriers (3). These tasks, at least in part, are regulated by the hippocampus (57–60). Hippocampal-dependent behaviors depend on changes in synaptic function and plasticity (61), and mouse models of HD have alterations in hippocampal excitatory synaptic plasticity (5, 7–10, 62–64). Electrophysiological assessment of hippocampal function has shown that basal neurotransmission at hippocampal synapses (CA3–CA1 field excitatory postsynaptic potentials) appears normal, whereas long-term potentiation (LTP) is reduced in transgenic (5) and knock-in (6, 7) models of HD. Our study provides direct evidence that reduced inhibition contributes to the learning and memory deficits in the early stages of HD. KCC2-mediated Cl− regulation directly controls synapse specificity of LTP at CA1 synapses in mature animals (23); thus, it is possible that the reduction in inhibition we observe in the hippocampus of R6/2 mice leads to a reduction of synapse-specific LTP, which in turn is responsible for the reduced performance of R6/2 mice on hippocampal-dependent learning and memory tests.

The alterations in cation-chloride cotransporters in R6/2 hippocampal neurons resulted in a depolarization of E_{GABA} that was significant enough to render GABA excitatory. By performing a pharmacological occlusion experiment, we determined that excitatory GABA primarily resulted from the NKCC1-mediated transport of Cl− into the neuron. Consistently, bumetanide treatment decreased spontaneous and exogenous GABA-induced spiking in R6/2 neurons and rescued hippocampal-associated learning and memory test performance. This is line with previous literature showing that bumetanide reversed excitatory GABA and improves behavioral outcomes in animal models of Down syndrome (21), ASD (22), epilepsy (38), and seizure (65).

Bumetanide has been used previously to improve behavioral phenotypes in patients with various neurological disorders where intracellular Cl− is high and inhibition is disrupted, including ASD (66), schizophrenia (67), and Parkinson’s disease (68). Although bumetanide effectiveness in humans may be further improved with regard to target specificity and blood–brain barrier penetration (47), our behavioral results suggest that a systemic i.p. injection is sufficient to improve learning and memory deficits seen in R6/2 mice. HD is classically considered a motor disorder, however the cognitive and behavioral impairments emerge in the early stages of the disease and precede the motor impairments by ~15 y, producing a significant burden on caregivers (4). Our findings describe a safe pharmacological approach to reduce cognitive dysfunction in HD that can improve patient quality of life and reduce caregiver burden.

Materials and Methods

More detailed information on materials and methods is provided in SI Appendix, SI Materials and Methods.

Animals. All animal procedures were approved by the University of Toronto Animal Care Committee in accordance with the Canadian Council for Animal Care guidelines. All efforts were made to minimize animal suffering and to reduce the number of animals used. Two HD mouse models were used in this study: transgenic R62 mice containing the mutated Htt gene expressing exon 1 of the human Htt gene carrying ~120 ± 5 CAG repeat expansions (31) and YAC128 mice containing full-length human Htt with 128 CAG repeats (37). Both males and females were used for biochemistry, imaging, electrophysiology, and behavioral experiments.

Biochemistry and Imaging. Antibodies used in this study have been described previously, as have the methods for immunoblotting, imaging, and immunoprecipitation (69).

Electrophysiology. To estimate GABA reversal potential (E_{GABA}), we performed whole-cell and perforated patch-clamp recordings. To record GABA spiking activity, we used cell-attached voltage-clamp configuration and perfused the slices with ACSF and GABA at increasing concentration (21).

Behavioral Testing. For behavioral experiments, R62 and WT littermates were randomly assigned to bumetanide (0.2 mg/kg body weight or 2% DMSO in saline; Sigma) for daily i.p. injection or (6 mg/mL bumetanide in 50% DMSO/15% EtOH or 50% DMSO/15% EtOH in ddH2O) for micro-osmotic infusion pumps. On the day of behavioral testing, i.p. injections were given at least 1 h before the beginning of the task. For micro-osmotic pump implantations, we targeted the lateral ventricle to deliver bumetanide via ALZET brain infusion kit cannula (#0008851; ALZET). The pumps were then surgically implanted s.c. on the animal’s back.

Acknowledgments. We thank Michael Hayden (University of British Columbia) for Htt constructs and Thanh Nguyen for technical assistance. This work was supported by the following funding sources: a Canadian Institutes of Health Research (CIHR) grant (to M.A.W.); Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant MOP 491009 and CIHR Grant MOP 496401 (to J.C.K.); an Ontario Graduate Scholarship (OGS) (Z.D.); and a Brazilian National Council for Scientific and Technological Development (CNPq, Conselho Nacional de Desenvolvimento Científico e Tecnológico) postdoctoral fellowship (G.M.P.).

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