Allosteric activation of M_4 muscarinic receptors improve behavioral and physiological alterations in early symptomatic YAC128 mice

Tristano Pancani[a,b], Daniel J. Foster[a,b], Mark S. Moehle[a,b], Terry Jo Bichell[c], Emma Bradley[c], Thomas M. Bridges[a,b], Rebecca Klar[a,b], Mike Poslusney[d], Jerri M. Rook[a,b], J. Scott Daniels[a,b], Colleen M. Niswender[a,b], Carrie K. Jones[a,b], Michael R. Wood[a,b], Aaron B. Bowman[c], Craig W. Lindsley[a,b], Zixiu Xiang[a,b,1], and P. Jeffrey Conn[a,b,1]

[a]Department of Pharmacology, Vanderbilt University, Nashville, TN 37232; [b]Vanderbilt Center for Neuroscience Drug Discovery, Vanderbilt University, Nashville, TN 37232; [c]Department of Neurology, Vanderbilt University, Nashville, TN 37232; and [d]Lieber Institute for Brain Development, Baltimore, MD 21205

Edited by Solomon H. Snyder, Johns Hopkins University School of Medicine, Baltimore, MD, and approved September 29, 2015 (received for review June 29, 2015)

Mutations that lead to Huntington's disease (HD) result in increased transmission at glutamatergic corticostriatal synapses at early presymptomatic stages that have been postulated to set the stage for pathological changes and symptoms that are observed at later ages. Based on this, pharmacological interventions that reverse excessive corticostriatal transmission may provide a novel approach for reducing early physiological changes and motor symptoms observed in HD. We report that activation of the M_4 subtype of muscarinic acetylcholine receptor reduces transmission at corticostriatal synapses and that this effect is dramatically enhanced in presymptomatic YAC128 HD and BACHD relative to wild-type mice. Furthermore, chronic administration of a novel highly selective M_4 positive allosteric modulator (PAM) beginning at presymptomatic ages improves motor and synaptic deficits in 5-mo-old YAC128 mice. These data raise the exciting possibility that selective M_4 PAMs could provide a therapeutic strategy for the treatment of HD.

neurodegenerative | basal ganglia | movement disorder | trinucleotide repeat disorder

Huntington's disease (HD) is a rare and fatal neurodegenerative disease caused by an expansion of a CAG triplet repeat in Htt, the gene that encodes for the protein huntingtin (1, 2). HD is characterized by a prediagnostic phase that includes subtle changes in personality, cognition, and motor function, followed by a more severe symptomatic stage initially characterized by hyperkinesia (chorea), motor incoordination, deterioration of cognitive abilities, and psychiatric symptoms. At later stages of disease progression, patients experience dystonia, rigidity, and bradykinesia, and ultimately death (3–7). The cortex and striatum are the most severely affected brain regions in HD and, interestingly, an increasing number of reports suggest that alterations in cortical and striatal physiology are present in prediagnostic individuals and in young HD mice (6–16).

Striatal spiny projection neurons (SPNs) receive large glutamatergic inputs from the cortex and thalamus, as well as dopaminergic innervation from the substantia nigra. In the healthy striatum, the interplay of these neurotransmitters coordinates the activity of SPNs and striatal interneurons, regulating motor planning and execution as well as cognition and motivation (17, 18). Htt mutations lead to an early increase in striatal glutamatergic transmission, which begins during the asymptomatic phase of HD (12–14) and could contribute to synaptic changes observed in later stages of HD (19, 20). Based on this, pharmacological agents that reduce excitatory transmission in the striatum could reduce or prevent the progression of alterations in striatal synaptic function and behavior observed in symptomatic stages of HD.

Muscarinic acetylcholine receptors (mAChRs), particularly M_4, can inhibit transmission at corticostriatal synapses (21–25). Therefore, it is possible that selective activation of specific mAChR subtypes could normalize excessive corticostriatal transmission in HD. Interestingly, previous studies also suggest that HD is associated with alterations of striatal cholinergic markers, including mAChRs (26–29). We now provide exciting new evidence that M_4-mediated control of corticostriatal transmission is increased in young asymptomatic HD mice and that M_4 positive allosteric modulators (PAMs) may represent a new treatment strategy for normalizing early changes in corticostriatal transmission and reducing the progression of HD.

Results

Age-Dependent Changes in Corticostriatal Glutamatergic Transmission in YAC128 Mice. Consistent with previous studies in YAC128 mice maintained on an FVB background ($YAC128_{FVB}$), BACHD mice, and R6/2 mice (12, 14), we found that YAC128 mice maintained on a C57BL/6 background (hereafter referred to simply as YAC128) display alterations of glutamatergic transmission onto SPNs that follow an age-dependent biphasic pattern. Specifically,

Significance

Huntington's disease (HD) is a devastating neurodegenerative genetic disorder characterized by progressive decline of motor control. Although the genetic mutation responsible for the syndrome associated with HD has been clearly identified, a specific treatment for HD is not yet available. Therefore, there is a tremendous need for new therapeutic approaches and new molecular therapeutic targets. Using HD mouse models, we show age-dependent alterations of corticostriatal transmission paralleled by alterations of M_4 muscarinic acetylcholine receptor (mAChR)-mediated control of corticostriatal glutamate signaling. Also, chronic treatment with the selective M_4 mAChR positive allosteric modulator VU0467154 improves motor and synaptic deficits in 5-mo-old YAC128 mice. This suggests that M_4 may represent a therapeutic target for the treatment of HD.

Author contributions: T.P., T.J.B., E.B., C.K.J., A.B.B., Z.X., and P.J.C. designed research or research tools; T.P., D.J.F., M.S.M., T.M.B., R.K., and J.M.R. performed research; T.M.B., M.P., J.S.D., C.M.N., M.R.W., and C.W.L. contributed new reagents/analytic tools; T.P., D.J.F., and M.S.M. analyzed data; and T.P. and P.J.C. wrote the paper.

Conflict of interest statement: P.J.C. has been funded by the NIH, Johnson & Johnson, AstraZeneca, Bristol-Myers Squibb, Michael J. Fox Foundation, and Seaside Therapeutics. Over the past 3 years, he has served on the Scientific Advisory Boards of Seaside Therapeutics, Michael J. Fox Foundation, Stanley Center for Psychiatric Research at Broad Institute (MIT/Harvard), Karuna Pharmaceuticals, Lieber Institute for Brain Development (Johns Hopkins University), Clinical Mechanism and Proof of Concept Consortium, and Neurobiology Foundation for Schizophrenia and Bipolar Disorder. He is an inventor on patents that protect different classes of muscarinic receptor allosteric modulators.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

[1]To whom correspondence may be addressed. Email: zixiu.xiang@vanderbilt.edu or jeff.conn@vanderbilt.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1512812112/-/DCSupplemental.

we examined input–output (I/O) relations of evoked excitatory postsynaptic currents (eEPSCs) elicited by stimulation of corticostriatal afferents onto SPNs. In postweanlings (20- to 30-d-old mice), the peak amplitude of eEPSCs recorded from YAC128 mice was not significantly different from eEPSCs recorded from WT controls [Fig. 1*A*; no significant effect of genotype in a two-way repeated measures (RM) ANOVA; WT, $n = 5$; YAC128, $n = 5$]. In contrast, there was a significant increase in the amplitudes of eEPSCs recorded from SPNs in slices from 2-mo-old YAC128 mice compared with WT controls [Fig. 1*B*; WT, $n = 11$; YAC128, $n = 18$; main effect of genotype in a two-way RM ANOVA, $F_{(1,112)} = 12.86$, $P = 0.001$]. Similar results were seen in slices from young presymptomatic 2-mo-old BACHD mice and $YAC128_{FVB}$ (Fig. S1 *A* and *B*), suggesting that this change is common to multiple HD mouse strains. Additionally, there was a significant increase in the amplitudes (Fig. S2; Mann–Whitney nonparametric test, $P < 0.05$) but not frequency (Fig. S2) of spontaneous EPSCs in SPNs from YAC128 compared with WT mice. Interestingly, SPNs recorded from 2-mo-old YAC128 also displayed significantly reduced somatic afterhyperpolarization (AHP) current amplitudes compared with WT (Fig. S3*A*; WT, $n = 7$; YAC128, $n = 9$; *t* test, $P < 0.05$), an effect that could also increase excitability of YAC128 SPNs at this age. Consistent with previous reports in other HD models (8, 12, 14), this early alteration of EPSCs was reversed in older animals. Thus, in SPNs recorded in slices from 5- to 6-mo-old YAC128 mice, there was a significant decrease in peak amplitudes of eEPSCs compared with SPNs from WT mice [Fig. 1*C*; WT, $n = 7$; YAC128, $n = 10$; main effect of genotype in a two-way RM ANOVA, $F_{(1,72)} = 4.74$, $P = 0.043$]. AHP amplitude was unchanged in YAC128 at this age (Fig. S3*B*).

The increase in excitatory transmission in the striatum observed here and in other rodent models of HD has been postulated to have excitotoxic effects and induce aberrant synaptic remodeling that could play an important role in the changes in striatal function and motor behavior observed in older HD mice (8, 14). Furthermore, the reduced excitatory synaptic transmission observed in 5-mo-old YAC128 mice (Fig. 1*C*) is consistent with previous studies showing loss of corticostriatal transmission in older HD animals, which coincides with measurable deficits in motor function. Interestingly, we found that 5-mo-old YAC128 mice also show motor deficits in an accelerated rotarod test (Fig. S4*A*; *t* test, $P < 0.01$) that are correlated with eEPSC amplitudes at the corticostriatal synapse (Fig. S4 *B* and *C*; $R^2 = 0.47$, $P = 0.03$). These data are consistent with the hypothesis that loss of corticostriatal transmission represents a pathophysiological deficit in striatal function that coincides with the appearance of deficits in motor coordination in older HD mice.

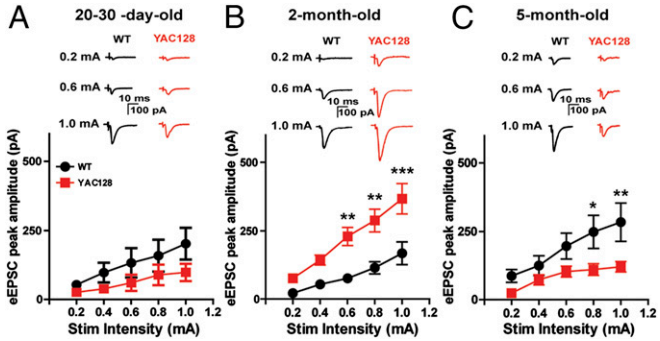

Fig. 1. Age-dependent biphasic alteration in corticostriatal glutamatergic transmission. (*Top*) Representative traces of EPSCs evoked with increasing stimulus intensities (0.2, 0.6, and 1.0 mA) in dorsal SPNs from 20- to 30-d-old (*A*), 2-mo-old (*B*), and 5-mo-old (*C*) WT (black) and YAC128 (red) mice. (*Bottom*) Input–output graphs showing increased eEPSC amplitude in SPNs from 2-mo-old YAC128 (*B*) and a decrease in 5-mo-old YAC128 compared with WT (*C*). No alterations are seen in 20- to 30-d-old animals (*A*) (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Bonferroni posttest). Data are reported as mean ± SEM.

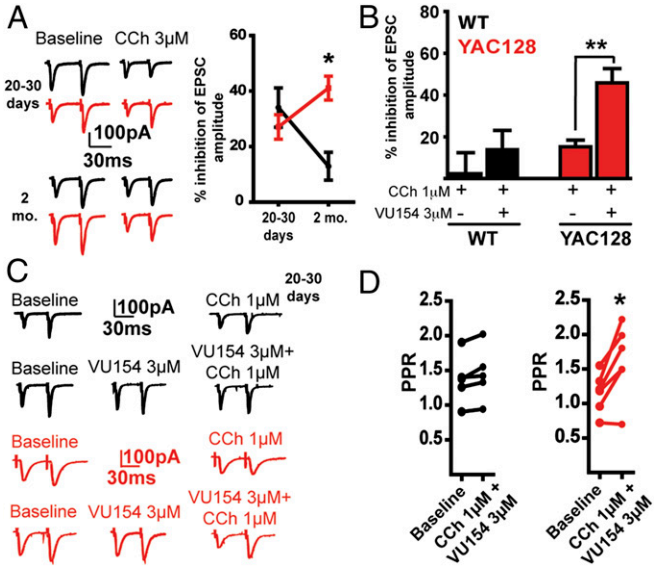

Fig. 2. Age-dependent increase in M_4-mediated modulation of corticostriatal glutamatergic transmission. (*A*, *Left*) Representative traces showing the inhibitory effect of 3 μM CCh on eEPSC peak amplitude in SPNs from WT (black) and YAC128 (red) in 20- to 30-d-old and 2-mo-old mice. (*A*, *Right*) Graph summarizing the effects of 3 μM CCh on eEPSCs recorded in SPNs from 20- to 30-d-old and 2-mo-old mice (*$P < 0.05$, paired *t* test). (*B*) Bar graph summarizing the potentiating effect of 3 μM VU0467154 on the CCh (1 μM)-mediated inhibition of eEPSC amplitude in WT and YAC128 mice in SPNs from WT and YAC128 mice (**$P < 0.01$, *t* test). (*C*) Representative traces showing the inhibitory effect of CCh alone or in combination with 3 μM VU0467154 (VU154) on eEPSC peak amplitude. (*D*) Graph showing the effect of 1 μM CCh in combination with 3 μM VU0467154 on the paired-pulse ratio in SPNs from WT (black) and YAC128 (red) (*$P < 0.05$, paired *t* test). Data are reported as mean ± SEM.

Age-Dependent Increase in Corticostriatal Glutamatergic Transmission Can Be Modulated by Potentiation of M_4. Activation of M_4 mAChRs induces a robust inhibition of transmission at corticostriatal synapses in postweanling/prepubertal (20- to 30-d-old) mice (25). We now report that the nonselective mAChR agonist carbachol (CCh) has a similar effect on excitatory transmission in striatal slices from 20- to 30-d-old YAC128 mice, and the inhibitory effect of CCh (3 μM) on eEPSC peak amplitude is not significantly different in SPNs from YAC128 mice compared with the inhibition observed in age-matched WT mice (Fig. 2*A*; WT, $n = 6$; YAC128, $n = 8$; *t* test, $P > 0.05$). Interestingly, we found that mAChR-mediated inhibition of excitatory transmission undergoes a developmental decline in striatal slices from WT mice such that mAChR activation has minimal effect on corticostriatal transmission at 2 mo [Fig. 2*A*; WT, $n = 8$; YAC128, $n = 5$; two-way ANOVA reports statistically significant interaction between age and genotype, $F_{(1,22)} = 9.49$, $P = 0.005$ and a main effect of genotype, $F_{(1,22)} = 5.94$, $P = 0.02$]. Surprisingly, this developmental decline is completely lost in YAC128 mice (Fig. 2*A*). In contrast to effects in WT mice, CCh induced a more robust decrease in eEPSC amplitude in SPNs from 2-mo-old relative to 20- to 30-d-old YAC128 mice. Thus, at 2 mo of age, the inhibitory effect of 3 μM CCh was significantly greater in YAC128 mice compared with WT (*t* test, $P < 0.05$). Similar results were seen in slices from 2-mo-old BACHD and $YAC128_{FVB}$ mice (Fig. S1). Increasing the CCh concentration resulted in larger inhibition in YAC128 compared with WT (10 μM CCh, Fig. S5*A*).

Although M_4 is responsible for regulation of corticostriatal transmission in prepubertal WT mice (25), it is possible that M_4 is not the predominant mAChR subtype responsible for the increased effect of CCh in older YAC128 mice. To determine whether the enhanced inhibitory effect of CCh in SPNs from 2-mo-old YAC128 mice was mediated by M_4, we determined the potentiating effect of VU0467154, a highly selective M_4 PAM discovered in our laboratory (30), on the CCh-mediated inhibition of glutamatergic transmission. Bath application of VU0467154 (3 μM) potentiated the inhibitory effect of a submaximal concentration of CCh (1 μM) on peak eEPSC amplitude in YAC128 SPNs (Fig. 2 *B* and *C*; $N_{CCh} = 7$; $N_{CCh+VU154} = 6$; *t* test, $P < 0.01$) but not in neurons from WT mice ($N_{CCh} = 5$; $N_{CCh+VU154} = 5$; *t* test, $P > 0.05$). Moreover, as shown in younger mice (25), coapplication of CCh and VU0467154 induced a significant increase in the paired-pulse ratio (PPR) of eEPSCs in SPNs from YAC128 mice (Fig. 2*D*; paired *t* test, $P < 0.05$), suggesting that the M_4-mediated inhibition of eEPSCs is due to a decrease in presynaptic release probability. Similar results were seen in 2-mo-old BACHD mice, where 3 μM VU0467154 was able to potentiate the inhibitory effect of 3 μM CCh on corticostriatal transmission (Fig. S1 *C* and *D*). To definitively confirm a role for M_4 in mediating this response, we crossed YAC128 mice with M_4 knockout mice (YAC128/$M_4^{-/-}$ mice) and performed similar experiments in YAC128 mice in which M_4 had been genetically deleted. CCh had no effect on eEPSCs in slices from 2-mo-old YAC128/$M_4^{-/-}$ mice (Fig. 3*B*; $n = 6$; *t* test baseline vs. 3 μM CCh, $P > 0.05$). This is similar to our previous finding showing that CCh has no effect in $M_4^{-/-}$ mice on a non-HD C57BL/6 background (25), and confirms that M_4 is the mAChR subtype responsible for modulation of glutamatergic transmission at corticostriatal synapses in young adult YAC128 mice (25). Interestingly, in addition to a lack of response to CCh, eEPSC peak amplitude was significantly increased in slices from YAC128/$M_4^{-/-}$ animals compared with YAC128 mice [Fig. 3 *A* and *B*; YAC128, $n = 10$; YAC128/$M_4^{-/-}$, $n = 7$; main effect of genotype, $F_{(1,60)} = 6.58$, $P = 0.02$ in a two-way RM ANOVA]. This raises the possibility that the increase in M_4 modulation of excitatory transmission in YAC128 mice could represent a compensatory change that helps dampen the increased excitatory transmission at this synapse. There was no significant difference in CCh-induced inhibition in SPNs from YAC128 mice compared with WT age-matched controls in slices from 5- to 6-mo-old mice (Fig. S5*B*; *t* test, $P > 0.05$), an age when EPSCs were reduced rather than increased in YAC128 relative to WT mice.

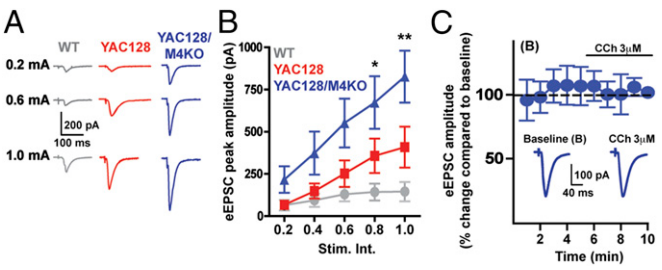

Fig. 3. Corticostriatal glutamatergic transmission is increased in 2-mo-old YAC128/$M_4^{-/-}$ mice. (*A*) Representative eEPSC traces elicited with 1-mA stimulation intensity in SPNs from YAC128 (red) and YAC128/$M_4^{-/-}$ (blue), as well as in WT (gray) shown for comparison. (*B*) I/O graph showing increased eEPSCs elicited with increasing stimulation intensities (0.2–1 mA) in SPNs from YAC128/$M_4^{-/-}$ compared with YAC128 mice (*$P < 0.05$, **$P < 0.01$, Bonferroni posttest). WT I/O curve is shown for comparison. (*C*) No effect of 3 μM CCh on peak eEPSC amplitude was seen in SPNs from YAC128/$M_4^{-/-}$. (*Insets*) Representative traces recorded from baseline and during CCh application. Data are reported as mean ± SEM.

Chronic Treatment with VU0467154 Rescues Synaptic and Motor Alterations in Early Symptomatic YAC128 Animals. If an early and prolonged increase in excitatory synaptic transmission in the striatum is responsible for initiating the progressive deficits in synaptic function and behavioral deficits seen in YAC128 mice at later ages, inhibiting this excessive glutamate release by chronic administration of an M_4 PAM such as VU0467154 may prevent the development of deficits in excitatory synaptic function and motor performance observed at 5 mo of age in these mice. To test this hypothesis, we determined the effects of chronic administration of the M_4 PAM VU0467154 [10 mg/kg, intraperitoneally (i.p.), once daily] on the appearance of synaptic and motor deficits in YAC128 mice. The dose and dosing interval were chosen based on the compound's predicted pharmacokinetics (PK) at steady state using previously reported acute PK data together with concentration–efficacy relationship data (30). Based on this, we determined that dosing animals once daily at a dose of 10 mg/kg should provide constant central nervous system (CNS) exposure for the duration of the dosing period. WT and YAC128 mice were administered vehicle (Vh; 10% vol/vol Tween 80 in saline) or VU0467154 (10 mg/kg) once daily starting at 2 mo of age, when YAC128 display increased corticostriatal glutamatergic transmission but no signs of motor incoordination or decreased locomotion [Fig. S6*B*; *t* test, $P > 0.05$ (n.s., not significant) for both behavioral tests in YAC128 compared with WT], and daily administration was continued until animals were tested at 5 mo of age. WT and YAC128 animals received their last administration of VU0467154 or Vh at least 48 h before testing to allow clearance of the M_4 PAM. Previous PK analysis of VU0467154 predicts that the compound should be cleared from the brain and plasma by 48 h and that residual levels will be well below those required to potentiate M_4 responses at this time point (30). To directly determine the effective residual brain levels of VU0467154, 48 h after the last dose, mice were injected daily for 14 d with 10 mg/kg VU0467154. Using mass spectrometry, we determined that, at the 48-h time point, the measured unbound concentrations of VU0467154 were 3.15 ± 1.7 nM, which is below the active concentration of this compound at potentiating M_4 (30).

To determine whether chronic treatment with VU0467154 counteracts the loss of corticostriatal glutamatergic transmission observed in 5-mo-old mice, we monitored eEPSC peak amplitude elicited at increasing stimulus intensities onto SPNs in slices obtained from these chronically treated animals. Interestingly, chronic treatment with VU0467154 completely prevented the loss of corticostriatal transmission in slices from 5-mo-old YAC128 [Fig. 4 *A* and *B*; WT_{Vh}, $n = 9$; WT_{VU154}, $n = 12$; $YAC128_{Vh}$, $n = 10$; $YAC128_{VU154}$, $n = 12$; main effect of treatment in a two-way RM ANOVA, Vh-treated YAC128 vs. VU0467154-treated YAC128, $F_{(1,60)}$, $P = 0.037$] but had no significant effect on eEPSCs recorded from WT mice. In the same mice, the effect of 3 μM CCh on eEPSC amplitude was similar in SPNs from all treatment groups (Fig. S7; no main effect in one-way ANOVA) and slightly but not significantly increased in YAC128 mice compared with WT. Additionally, previous studies have shown that dopamine (DA) release is impaired in HD models when the animals reach ages where they show motor symptoms (31–33). Thus, we used fast-scanning cyclic voltammetry (FSCV) to monitor evoked DA release in striatal slices from WT and YAC128 mice. Consistent with previous studies in other mouse models of HD, we found that in slices from Vh-treated YAC128 mice, evoked dopamine release (overflow) was significantly reduced compared with Vh-treated WT [Fig. 4*E*; Bonferroni posttest, $P < 0.05$; significant differences between treatment groups were detected at 800-μA stimulation intensity in one-way ANOVA, $F_{(3,22)} = 3.73$, $P = 0.03$]. In YAC128 mice chronically treated with VU0467154, dopamine overflow was increased compared with Vh-treated YAC128 (Fig. 4 *E* and *F*; Bonferroni posttest, $P < 0.05$), whereas no difference was noted in recordings from Vh- compared with VU0467154-treated WT mice. These findings suggest that chronic administration of

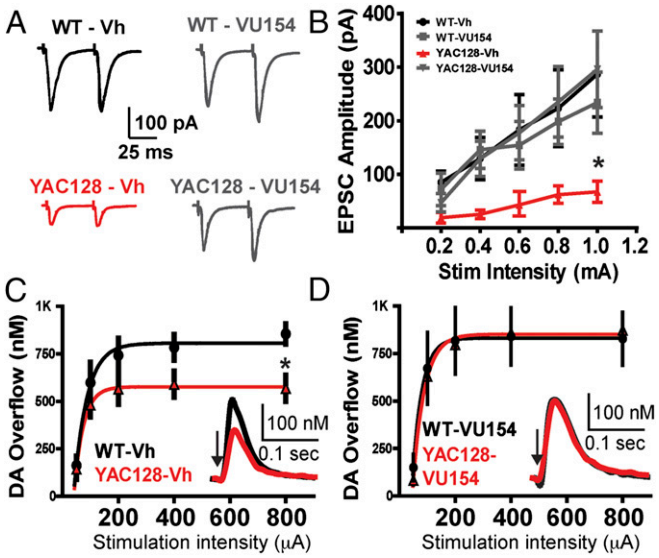

Fig. 4. Chronic VU0467154 improves striatal glutamatergic and dopaminergic signaling in early symptomatic YAC128 mice. (*A*) Representative traces showing eEPSCs evoked with 1-mA stimulus intensity recorded from dorsal SPNs from mice chronically treated with either VU0467154 (VU154) or vehicle. (*B*) I/O graph showing decreased corticostriatal glutamatergic transmission in YAC128 mice chronically treated with VU0467154 (YAC128-VU154; $^*P < 0.05$, Bonferroni posttest). (*C* and *D*) Graphs showing DA release (overflow) evoked with increasing stimulus intensities (25–800 μA) in Vh-treated (*C*) and VU154-treated (*D*) WT and YAC128 mice. Vh-treated YAC128 mice display a consistent decrease in DA release across all stimulation intensities, with a significant decrease observed at 800-μA stimulation intensity ($^*P < 0.05$, Bonferroni posttest) compared with Vh-treated WT. No statistical difference was seen in VU154-treated WT or YAC128 mice (*D*). (*Insets*) Representative traces showing an electrically evoked (800-μA stimulation; arrows) DA rise in slices from Vh-treated WT (black) and YAC128 mice (red) and from VU0467154-treated WT and YAC128 mice. Data are reported as mean ± SEM.

VU0467154 completely counteracts the appearance of deficits in both glutamatergic and dopaminergic transmission that are observed in early symptomatic YAC128 mice.

We next performed behavioral studies to determine whether the M_4 PAM would prevent the onset of behavioral motor deficits in 5-mo-old YAC128 mice. At 5 mo, Vh-treated YAC128 mice displayed a significant deficit in the accelerated rotarod test, as assessed by time to fall (TTF), compared with Vh-treated WT mice [Fig. 5*A*; WT_{Vh}, $n = 9$; WT_{VU154}, $n = 11$; $YAC128_{Vh}$, $n = 11$; $YAC128_{VU154}$, $n = 10$; first trial on test day, main effect of genotype in a two-way ANOVA, $F_{(1,37)} = 7.57$, $P = 0.009$]. Worsening on the rotarod test cannot be attributed to an increase in weight often described in older YAC128 animals, because mouse weight was comparable among all four treatment groups at this age (Table S1; no significant effect in a two-way ANOVA). Remarkably, 5-mo-old YAC128 mice chronically treated with VU0467154 at 10 mg/kg performed significantly better in the accelerated rotarod test compared with Vh-treated YAC128 mice (Fig. 5*A*, *Right*; Bonferroni posttest, $P < 0.05$) and showed no significant deficits compared with WT controls (Bonferroni posttest, $P > 0.05$). As discussed above, residual levels of VU0467154 are below pharmacologically active concentrations at the time of testing. However, to confirm that the reversal of rotarod deficits could not be attributed to residual VU0467154, we determined the effect of acute administration of 10 mg/kg (i.p.), VU0467154, given at least 45 min before testing, a time when VU0467154 levels in the CNS are at their peak (30). As in the original cohort of WT and YAC128 mice, Vh-treated YAC128 mice showed deficits in rotarod performance relative to WT controls. Acute administration of VU0467154 does not affect accelerated rotarod performance in symptomatic YAC128 mice [Fig. S8; t test, $P > 0.05$ between Vh- ($n = 4$) and VU0467154-treated ($n = 4$) mice], suggesting that acute exposure to the M_4 PAM is without effect.

In addition to the accelerated rotatod test, we also monitored performance in the open-field test. We observed a significant decline in locomotor activity with age in both WT and YAC128 mice (Fig. 5*B* and Fig. S6*B*, *Right*; *t* test, $P < 0.0001$). Additionally, at 5 mo of age, significantly lower levels of locomotor activity were detected in Vh-treated YAC128 compared with Vh-treated WT [Fig. 5*B*; WT_{Vh}, $n = 10$; WT_{VU154}, $n = 10$; $YAC128_{Vh}$, $n = 9$; $YAC128_{VU154}$, $n = 11$; main effect of genotype in a two-way ANOVA, $F_{(1,36)} = 4.51$, $P = 0.047$]. A significant interaction between treatment and genotype was also noted [two-way ANOVA, $F_{(1,36)} = 7.84$, $P = 0.008$]. Remarkably, 5-mo-old YAC128 mice chronically treated with VU0467154 displayed a significantly greater locomotor mutant huntingtin (mHtt) activity compared with Vh-treated YAC128 mice (Fig. 5*B*; Bonferroni posttest, $P < 0.05$). Interestingly, and in accordance with the locomotor activity data, chronic treatment with VU0467154 was also able to improve exploratory behavior, monitored as rearing counts. The effect was evident only in VU0467154-treated YAC128 mice, and the M_4 PAM did not significantly alter rearing behavior in 5-mo-old WT mice (Fig. S6*D*; significant interaction in a two-way ANOVA, $P < 0.05$; Bonferroni posttest shows a significant effect of VU467154 in YAC128 but not in WT mice, $P < 0.05$).

No Detectable Pathological Alterations in Striatum from YAC128 Mice. The data presented above suggest that chronic exposure to VU0467154 may reduce the early deficits in synaptic transmission and motor function that appear in 5-mo-old YAC128 mice. Interestingly, previous studies suggest that these deficits occur at an age when there are no clear histopathological changes in the striatum (34). We performed additional histology studies to confirm a lack of histopathological changes at this age. Neuron densities (NeuN-positive cells per field) were similar between Vh-treated WT and YAC128 mice at 5 mo of age. VU0467154 chronic treatment did not change the number of NeuN-positive neurons compared with Vh-treated animals (Figs. S9 and S10; one-way ANOVA with a Tukey's post hoc test). Moreover, we did not detect a significant increase in Fluoro-Jade C–positive cells in the striatum from 5-mo-old Vh-treated YAC128 mice compared

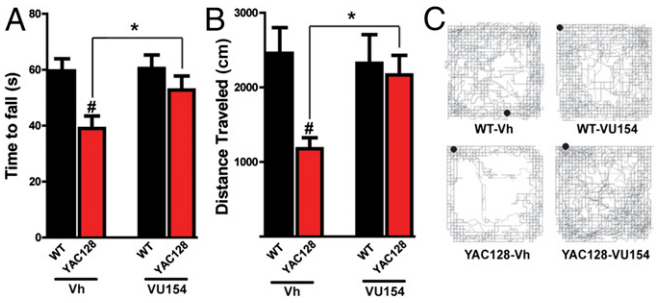

Fig. 5. Chronic administration of VU0467154 improves motor coordination and locomotor activity in 5-mo-old YAC128 mice. (*A*) Vh-treated YAC128 mice displayed reduced time spent on the rotarod compared with Vh-treated WT animals ($^{\#}P < 0.05$, *t* test). Chronic treatment with VU0467154 significantly increased the time spent on the rotarod in YAC128 but not in WT mice ($^*P < 0.05$, Bonferroni posttest). (*B*) Five-month-old Vh-treated YAC128 animals displayed reduced total distance traveled ($^{\#}P < 0.05$, *t* test) compared with Vh-treated WT mice. Chronic treatment with VU0467154 (10 mg/kg) improved locomotor activity in YAC128 but not in WT animals ($^*P < 0.05$, Bonferroni posttest). (*C*) Representative locomotor activity paths from one representative 5-mo-old animal per treatment group. Dots represent the position of the mouse at the end of the experiment. Data are reported as mean ± SEM.

with WT (Figs. S9 and S10) or YAC125 mice chronically treated with VU0467154 (Figs. S9 and S10). Finally, nuclei from Vh-treated YAC128 striata did not reveal significant nuclear mHtt inclusions. Also, there was no effect of chronic VU0467154 treatment on mHtt inclusions (Figs. S10 and S11).

Discussion

The striatum serves as a major relay center of cortical information through the basal ganglia and is critically involved in regulating motor function and cognition (17, 18). Due to its pivotal importance in these domains, changes in striatal physiology can contribute to multiple neurological diseases, including Huntington's disease, Parkinson's disease, dystonia, and others. An increasing number of reports suggest that changes in striatal synaptic physiology, including changes at glutamatergic, dopaminergic, and cholinergic synapses, are evident beginning in the prediagnostic phase of HD (5, 6, 8–14, 35–37). These changes may contribute to early motor, cognitive, and psychiatric abnormalities, and could set the stage for the extensive pathophysiological alterations appearing later in the striatum.

We report that changes in glutamatergic transmission in the striatum follow an age-dependent biphasic pattern in YAC128 mice maintained on a C57BL/6 background, as previously described in $YAC128_{FVB}$ mice (12, 14). Interestingly, we found that the large increase in excitatory transmission observed in young adult YAC128 and BACHD (but also in $YAC128_{FVB}$) animals is accompanied by an increase in the ability of M_4 to inhibit excitatory synaptic responses in the striatum. Additionally, genetic deletion of M_4 in YAC128 mice leads to a further increase in excitatory synaptic responses, suggesting that increased activity of M_4 at the corticostriatal synapse could provide a compensatory mechanism that helps reduce excessive excitatory transmission in young HD mice. Finally, we also found that chronic treatment with the highly selective M_4 PAM VU0467154 prevents or delays the onset of deficits in both striatal glutamatergic and dopaminergic signaling that are observed in 5-mo-old YAC128 mice and also improves motor symptoms detected at this age. An early increase in corticostriatal glutamatergic transmission has been described previously in multiple mouse models of HD (8, 11–14). These changes could contribute to early vulnerability of SPNs to excitotoxicity and synaptic alterations (11).

We and others have shown that M_4 receptors play an essential role in the mAChR-mediated modulation of corticostriatal glutamatergic transmission in postweanling mice by a presynaptic mechanism (24, 25). Based on this and previous findings describing alterations in striatal cholinergic activity in HD (26–29), we hypothesized that activation of M_4 could reduce excessive excitatory transmission in YAC128 mice. It was especially interesting to find that M_4-mediated modulation of transmission at the corticostriatal synapse is developmentally regulated in adult WT mice. This age-dependent decrease in M_4 function seen in WT mice is reminiscent of the developmental switch between metabotropic glutamate receptor 8 (mGlu8) and mGlu7 in the hippocampus (38). However, the loss of M_4 function at the corticostriatal synapse does not likely represent a developmental switch to a role for another mAChR subtype, because the response to a nonselective mAChR agonist is absent in $M_4^{-/-}$ mice. Other neurotransmitters could be involved.

One of the most exciting aspects of our studies was that the developmental regulation of M_4 function is not present in age-matched 2-mo-old YAC128 mice. Thus, cholinergic inputs, and specifically M_4 activation, continue to play a major role in regulating corticostriatal transmission in young adult YAC128 mice. WT Htt is involved in the transcription of many repressor element 1- or neuron-restrictive silencer element (RE1/NRSE)–controlled genes, including M_4 (39), and it is possible that the increased inhibitory role of M_4 could be mediated by transcriptional alterations due to the Htt mutation.

Although the current data raise the exciting possibility that M_4 may provide a target for reducing and/or delaying the development of early physiological alterations associated with Htt mutations, a causal link between the increased glutamatergic transmission at early ages and the loss of striatal glutamatergic and dopaminergic transmission in older symptomatic YAC128 mice has not been firmly established. Sustained increases in corticostriatal excitatory activity can trigger excitotoxic damage in the striatum of WT mice (40); however, no significant cell death has been demonstrated in YAC128 mice at early asymptomatic age and up to 12 mo (14). Likewise, it is important to point out that M_4 has multiple actions in the striatum that are unrelated to effects on corticostriatal transmission, including direct effects on striatal SPNs (41), effects on DA release (30, 42), and effects on cholinergic interneurons. These actions of M_4 PAMs could also contribute to the ability of M_4 PAMs to reduce age-related progression of HD-associated physiological alterations. Nonetheless, the increase in corticostriatal transmission could contribute to alterations of both glutamatergic (43) and dopaminergic (44) synaptic transmission. Moreover, loss of striatal DA activity seen in symptomatic animals has been associated with aberrant plasticity (45), alterations of dendritic spines, and also alteration of glutamatergic signaling (46, 47). Regardless of the exact mechanisms by which M_4 PAMs elicit their effects, the present studies clearly establish utility of these compounds in reversing both early changes in corticostriatal transmission and age-related development of synaptic and behavioral deficits in the initial phase of the symptomatic stage in YAC128 mice. This provides a potential therapeutic approach that could slow the progression of this devastating disorder.

Importantly, the late "hypoglutamatergic" state has been described in previous studies, showing that symptomatic stages are characterized by loss of corticostriatal presynaptic and postsynaptic glutamatergic function and also by alteration in synaptic plasticity (8, 11, 14, 48–50). This corticostriatal "disconnection" (8) could be responsible, at least in part, for motor and cognitive dysfunction seen at later ages. Likewise, deficits in DA signaling in HD patients (51, 52) and mouse models (31–33) have been postulated to be partially responsible for the decreases in locomotor activity and motor coordination observed in later stages of HD and in HD models. Thus, it is especially encouraging to find that M_4 PAMs reverse each of these electrophysiological and behavioral alterations in a mouse model of HD.

Whereas the current study focused on assessment of the effects of chronic VU0467154 on behavioral and electrophysiological markers only in early symptomatic mice (5-mo-old mice), in future studies it will be important to extend these exciting findings by determining the time course of the effects seen with chronic VU0467154 treatment on behavioral, electrophysiological, and pathological markers of the disease. It is also important to consider the possibility that early increases in striatal glutamatergic transmission could contribute to some of the subtle motor and cognitive aberrations seen in early-stage HD patients (14, 53–55). If so, it is possible that M_4 PAMs could provide symptomatic benefits also at early stages of HD.

The risk of developing HD can be clearly determined via early genetic testing, and recent research is aimed at determining objective biomarkers to improve the diagnosis of HD (7, 15, 16, 56, 57). HD patients could represent an ideal target for a therapeutic strategy involving chronic exposure to M_4 PAMs beginning in the prodromal stages of the disease.

Materials and Methods

All experimental procedures were approved by the Vanderbilt University Institutional Animal Care and Use Committee. All efforts were made to minimize animal suffering. We used male YAC128 mice maintained on the C57BL/6 background (YAC128 in the text). For detailed description of the methods, see *SI Materials and Methods*.

ACKNOWLEDGMENTS. This work is supported by the National Institutes of Health (U01MH067965; to P.J.C.), National Institute of Neurological Disorders and Stroke (R01 NS065867; to Z.X.), National Institute of Mental Health (5P56-NS071669; to C.W.L.), and NIH/National Institute of Environmental Health Sciences (R01 ES016931; to A.B.B.).

1. The Huntington's Disease Collaborative Research Group (1993) A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. *Cell* 72(6):971–983.
2. Beglinger LJ, et al.; Huntington Study Group Investigators (2010) Earliest functional declines in Huntington disease. *Psychiatry Res* 178(2):414–418.
3. Siemers E, et al. (1996) Motor changes in presymptomatic Huntington disease gene carriers. *Arch Neurol* 53(6):487–492.
4. Kirkwood SC, et al. (2000) Subtle changes among presymptomatic carriers of the Huntington's disease gene. *J Neurol Neurosurg Psychiatry* 69(6):773–779.
5. Walker FO (2007) Huntington's disease. *Semin Neurol* 27(2):143–150.
6. Paulsen JS, et al.; Predict-HD Investigators and Coordinators of the Huntington Study Group (2008) Detection of Huntington's disease decades before diagnosis: The Predict-HD study. *J Neurol Neurosurg Psychiatry* 79(8):874–880.
7. Ross CA, et al. (2014) Huntington disease: Natural history, biomarkers and prospects for therapeutics. *Nat Rev Neurol* 10(4):204–216.
8. Cepeda C, et al. (2003) Transient and progressive electrophysiological alterations in the corticostriatal pathway in a mouse model of Huntington's disease. *J Neurosci* 23(3):961–969.
9. DiProspero NA, et al. (2004) Early changes in Huntington's disease patient brains involve alterations in cytoskeletal and synaptic elements. *J Neurocytol* 33(5):517–533.
10. André VM, Cepeda C, Venegas A, Gomez Y, Levine MS (2006) Altered cortical glutamate receptor function in the R6/2 model of Huntington's disease. *J Neurophysiol* 95(4):2108–2119.
11. Graham RK, et al. (2009) Differential susceptibility to excitotoxic stress in YAC128 mouse models of Huntington disease between initiation and progression of disease. *J Neurosci* 29(7):2193–2204.
12. Joshi PR, et al. (2009) Age-dependent alterations of corticostriatal activity in the YAC128 mouse model of Huntington disease. *J Neurosci* 29(8):2414–2427.
13. Milnerwood AJ, et al. (2010) Early increase in extrasynaptic NMDA receptor signaling and expression contributes to phenotype onset in Huntington's disease mice. *Neuron* 65(2):178–190.
14. André VM, et al. (2011) Differential electrophysiological changes in striatal output neurons in Huntington's disease. *J Neurosci* 31(4):1170–1182.
15. Tabrizi SJ, et al.; TRACK-HD Investigators (2013) Predictors of phenotypic progression and disease onset in premanifest and early-stage Huntington's disease in the TRACK-HD study: Analysis of 36-month observational data. *Lancet Neurol* 12(7):637–649.
16. Paulsen JS, et al.; PREDICT-HD Investigators and Coordinators of the Huntington Study Group (2014) Prediction of manifest Huntington's disease with clinical and imaging measures: A prospective observational study. *Lancet Neurol* 13(12):1193–1201.
17. Graybiel AM, Aosaki T, Flaherty AW, Kimura M (1994) The basal ganglia and adaptive motor control. *Science* 265(5180):1826–1831.
18. Crossman AR (2000) Functional anatomy of movement disorders. *J Anat* 196(Pt 4): 519–525.
19. Canales JJ, et al. (2002) Shifts in striatal responsivity evoked by chronic stimulation of dopamine and glutamate systems. *Brain* 125(Pt 10):2353–2363.
20. Mark KA, Soghomonian JJ, Yamamoto BK (2004) High-dose methamphetamine acutely activates the striatonigral pathway to increase striatal glutamate and mediate long-term dopamine toxicity. *J Neurosci* 24(50):11449–11456.
21. Malenka RC, Kocsis JD (1988) Presynaptic actions of carbachol and adenosine on corticostriatal synaptic transmission studied in vitro. *J Neurosci* 8(10):3750–3756.
22. Pakhotin P, Bracci E (2007) Cholinergic interneurons control the excitatory input to the striatum. *J Neurosci* 27(2):391–400.
23. Pisani A, Bernardi G, Ding J, Surmeier DJ (2007) Re-emergence of striatal cholinergic interneurons in movement disorders. *Trends Neurosci* 30(10):545–553.
24. Higley MJ, Soler-Llavina GJ, Sabatini BL (2009) Cholinergic modulation of multivesicular release regulates striatal synaptic potency and integration. *Nat Neurosci* 12(9):1121–1128.
25. Pancani T, et al. (2014) M4 mAChR-mediated modulation of glutamatergic transmission at corticostriatal synapses. *ACS Chem Neurosci* 5(4):318–324.
26. Spokes EG (1980) Neurochemical alterations in Huntington's chorea: A study of post-mortem brain tissue. *Brain* 103(1):179–210.
27. Vetter JM, et al. (2003) Mice transgenic for exon 1 of Huntington's disease: Properties of cholinergic and dopaminergic pre-synaptic function in the striatum. *J Neurochem* 85(4):1054–1063.
28. Smith R, et al. (2006) Cholinergic neuronal defect without cell loss in Huntington's disease. *Hum Mol Genet* 15(21):3119–3131.
29. Farrar AM, Callahan JW, Abercrombie ED (2011) Reduced striatal acetylcholine efflux in the R6/2 mouse model of Huntington's disease: An examination of the role of altered inhibitory and excitatory mechanisms. *Exp Neurol* 232(2):119–125.
30. Bubser M, et al. (2014) Selective activation of M4 muscarinic acetylcholine receptors reverses MK-801-induced behavioral impairments and enhances associative learning in rodents. *ACS Chem Neurosci* 5(10):920–942.
31. Hickey MA, Reynolds GP, Morton AJ (2002) The role of dopamine in motor symptoms in the R6/2 transgenic mouse model of Huntington's disease. *J Neurochem* 81(1): 46–59.
32. Ortiz AN, Kurth BJ, Osterhaus GL, Johnson MA (2010) Dysregulation of intracellular dopamine stores revealed in the R6/2 mouse striatum. *J Neurochem* 112(3):755–761.
33. Callahan JW, Abercrombie ED (2011) In vivo dopamine efflux is decreased in striatum of both fragment (R6/2) and full-length (YAC128) transgenic mouse models of Huntington's disease. *Front Syst Neurosci* 5:61.
34. Bayram-Weston Z, Jones L, Dunnett SB, Brooks SP (2012) Light and electron microscopic characterization of the evolution of cellular pathology in YAC128 Huntington's disease transgenic mice. *Brain Res Bull* 88(2-3):137–147.
35. Modregger J, DiProspero NA, Charles V, Tagle DA, Plomann M (2002) PACSIN 1 interacts with huntingtin and is absent from synaptic varicosities in presymptomatic Huntington's disease brains. *Hum Mol Genet* 11(21):2547–2558.
36. Kipps CM, et al. (2005) Progression of structural neuropathology in preclinical Huntington's disease: A tensor based morphometry study. *J Neurol Neurosurg Psychiatry* 76(5):650–655.
37. Starling AJ, et al. (2005) Alterations in N-methyl-D-aspartate receptor sensitivity and magnesium blockade occur early in development in the R6/2 mouse model of Huntington's disease. *J Neurosci Res* 82(3):377–386.
38. Ayala JE, Niswender CM, Luo Q, Banko JL, Conn PJ (2008) Group III mGluR regulation of synaptic transmission at the SC-CA1 synapse is developmentally regulated. *Neuropharmacology* 54(5):804–814.
39. Cattaneo E, Zuccato C, Tartari M (2005) Normal huntingtin function: An alternative approach to Huntington's disease. *Nat Rev Neurosci* 6(12):919–930.
40. Chiarlone A, et al. (2014) A restricted population of CB1 cannabinoid receptors with neuroprotective activity. *Proc Natl Acad Sci USA* 111(22):8257–8262.
41. Hernández-Flores T, et al. (2015) Modulation of direct pathway striatal projection neurons by muscarinic M_4-type receptors. *Neuropharmacology* 89:232–244.
42. Tzavara ET, et al. (2004) M4 muscarinic receptors regulate the dynamics of cholinergic and dopaminergic neurotransmission: Relevance to the pathophysiology and treatment of related CNS pathologies. *FASEB J* 18(12):1410–1412.
43. Ahmari SE, et al. (2013) Repeated cortico-striatal stimulation generates persistent OCD-like behavior. *Science* 340(6137):1234–1239.
44. Avshalumov MV, Chen BT, Marshall SP, Peña DM, Rice ME (2003) Glutamate-dependent inhibition of dopamine release in striatum is mediated by a new diffusible messenger, H_2O_2. *J Neurosci* 23(7):2744–2750.
45. Calabresi P, Picconi B, Tozzi A, Di Filippo M (2007) Dopamine-mediated regulation of corticostriatal synaptic plasticity. *Trends Neurosci* 30(5):211–219.
46. Day M, et al. (2006) Selective elimination of glutamatergic synapses on striatopallidal neurons in Parkinson disease models. *Nat Neurosci* 9(2):251–259.
47. Picconi B, Centonze D, Rossi S, Bernardi G, Calabresi P (2004) Therapeutic doses of L-dopa reverse hypersensitivity of corticostriatal D2-dopamine receptors and glutamatergic overactivity in experimental parkinsonism. *Brain* 127(Pt 7):1661–1669.
48. Klapstein GJ, et al. (2001) Electrophysiological and morphological changes in striatal spiny neurons in R6/2 Huntington's disease transgenic mice. *J Neurophysiol* 86(6): 2667–2677.
49. Marco S, et al. (2013) Suppressing aberrant GluN3A expression rescues synaptic and behavioral impairments in Huntington's disease models. *Nat Med* 19(8):1030–1038.
50. Kung VW, Hassam R, Morton AJ, Jones S (2007) Dopamine-dependent long term potentiation in the dorsal striatum is reduced in the R6/2 mouse model of Huntington's disease. *Neuroscience* 146(4):1571–1580.
51. McCormack MK, Lazzarini A (1982) Attitudes of those at risk for Huntington's disease toward presymptomatic provocative testing. *N Engl J Med* 307(22):1406.
52. Garrett MC, Soares-da-Silva P (1992) Increased cerebrospinal fluid dopamine and 3,4-dihydroxyphenylacetic acid levels in Huntington's disease: Evidence for an overactive dopaminergic brain transmission. *J Neurochem* 58(1):101–106.
53. Brooks S, Higgs G, Janghra N, Jones L, Dunnett SB (2012) Longitudinal analysis of the behavioural phenotype in YAC128 (C57BL/6J) Huntington's disease transgenic mice. *Brain Res Bull* 88(2-3):113–120.
54. Menalled LB, Sison JD, Dragatsis I, Zeitlin S, Chesselet MF (2003) Time course of early motor and neuropathological anomalies in a knock-in mouse model of Huntington's disease with 140 CAG repeats. *J Comp Neurol* 465(1):11–26.
55. Van Raamsdonk JM, et al. (2005) Cognitive dysfunction precedes neuropathology and motor abnormalities in the YAC128 mouse model of Huntington's disease. *J Neurosci* 25(16):4169–4180.
56. Sampaio C, Borowsky B, Reilmann R (2014) Clinical trials in Huntington's disease: Interventions in early clinical development and newer methodological approaches. *Mov Disord* 29(11):1419–1428.
57. Ross CA, Tabrizi SJ (2011) Huntington's disease: From molecular pathogenesis to clinical treatment. *Lancet Neurol* 10(1):83–98.
58. Gomeza J, et al. (1999) Enhancement of D1 dopamine receptor-mediated locomotor stimulation in M(4) muscarinic acetylcholine receptor knockout mice. *Proc Natl Acad Sci USA* 96(18):10483–10488.
59. Zhao S, et al. (2011) Cell type–specific channelrhodopsin-2 transgenic mice for optogenetic dissection of neural circuitry function. *Nat Methods* 8(9):745–752.
60. Foster DJ, et al. (2014) M5 receptor activation produces opposing physiological outcomes in dopamine neurons depending on the receptor's location. *J Neurosci* 34(9): 3253–3262.