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Genetic rescue of CB₁ receptors on medium spiny neurons prevents loss of excitatory striatal synapses but not motor impairment in HD mice

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Abstract

Huntington's disease (HD) is caused by an expanded polyglutamine repeat in huntingtin protein that disrupts synaptic function in specific neuronal populations and results in characteristic motor, cognitive and affective deficits. Histopathological hallmarks observed in both HD patients and genetic mouse models include the reduced expression of synaptic proteins, reduced medium spiny neuron (MSN) dendritic spine density and decreased frequency of spontaneous excitatory post-synaptic currents (sEPSCs). Early down-regulation of cannabinoid CB₁ receptor expression on MSN (CB_{1(MSN)}) is thought to participate in HD pathogenesis. Here we present a cell-specific genetic rescue of CB_{1(MSN)} in R6/2 mice and report that treatment prevents the reduction of excitatory synaptic markers in the striatum (synaptophysin, vGLUT1 and vGLUT2), of dendritic spine density on MSNs and of sEPSCs, but does not prevent motor impairment. We conclude that loss of excitatory striatal synapses in HD mice is controlled by CB_{1(MSN)} and can be uncoupled from the motor phenotype.

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CONFLICTS OF INTEREST:

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INTRODUCTION

The early down-regulation of CB₁ (MSN) was first observed in post-mortem tissue of HD patients and occurs in most mouse models of HD, including R6/2, R6/1, YAC128 and HdhQ150 mice (Dowie et al., 2009; Glass et al., 2000; Pouladi et al., 2012; Woodman et al., 2007). It was recently reported that mutant huntingtin acts at the *Cnr1* promoter and inhibits CB₁ expression in neurons (Blázquez et al., 2010). Down-regulation of CB₁ (MSN) – the neuronal population expressing the highest amount of CB₁ receptors (Herkenham et al., 1990) – precedes the loss of other synaptic proteins, including dopamine D2 and D1 receptors and striatal synaptophysin (Glass et al., 2000). Furthermore, it is known that functional CB₁ receptor signaling allows neurons to better resist various insults and pathological events by, for example, reducing excitotoxicity and regulating growth factor expression (Katona and Freund, 2008). Together, this evidence led to the hypothesis that CB₁(MSN) dysfunction controls downstream pathological events occurring in HD patients and mouse models (Maccarrone et al., 2007). For example, the genetic deletion of CB₁ receptors in HD mice models both accelerates the motor phenotype and aggravates HD pathogenesis measured in the striatum, suggesting that CB₁ signaling might participate through an unknown mechanism in the development of HD (Blázquez et al., 2010; Mievis et al., 2011). In line with this result, environmental enrichment both improves the phenotype of HD mice models and increases CB₁ receptor expression in MSNs, without rescuing the expression of other proteins (D1, D2, and GABA_A receptors) (Glass et al., 2004). Nevertheless, other groups have suggested that CB₁ loss in HD may in fact be a compensatory event that corrects impaired D2 receptor signaling (Eidelberg and Surmeier, 2011).

Several attempts to increase CB₁ signaling by pharmacological treatment in HD mouse models led to divergent conclusions. One study treated R6/1 mice with a full CB₁ agonist (HU210), a partial CB₁ agonist (THC) and an inhibitor (URB597) of fatty acid amide hydrolase, an enzyme known to inactivate the prototypical endocannabinoid anandamide, and found that none of these treatments improved the motor phenotype of R6/1 mice (Dowie et al., 2010). Conversely, another study treated R6/2 mice with a lower concentration of THC (2 mg/kg, compared with 10 mg/kg) and found improvement in several motor and neuropathological measures (Blázquez et al., 2010). A caveat to pharmacological manipulation of CB₁ receptors in HD mouse models is that systemic injection of agonists activates large populations of CB₁ receptors irrespective of cell type, whereas CB₁ signaling is lost only in select neuronal types. Specifically, in R6/2 mice, presynaptic CB₁ signaling is down-regulated in MSN axon terminals, while it is preserved on corticostriatal terminals (Chiodi et al., 2012). Furthermore, in striatal interneurons, CB₁ receptor expression and signaling is lost on NPY interneurons but not on cholinergic, parvalbumin or somatostatin interneurons (Horne et al., 2012). Therefore, the effects of delivering cannabinoid agonists systemically are difficult to interpret because of their action on multiple populations of CB₁ receptors. Adding to this difficulty, the selectivity of cannabinoid drugs is limited by off-target responses, including through CB₂ receptors, TRPV1 and GPR55 (Abood et al., 2012). To circumvent these issues and directly test the physiopathological relevance of CB₁(MSN) dysfunction in HD, we generated a conditional flox-stop CB₁ knock-in mouse and

backcrossed it to both the *Gpr88*^{+/Cre} mouse line and to R6/2 mice, generating a new R6/2 mouse line in which an extra copy of *Cnr1* is selectively expressed in MSNs. Using this new genetic tool, we studied whether cell selective genetic rescue of CB₁ signaling in MSNs affects characteristic motor behaviors and pathogenesis in R6/2 mice.

RESULTS

Generation of R6/2 mice with genetic rescue of CB₁ (MSN)

We inserted a mouse *Cnr1* open reading frame between a loxP-flanked *Pgk-Neo* resistance gene and ires EGFP, and then moved that cassette into a *Gt(Rosa26)Sor* targeting vector with the CBA promoter (CMV-chicken β -actin) at the transcription start (Figure 1A, Supplementary Figure 1). We refer to this modified *Gt(Rosa26)Sor* locus as *R26*^{fsCB1}. Females with this gene were bred with *Gpr88*^{+/Cre} males that express Cre recombinase predominantly in MSNs to activate CB₁ expression in MSNs (Quintana et al., 2012). Because low levels of GPR88 expression have been reported in other brain areas including the cortex, we performed PCR to verify that the excision of *Pgk-Neo*, and thus activation of the fsCB₁ insert, does not occur in other brain regions. Confirming the regional selectivity of our construct, DNA from dissected striatum showed a band indicating *Pgk-Neo* excision, whereas DNA from tail, cortex and thalamus did not (Figure 1B).

Down-regulation of CB₁ protein occurs first in the indirect pathway and later in the direct pathway in HD patients and mouse models (Glass et al., 2000). To verify the rescue of CB₁ protein expression in R6/2 mice, we performed semi-quantitative IHC in the GP and SNr, the respective targets of indirect- and direct-pathway MSNs. By co-staining for either Leu-Enkephalin or Substance P, we delineated regions of interest and measured CB₁ protein expression as previously described (Horne et al., 2012). In the GP of R6/2 mice, CB₁ protein was reduced to 64% $\pm$ 12 of wild-type (WT) levels ($p=0.003$) and this down-regulation did not occur in R6/2-CB₁(MSN) mice ($F_{(2,30)}=7.2$, $p=0.003$, 91% $\pm$ 10 of WT levels (Figure 1C,E, see Supplementary Figure 2 for details on image analysis). In the SNr, we measured a trend towards loss of CB₁ protein in R6/2 mice compared with WT or R6/2-CB₁(MSN) mice ($F_{(2,28)}=1.9$, $p=0.16$) (Figure 1D). These results indicate that expression of the *R26*^{fsCB1} allele rescues the expression of CB₁ protein in MSNs. Because of the rapid disease progression of R6/2 mice, it is possible that these mice die before the full expression of the molecular phenotype (*i.e.* before CB₁ protein loss occurs in the direct pathway).

Knock-In CB₁ receptors are functional

To examine the functionality of CB₁ receptors expressed from the *R26*^{fsCB1} allele, we bred this and the *Gpr88*^{+/Cre} allele into a *Cnr1*^{-/-} background. Here we used *Gpr88*^{+/Cre} females, which express Cre recombinase in the ova, to generate mice with global expression of *R26*^{fsCB1} allele as verified for all animals by PCR reaction from DNA dissected from tail, cortex, thalamus, or striatum (Figure 2A). To test whether these CB₁ receptors functionally couple to G proteins, we performed a GTP γ S-binding assay. First, we established the EC₈₀ response induced by the CB₁ receptor agonist CP55,940, as measured by increased GTP γ S binding in freshly harvested SNr tissue from 5 WT mice (Figure 2B). In the next experiment, we harvested SNr tissue from WT, *Cnr1*^{-/-} or Global-fsCB₁ mice and

performed GTP γ S binding using the EC₈₀ dose of CP55,940 (*i.e.* 140 nM, Figure 2C). Stimulation of GTP γ S binding in response to CP55,940 in Global-fsCB₁ mice was similar to the response measured in WT levels when compared to *Cnr1*^{-/-} mice (WT: 148.5% $\pm$ 7.3, *Cnr1*^{-/-} = 104.5% $\pm$ 10.5, Global-fsCB₁ = 133.4% $\pm$ 2.7; $F_{(2,10)}=9.48$, $p=0.005$). As a secondary verification of fsCB₁ receptor functionality, we tested the ability of Global-fsCB₁ mice to reproduce characteristic motor response induced by cannabinoid agonists. Specifically, CP55,940-induced catalepsy was absent in *Cnr1*^{-/-} mice and rescued in Global-fsCB₁ mice (genotype: $F_{(2,28)}=111.3$, $P<0.001$; time: $F_{(3,28)}=49.2$, $p<0.001$, interaction: $F_{(6,28)}=15.1$, $p<0.001$) ($p<0.001$ at 5 and 30 min, $p=0.02$ at 60 min, Figure 2D). Similarly, CP55,940 induced hypolocomotion in both WT and Global-fsCB₁ mice, whereas this response was absent in *Cnr1*^{-/-} mice (interaction of genotype $\times$ treatment: $F_{(2,18)}=29.58$, $p<0.001$, Figure 2E). We conclude that fsCB₁-derived CB₁ receptors functionally couple to G proteins and are sufficient to restore CP55,940-induced behaviors in mice lacking endogenous CB₁ receptors.

The R6/2 motor phenotype is unchanged by genetic rescue of CB_{1(MSN)} receptors

The effect of CB_{1(MSN)} rescue on R6/2 mice was tested using several indices of motor impairment. Rotarod performance showed a trend towards impairment at 4 wk of age in both R6/2 and R6/2-CB_{1(MSN)} mice compared to WT mice, and was similarly impaired in both these lines at 8 weeks ($F_{(2,8)}=25.7$, $p<0.001$) and 12 weeks of age ($F_{(2,10)}=285.7$, $p<0.001$, Figure 3A). Locomotion was measured for 10 min in an open field chamber, and both R6/2 and R6/2-CB_{1(MSN)} rescue mice were similarly hypolocomotive compared to WT mice beginning at 10 wk of age (Figure 3B). At 4 and 6 wk, R6/2 mice performed fewer rearings than R6/2-CB_{1(MSN)} mice and WT mice (pooled 4 and 6 wk: WT = 16.5 $\pm$ 2.2, R6/2 = 5.8 $\pm$ 1.2, R6/2-CB_{1(MSN)} = 12.5 $\pm$ 2.0), but at 8 weeks of age, R6/2-CB_{1(MSN)} mice were indistinguishable from R6/2 mice (as indicated by the fact that both groups reared much less than WT mice) (pooled 8, 10, and 12 wk: WT = 16.25 $\pm$ 0.8, R6/2 = 2.8 $\pm$ 1.4, R6/2-CB_{1(MSN)} = 2.6 $\pm$ 1.0, Figure 3C). General measures of HD mouse impairment were scored biweekly by blinded observers for nine indices commonly measured in R6/2 mice (factors on a 1–2 scale, see methods). Two-factor ANOVA showed an effect of genotype ($F_{(2,114)}=81.6$, $p<0.001$), and of age ($F_{(4,114)}=24.6$, $p<0.001$) when comparing WT and R6/2 mice, and the interaction between genotype and age was also significant ($F_{(8,114)}=6.4$, $P<0.001$), reflecting a progressive motor phenotype in R6/2 mice. This measure of disease progression was unchanged between R6/2 and R6/2-CB_{1(MSN)} mice (Figure 3D). Similarly, clasping behavior showed an interaction between age and genotype ($F_{(8,111)}=3.1$, $p=0.003$) and this measure was similarly impaired in R6/2 and R6/2-CB_{1(MSN)} mice compared to WT mice (Figure 3E).

To complement these measures of motor behavior, we performed automated gait analysis using the Noldus Catwalk XT system (Wageningen, The Netherlands). R6/2-CB_{1(MSN)} mice showed mild improvement in base of support ($F_{(2,14)}=4.4$, $p=0.03$), step cycle ($F_{(2,14)}=6.0$, $p=0.01$) and print position ($F_{(2,13)}=6.8$, $p=0.01$) compared with R6/2 mice (Figure 3F–H) but no improvement in swing speed (forelimbs: $F_{(2,14)}=8.31$, $p<0.01$; hindlimbs: $F_{(2,14)}=10.86$, $p<0.01$), stride length (forelimbs: $F_{(2,14)}=4.7$, $p<0.03$; hindlimbs: $F_{(2,14)}=11.4$, $p<0.01$) or crossing speed ($F_{(2,14)}=8.1$, $p<0.01$) (Figure 3I–K). Based on the battery of motor behavior

testing analyzed here, we conclude that no appreciable motor rescue resulted from CB₁(MSN) in R6/2 mice.

CB₁(MSN) receptor rescue is sufficient to prevent loss of excitatory synapses in striatum of R6/2 mice

Previous studies have reported a loss of striatal synaptophysin in R6/2 mice (Cepeda et al., 2003). We found that CB₁(MSN) rescue was sufficient to prevent the loss of synaptophysin in both the dorsolateral and dorsomedial striatum (DL-STR: $F_{(2,28)}=4.7$, $p=0.018$; DM-STR: $F_{(2,29)}=5.0$, $p=0.013$) (Figure 4A–C). Furthermore, when levels of striatal synaptophysin were plotted against CB₁ receptors in the GP (WT mice were excluded to prevent genotype-driven bias), we calculated a positive correlation ($r^2=0.29$, $p=0.045$, slope= 0.67 ± 0.30) between loss of CB₁ receptors and reduction in synaptophysin staining within individual mice (Figure 4D). To determine in greater detail which populations of synapses lose synaptophysin, we co-stained for either vesicular glutamate transporter 1 (vGLUT1) and vesicular glutamate transporter 2 (vGLUT2), markers of corticostriatal and thalamocortical synapses, or vesicular GABA transporter (vGAT) and Neuroligin-2, markers of GABAergic synapses. We observed decreased immunostaining in both vGLUT1 ($F_{(2,25)}=5.45$, $p=0.011$) and vGLUT2 ($F_{(2,25)}=7.01$, $p=0.004$) in the striatum of R6/2 mice compared to WT (vGLUT1: $p=0.004$, vGLUT2: $p=0.002$), and found that both proteins remained at WT levels in R6/2-CB₁(MSN) rescue mice (vGLUT1: $p=0.012$; vGLUT2: $p=0.005$, Fig. 5A–C). As predicted by previous reports, vGAT and Neuroligin-2 levels were similar in WT and R6/2, and remained unchanged in R6/2-CB₁(MSN) rescue mice (Figure 5D–F) (Nithianantharajah et al., 2008). We conclude that excitatory, but not inhibitory, presynaptic proteins are lost in the striatum of R6/2 mice, and that genetic rescue of CB₁(MSN) is sufficient to prevent the selective loss of presynaptic glutamatergic markers.

To extend our IHC findings, we measured dendritic spine density in Golgi-stained sections. Indeed, prior work has shown that R6/2 mice exhibit a reduction in spine number which is most pronounced in higher-order dendrites (Klapstein et al., 2001; Spires et al., 2004). We reproduced this result, and extended it to show that genetic rescue of CB₁(MSN) is sufficient to prevent the reduction in MSN dendritic spine density in R6/2 mice (Figure 6). Dendritic spine density was different between genotypes ($F_{(2,24)}=11.58$, $p<0.001$) and these differences were more pronounced with increasing branch order ($F_{(6,72)}=3.81$, $p=0.002$). Specifically, spine density was unchanged between genotypes in first and second order dendrites, but spine density was reduced in R6/2 mice in third and fourth order dendrites compared to WT. This loss of spine density did not occur in R6/2-CB₁(MSN) mice (see supplementary Table 1 for specific values).

Rescued excitatory striatal synapses are functional

To determine if rescued excitatory striatal synapses are functional, we recorded spontaneous synaptic events by whole-cell voltage-clamp at -70mV in the presence of $50\text{ }\mu\text{M}$ picrotoxin to block GABA_A mediated inhibitory events and isolate sEPSCs. As previously reported, we found that the frequency of sEPSCs in R6/2 MSNs was significantly reduced compared to WT ($p<0.05$, Figure 7A) (Cepeda et al., 2003). We also found that sEPSC frequency in R6/2-CB₁(MSN) mice was significantly greater than R6/2 ($p<0.05$) and indistinguishable from WT.

Note that we found no difference in the amplitude of sEPSC across genotypes indicating that the excitatory receptors mediating this response were not affected. Together, these results suggest that the number of functional excitatory synapses measured in R6/2-CB_{1(MSN)} is rescued to WT levels.

We measured MSN capacitance and input resistance by a 10mV depolarizing step from the membrane potential clamped at -70mV after seal rupture. In concordance with previous studies (Cepeda et al., 2003), R6/2 MSN displayed reduced capacitance and increased input resistance compared to WT ($p<0.001$ and $p<0.01$ respectively Figure 8 A and B). In contrast, the capacitance of MSNs from R6/2-CB_{1(MSN)} was not reduced compared to WT and was greater than the capacitance of R6/2 MSN ($p<0.05$). The higher input resistance of R6/2 MSNs was also restored to WT levels in MSNs from R6/2-CB_{1(MSN)} mice ($p<0.001$). Resting membrane potentials of MSNs and current-voltage curve were recorded in current clamp mode. Whereas R6/2 MSN were depolarized at rest compared to WT ($p<0.01$, Fig 8C), R6/2-CB_{1(MSN)} resting membrane potentials were similar to WT and had a lower membrane potential than R6/2 MSN ($p<0.05$). Somatic input current steps resulted in changes in membrane potential that were greater magnitude in MSNs of both R6/2 and R6/2-CB_{1(MSN)} mice compared to WT mice, indicating no rescue of this measure (Fig 8E). Specifically, the relationship between spiking frequency and injected current was shifted leftward in R6/2 and R6/2-CB_{1(MSN)} genotypes compared to WT (Figure 8 D, $P<0.001$), but only intrasomatic injection of a 150pA current step produced more action potentials over 1 sec in R6/2 MSN (mean= 19.6 $\pm$ 1.7) compared to WT MSN (mean=11.5 $\pm$ 2.6 $p<0.05$). Additionally, Rheobase current was significantly lower in both R6/2 (78.26 $\pm$ 6.15, $n=23$, $p<0.001$) and R6/2-CB_{1(MSN)} (81.82 $\pm$ 10.16, $n=11$, $p<0.05$) compared to WT (116.7 $\pm$ 8.33, $n=9$). In summary, passive membrane properties including capacitance, input resistance and membrane potential of R6/2-CB_{1(MSN)} were restored to WT levels, whereas the response to somatic current injection and action potential thresholds in MSNs of R6/2-CB_{1(MSN)} mice remained impaired compared to WT mice.

DISCUSSION

Here we show that the genetic rescue of CB_{1(MSN)} receptors in R6/2 mice prevents the loss of excitatory input to the striatum as indicated by the sparing of excitatory synaptic proteins (synaptophysin, vGLUT1 and vGLUT2), as well as dendritic spine density on MSNs, as measured by Golgi staining. We confirm these anatomical findings by demonstrating a rescue of sEPSC frequency in MSNs, suggesting that rescued excitatory synapses are functional. Additionally, we measured changes in the intrinsic properties of MSNs that are consistent with changes in the density of dendritic spines, since neither soma size nor dendritic arbors are affected in R6/2 mice (Spires et al., 2004). However, genetic rescue of CB_{1(MSN)} did not rescue all impairments of MSN physiology, as active properties (I-V relationship, rheobase) remained compromised in R6/2-CB_{1(MSN)} mice. We also found that inhibitory synaptic proteins are unaffected in R6/2 mice, confirming previous studies reporting reductions in both GluR1 and PSD95, but not gephyrin, in the striatum of R6/1 mice (Nithianantharajah et al., 2008) and reduction of PSD95 in R6/2 mice (Luthi-Carter et al., 2003).

Despite the reversal of synaptopathy among striatal inputs, the R6/2-CB₁(MSN) mice did not show improvement in motor phenotype among a battery of behavioral measures. Importantly, reduced sEPSCs are only one of many electrophysiological disturbances that have been described in the striatum of R6/2 mice (Raymond et al., 2011). Specifically, reduced connectivity between MSNs (Cepeda et al., 2013), abnormal increase in MSN responses to input from fast-spiking interneurons (Cepeda et al., 2013), alterations in GABA synaptic transmission (Centonze et al., 2005) and impaired dopamine-dependent long-term potentiation (Kung et al., 2007) have been reported in R6/2 mice, any combination of which are likely to contribute to the motor phenotype observed in this model. This body of evidence emphasizes the notion that multiple circuits are impaired in R6/2 mice and that it is likely their summation that produces a behavioral phenotype. Our results extend this notion by showing that striatal excitatory synaptic loss and rescue can be uncoupled from motor deficits in HD.

Several likely mechanisms might be considered with regard to the CB₁(MSN)-mediated sparing of excitatory striatal synapses. Activation of CB₁ receptors regulates the release of GABA from MSN terminals; therefore the early loss of CB₁ receptors from indirect-pathway MSN terminals in R6/2 mice may cause imbalanced output from the basal ganglia to the thalamus and cortex that likely alters excitatory input to the striatum (Kreitzer and Malenka, 2008). Additionally, it has been recently shown that silencing direct- or indirect-pathway MSNs controls the number of excitatory synapses onto MSNs while having no effect on their arborization (Kozorovitskiy et al., 2012). Thus CB₁(MSN) on MSN-MSN collaterals may affect the inhibitory tone of MSN recurrent network activity, which seems to control striatal inputs.

Interest in targeting the eCB system for therapeutic intervention in HD originally arose from the realization that CB₁(MSN) receptors are selectively lost in HD patients early in disease (Glass et al., 2000), a landmark observation that inspired the hypothesis that this loss constitutes a key pathogenic event which culminates in behavioral impairment (Blázquez et al., 2010). Our results do not support this hypothesis, as we did not observe an improvement of behavioral phenotype in response to CB₁(MSN) rescue. Instead, we report that rescue of CB₁(MSN) receptors prevents a very selective component of striatal synaptopathy, demonstrating that CB₁(MSN) signaling controls a subset of the anatomical and functional disturbances in HD mouse models. Our results also challenge the hypothesis that loss of CB₁ receptors in MSNs represents a compensatory mechanism that reduces neuronal damage associated with HD. Specifically, if CB₁ loss was a compensatory event, then the expectation would be that CB₁(MSN) rescue should exacerbate at least some elements of the disease phenotype. In sharp contrast to this assumption, the electrophysiological and neuropathological data presented in our study show a rescue of excitatory synapse loss. Importantly, our data do not rule out the therapeutic use of cannabinoid agonists in HD, since other populations of CB₁ receptors expressed by different neuronal types may be useful targets for controlling aberrant neurotransmission, especially CB₁ receptors on the corticostriatal synapse which are spared and likely to regulate excitotoxicity (Chiodi et al., 2012). Specifically, two independent groups found that knockout of CB₁ receptors in R6/2 mice worsened the motor phenotype (Blázquez et al., 2010; Mievis et al., 2011) and a recent study identified the population of CB₁ receptors expressed on glutamatergic terminals as

solely responsible for exacerbation of the R6/2 motor phenotype in response to CB₁ deletion (Chiarlone et al., 2014).

In summary, our study has addressed a long-standing question in the field regarding the pathogenic significance of the loss of CB_{1(MSN)} receptors in HD, by demonstrating a specific role for CB_{1(MSN)} dysregulation in the loss of excitatory synapses in the striatum. Further work remains to be done to elucidate the molecular, cellular and network details underlying the control of functional excitatory synapses in the striatum by CB_{1(MSN)} receptors.

MATERIALS AND METHODS

Mice

Mice were housed in a specific pathogen-free facility in accordance with the National Institutes of Health; the Institutional Animal Care and Use Committee at the University of Washington approved all experiments. Enrichment and *ad-libitum* access to food and water was provided and a 12-hr light/dark cycle was maintained in the facility. R6/2 and WT littermates were given a wet food mash in addition to dry pellets beginning at 10 weeks of age. Both female and male R6/2, R6/2-CB_{1(MSN)} rescue, and WT littermates were used in this study. The colony was maintained by breeding 6- to 8-week-old R6/2-*Gpr88*^{+/Cre} males with R26^{+/fsCB1} females, and all animals were on a 50:50 CBA, C57Bl/6 background. The average CAG repeat length of our colony is 114.1 ± 0.3 (n=10) and was determined by PCR from tail snips by Laragen, Inc [Culver City, CA, USA]. Genotyping was performed as follows: 1) fsCB₁ using primers aaagtcgctctgagttgttatcag (P1), ggagcgggagaaatggatatg (P2) and tcaatgcattctagttgtgtttg (P3) with ThermoPol Buffer (NE Biolabs, MA); 2) pgk-neo with primers ctctgctaaccatgttcacgcc (P4) and tctgcaaggccgtctaagat (P5) using ThermoPol Buffer (NE Biolabs, MA); 3) *Gpr88*^{Cre} with tggaggaacgaggagttccgc (1569) and agaaggaggcagtgccgagg (1570) using Phusion (NE Biolabs, MA); 4) R6/2 with primers cgcaggctgcagggttac and gctgcaccgaccgtgagt using Lightcycler 480 Master (Roche, Basel, Switzerland).

Generation of fsCB₁ mice

The open reading frame of mouse *Cnr1* was inserted between a loxP-flanked Pgk-Neo gene and ires EGFP in a transfer plasmid. This floxed *Pgk-Neo-Cnr1-ires-EGFP* insert was then moved into a targeting vector that has the CBA promoter inserted at the transcription start site of the *Gt(Rosa26)*^{Sor} locus and contains 7.7 kb of 5' flanking and 4.1 kb of 3' flanking *Gt(Rosa26)*^{Sor} sequence and a PgK-DTa gene for negative selection. This construct was linearized and electroporated into G4 embryonic stem cells. Correct gene targeting was determined by Southern blot of DNA digested with *NdeI* using a probe that lies outside of the targeting vector. Correctly targeting ES cells were injected into the blastocyst of C57Bl/6 recipients and chimeric pups were bred with C57Bl/6 mice.

[³⁵S]GTPγS Binding Assay

SNr tissue was rapidly dissected on ice and immediately homogenized in ice-cold 50 mM Tris-HCl (pH 7.4), 3 mM MgCl₂, and 1 mM EGTA. The homogenate was centrifuged at

45,000 × g for 10 min at 4°C, and then the pellet was homogenized in 50mM Tris-HCl, with 3 mM MgCl₂, 1 mM EGTA and 100 mM NaCl. The resulting membrane homogenate was pre-incubated with 3 mU/ml adenosine deaminase (Roche Applied Science, IN) for 10 min at 30°C, to inactivate endogenous adenosine. Then, 10 µg of protein per reaction was incubated in reaction buffer containing 0.1% BSA, 30 µM GDP, 0.1 nM [³⁵S]GTPγS (Perkin Elmer, OH) and 0.6 mU/ml adenosine deaminase, with either 140 µM CP55,940 or vehicle, for 45 min at 30°C. The incubation was terminated by vacuum filtration through Whatman filters (GE Healthcare), followed by three washes with 3 ml ice-cold Tris-HCl, pH 7.4. Bound [³⁵S]GTPγS was quantified using a liquid scintillation counter in vials containing isolated [³⁵S]GTPγS-bound filter paper along with 4ml of Ecoscint scintillation fluid (National Diagnostics).

Behavioral Studies

Balanced cohorts of males and females were used for behavioral studies. All behavioral tests were performed during the light phase of the light/dark cycle, between 9 am and 12 pm. All cages were cleaned with 70% ethanol between trials, and then dried with a paper towel. At 6 weeks of age, mice were tested for hypothermia, hypolocomotion, catalepsy and analgesia, both before and after injection of 0.3 mg/kg CP55,940. Core body temperature was measured by anal probe. Hypolocomotion was measured in an open field chamber (25 cm × 45 cm plexiglass cage) for 10 min starting 30 min after injection. Data were collected by vertically mounted video cameras and videos were analyzed in Ethovision 9.0 (Noldus, Wageningen, The Netherlands). Center-point tracking with dynamic- background subtraction was used to acquire the track for each animal. Catalepsy was measured by placing the animals' front paws on a horizon bar approximately 3 cm high and recording the latency to remove the paws from the bar. An average of four trials was used per animal, and measurements were made both before and 30 min post-injection. Tail flick analgesia was measured by placing 1 cm of the tail in a 55°C water bath (+/- 2°C), and measuring the latency to tail withdrawal. Rotarod (Columbus Instruments, OH) testing included a 5-min training period on the rotarod at 5 RPM, after which mice were rested and then began 7 consecutive trials, separated by 30-min resting periods. The rotarod rotational speed used for the test trials began at 4 rpm and increased to 40 rpm, with an acceleration of 0.2 rpm per every 4 sec. Catwalk testing was performed at 10 weeks of age: animals were placed at one end of the Catwalk apparatus (Noldus, Wageningen, Netherlands), with a vertically mounted camera beneath the catwalk and five crossings were analyzed for each animal. Crossings where the animal stopped, reared, or turned around were excluded. Phenotype was manually scored biweekly from 4–12 wk in an open-field chamber to generate a phenotype index and to count rearings. Animals were placed in a 25 cm × 45 cm plexiglass cage for 2 min and were scored from 1–2 on 9 measures (tremor, body position, tail position, piloerection, exploration of all 4 corners, observed seizures, presence of hindlimb claspings, palpebral closure, and grooming), such that the possible scores ranged from 9–18, with increasing score indicating pathological phenotype. Open field testing was performed in a and mice were allowed to explore for 10 min.

Immunohistochemistry and confocal microscopy

Mice were perfused with 20mL sterile PBS, followed by 10mL 4% paraformaldehyde. Brains were extracted, post-fixed overnight at 4°C in 4% paraformaldehyde, then successively dehydrated in 15% sucrose and 30% sucrose for 24 hr each, and finally frozen. Coronal sections were cut on a freezing microtome to a thickness of 30µm, placed in cryoprotectant and stored at -20°C. On the day of staining, 2 slices *per* mouse were removed from cryoprotectant, washed 3× in PBS, and then incubated in blocking buffer (1% Triton X-100, 5% donkey serum in PBS) for 90 min at room temperature, and then transferred to primary staining solution (0.5% Triton X-100, 2.5% donkey serum in PBS) for 72 hr at 4°C. Primary antibodies and dilutions used: CB₁, guinea pig, gift from Ken Mackie; leu-enkephalin, rabbit, Millipore AB5024; substance P, rabbit, Millipore AB1566; vGAT, mouse, Synaptic Systems 131011; vGlut1, mouse, Synaptic Systems 135311; vGlut2, rabbit, Invitrogen 42-7900; neuroligin2, rabbit, Synaptic Systems 129203. All primary antibodies were optimized by performing a dilution curve paired with quantitative analysis, and dilutions in the linear phase were chosen for further staining. After primary staining, sections were washed 8× in PBS-T for 5 min. Secondary staining was performed in 0.5% Triton X-100, 2.5% donkey serum in PBS, using Alexa secondary antibodies at a dilution of 1:500. After secondary staining, sections were washed 6 times in PBS-T for 10 min, once in PBS, and then mounted with Fluoromount (Sigma, St Louis, MO) and sealed with nail polish.

Images were collected on a Leica SL confocal microscope equipped with a 63× (N.A.=1.40) oil immersion objective and with a 64 mW argon laser with lines at 457 and 488, a 10 mW helium-neon laser with a 543 nm line, and a 10 mW helium-neon laser with a 633 nm line. Optical sections were taken with the pinhole set at 1.0 AU, or 1.25 µm, with a resulting xy resolution of 0.163 µm and a z resolution of 0.290 µm. Noise reduction was achieved by averaging three scans for each image, after verification that this method did not result in any measurable bleaching (Alexa dyes were used). Laser power was optimized to the brightest section for each stain, so that >99% of pixels were within the linear range, and 12-bit images were collected.

Semi-Quantitative Image Analysis and Statistics

All images were analyzed and quantified in ImageJ (National Institutes of Health), using custom written macros which were applied blindly to each batch of images, and analyzed as previously reported (Horne et al., 2012). Each channel was split into an individual image and the mean intensity and standard deviation of each fluorophore in each image was measured. Background signal was removed by thresholding images to mean + standard deviation, corresponding to the top third brightest pixels in the Gaussian distribution. After thresholding, final measurements were made by taking the mean intensity of the (top-third) pixels which passed thresholding. Threshold values were validated by a human observer for each stain, to verify that pixels passing threshold corresponded to actual staining (threshold was not too low), and that actual staining was not being lost in thresholding (threshold was not too high). Statistical analysis and graphs were generated using GraphPad PRISM (San Diego, CA, USA). One-way ANOVAs were used for group comparisons and Fisher's T-test was used for post-hoc tests unless otherwise specified.

Golgi Staining and analysis

Brains were cut in half at the midline and immediately placed in formaldehyde/glutaraldehyde fixative (BioEnno, Irvine CA) for 5 hours. Fixative was changed once after 30 min. After fixation, brains were stored in 0.1M phosphate buffer for a maximum of 5 days at 4°C. Brains were sliced to 100 µm at room temperature on a vibratome (EMS, Hatfield PA) and striatal slices were collected in PBS. On-slice Golgi staining was performed using a SliceGolgi kit (BioEnno, Irvine CA). Staining was performed according to the kit protocol, using a 6-day Golgi impregnation. Slices were mounted on glass slides and allowed to dry for 24 hrs, then dehydrated in 100% EtOH, and cleared in xylenes. Slides were coverslipped using a 1:1 mixture of xylenes and Permount, and were allowed to dry for 48 hrs. Slides were imaged immediately after the drying period on a Marianas Live Cell Imaging System (Intelligent Imaging Innovations, Denver, CO) equipped with an automated stage, using a 100× oil objective. Montage z-series images were collected and aligned in SlideBook V5.5, and then exported as 16 bit TIF files. Manual counting of dendritic spines was performed in ImageJ (National Institutes of Health) using the multi-point selection tool with zstacked montages, and x-y-z coordinates of each marked dendritic spine were recorded.

Electrophysiology

Procedures were performed in accordance with the Canadian Council on Animal Care and UBC Animal Care Committee regulations. Animals were deeply anesthetized with halothane vapor, decapitated and the brain rapidly removed. Acute sagittal brain slices (300µm) containing the dorso-lateral striatum were cut on a vibratome (Leica VT1000) in ice-cold aCSF (with Ca:Mg at 1:5) equilibrated with 95% O₂/5% CO₂. Slices were transferred to a holding chamber with aCSF (Ca:Mg 2:1) at 37°C containing (in mM): 125 NaCl, 2.5 KCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 1 MgCl₂, 2 CaCl₂, 25 glucose, pH 7.3–7.4, 300–310 mosmol L⁻¹ for 45 min then maintained at room temperature. In the recording chamber, slices equilibrated for 10 min continuously superfused at room temperature with oxygenated aCSF at 1–2 ml/min containing picrotoxin (50 µM, Tocris Bioscience, MO, USA) to block GABA_A receptor mediated inhibitory responses.

Whole cell patch-clamp recordings were performed from visualized MSN in the dorsal striatum. Glass electrodes (resistance 4–6 MOhms) were filled with internal solution as follows (mM): 128 K+Gluconate, 20 NaCl, 1 MgCl₂, 1 EGTA, 0.3 CaCl₂, 2 Na²⁺ATP, 0.3 Na⁺ GTP, buffered with 10 Hepes, pH 7.3, osmolarity 290–300 mOsm. Data were acquired with a Multiclamp700 amplifier and Clampfit 10 software (Molecular Devices), digitized at 20 kHz and filtered at 2 kHz. Immediately after breaking into the cell the capacitance, membrane resistance and access resistance was measured. Access resistance was uncompensated and cells were rejected if it was >25 MOhms. Resting membrane potential and current voltage (I-V) curves in MSNs were made in current clamp mode by a series of hyperpolarizing to depolarizing current steps. Spontaneous excitatory post-synaptic currents (sEPSCs) were recorded in whole-cell voltage clamp configuration for a minimum of 4 min at -70 mV. All data represents the mean ± SEM of n = neurons from a minimum of 4 animals. Statistical analyses were made by two-way ANOVA for repeated measures or one-way ANOVA for group comparisons as indicated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

CB₁(MSN)	refers to CB ₁ receptors expressed on medium spiny neurons
DL-STR	dorsal-lateral striatum
DM-STR	dorsal-medial striatum
HD	Huntington's disease
MSN	medium spiny neuron
sEPSC	spontaneous excitatory post-synaptic current
vGAT	vesicular GABA transporter
vGLUT1	vesicular glutamate transporter 1
vGLUT2	vesicular glutamate transporter 2

Highlights

- Genetic rescue of CB₁ receptors on medium spiny neurons in HD mice prevents loss of striatal excitatory synapses.
- Rescued excitatory synapses are functional.
- Loss of striatal excitatory synapses can be uncoupled from HD motor phenotype.

Figure 1. Knock-in and expression of $R26^{fsCB1}$ restores $CB1_{(MSN)}$ receptor expression in R6/2 mice

(A) An $fsCB1$ insert was generated and targeted to *Rosa26*, carrying *Cnr1-ires-EGFP* under a *CBA* promoter with a flox-stop containing *Pgk-neo*. (B) Tissue punches of cortex, thalamus and striatum were dissected on a freezing microtome and processed for DNA extraction. PCR was performed with primers flanking the *pgk-neo* sequence, such that the presence of a band would indicate that *Pgk-neo* has been removed and the insert has been activated. *Pgk-neo* excision by Cre was specific for striatal tissue, and was not observed in tail, cortex or thalamus. (C, D) Semi-quantitative IHC was used to verify that R6/2- $CB1_{(MSN)}$ rescue (n=8) resulted in restoration of $CB1$ protein to WT (n=14) levels in the GP and SNr. (C) Whereas 12-week R6/2 mice (n=11) exhibit significant loss of $CB1$ protein from the GP, R6/2- $CB1_{(MSN)}$ rescue mice expressed WT-levels of $CB1$ receptors. (D) Notably, $CB1$ protein loss is not observed in the SNr in 12-week R6/2 mice. (E) Representative images are shown from the GP of R6/2, WT, and R6/2- $CB1_{(MSN)}$ rescue mice. Images were acquired on a Leica confocal, using an oil-immersion 63 $\times$ objective. Scale bars indicate 25 μ m. All error bars indicate S.E.M. and post-hoc analyses were carried out with Fisher's T test as indicated by * $p < .05$, ** $p < .01$.

Figure 2. Receptors expressed from the $R26^{fsCB1}$ locus are functional

To test the functionality of fsCB₁-derived protein, we crossed $Cnr1^{-/-}$ $Gpr88^{+/Cre}$ females with $Cnr1^{-/-}$ $R26^{+/fsCB1}$ males, since $Gpr88$ is expressed transiently in the ova resulting in global excision of $Pgk-Neo$. Thus, the resulting mice had no endogenous CB₁ protein, but expressed fsCB₁-derived CB₁ protein in all tissues (global-fsCB₁ mice). (A) PCR demonstrating $Pgk-Neo$ excision in brain and peripheral tissue. (B) Using pooled SNr tissue from 4 WT mice, a dose response curve was established for CP55,940. (C) In harvested SNr tissue, GTPγS binding was induced by an EC₈₀ dose of 140 nM CP55,940 in WT (n=4) and Global-fsCB₁ mice (n=5), but not in $Cnr1^{-/-}$ mice (n=4). (D) After treatment with 0.3 mg/kg CP55,940, we measured catalepsy in WT (n=6), $Cnr1^{-/-}$ (n=7), and Global-fsCB₁ mice (n=7). The cataleptic response observed in WT mice was absent in $Cnr1^{-/-}$ mice, and restored in Global fsCB₁ mice. (E) Similarly, CP55,940 induced hypocomotion in WT mice, which was lost in $Cnr1^{-/-}$ mice, and restored in global-fsCB₁ mice. Post-hoc

comparisons were performed with Fisher's T-test, with $*p<.05$, $**p<.01$, $***p<.001$. Error bars depict S.E.M.

Figure 3. The rescue of functional $CB_1(MSN)$ receptors does not rescue the R6/2 motor phenotype

For all graphs, R6/2 mice are shown in black, R6/2- $CB_1(MSN)$ rescue mice are shown in grey, and WT mice are shown in white. (A) Rotarod performance was measured at 4, 8, and 12 wk, reflecting presymptomatic, early, and late symptomatic stages ($n=4-6$, all groups). (B) In an open field chamber, locomotion was scored using Noldus Ethovision software, and rearing (C) was manually recorded over the first two min (WT, $n=15$; R6/2, $n=13$; R6/2- $CB_1(MSN)$, $n=8$). (D) Manual scoring of the R6/2 motor phenotype along 9 measures (see methods), and clasping behavior (E). Gait analysis was performed at 10 wk of age, and base of support (F), step cycle (G), print position (H), swing speed (I), stride length (J), and

average speed (K) were measured in Noldus Ethovision. Error bars indicate S.E.M., and Fisher's T test was used for post-hoc analyses with $*p<0.05$, $**p<0.01$, and $***p<0.001$. For (B–E). Comparison between WT and R6/2 mice denoted by *; WT and R6/2-CB₁(MSN) mice denoted by #.

Figure 4. Loss of striatal synaptophysin in R6/2 mice is rescued by genetic restoration of $CB1_{(MSN)}$ receptors

(A) Representative images of dorsal medial striatal synaptophysin staining in sections from 12 week old mice are shown with background subtraction. R6/2 ($n=10$) sections showed both a decrease in overall intensity and fewer hyperintense foci in both dorsal-medial (B) and dorsal-lateral striatum (C), which was restored to WT ($n=14$) levels in R6/2- $CB1_{(MSN)}$ mice ($n=7$). (D) Within-subject plots of striatal synaptophysin staining against $CB1$ receptor staining in the GP show a positive linear correlation between $CB1$ receptors and synaptophysin intensities in 12-wk-old mice ($n=17$, R6/2 and R6/2- $CB1_{(MSN)}$ groups pooled). Error bars depict S.E.M., and Fisher's T-Test was used for post-hoc analyses, with $*p<.05$, and $**p<.01$. Scale bar = 10 μ m.

Figure 5. R6/2-CB₁(MSN) mice do not lose excitatory striatal synapses

To determine the specificity of synaptic loss, we co-stained slices from 12 week old mice for vGLUT1 and vGLUT2 (A–B, representative images, scale bar = 25 μ m) or vGAT and NL2 (C–D, representative images, scale bar = 25 μ m). There was a reduction in staining of both vGLUT2 (E) and vGLUT1 (F) in R6/2 mice (n=12) compared with WT (n=9), which did not occur in R6/2-CB₁(MSN) mice (n=7). No reduction in either vGAT or NL2 immunostaining was detected in either R6/2 or R6/2-CB₁(MSN) mice (G–H). All images were acquired with a Leica confocal microscope and are presented with the same settings. Insets represent a 4 $\times$

digital zoom, with vGLUT2 in green and vGLUT1 in red (B) or with vGAT in green and NL2 in red (D). Scale bars depict 25 μm and 10 μm (insets), and error bars show S.E.M. Post-hoc analyses were performed with Fisher's T Test, and are indicated by $*p<.05$, $**p<.01$.

Figure 6. Dendritic spine loss in R6/2 mice is rescued in R6/2-CB1(MSN) mice

We performed on-slice Golgi staining and quantified dendritic spines on up to four orders of dendrites on MSNs from 12 week old mice. Extended focus montage images of single MSNs or both 3rd and 4th order dendrites (insets) are presented for WT (A), R6/2 (B) and R6/2-CB1(MSN) (C) mice. (D) Density of dendritic spines from n=9 MSNs (from 3 mice per group) were manually counted from z-stack montage images in ImageJ. Scale bars depict 10 μm and 1 μm in main images and insets, respectively. Error bars show S.E.M. Post-hoc

analyses were performed with Fisher's T Test, and are indicated by $*p<.05$, $**p<.01$, $***p<.001$.

Figure 7. Functional excitatory synapses are rescued in R6/2-CB₁(MSN)

Representative traces of spontaneous excitatory post-synaptic currents (sEPSC) and summary group data show (A) reduction in sEPSC frequency in 12-week old R6/2 (number of cells indicated within bar, from n=9 mice) compared to WT (from n=6 mice). sEPSC frequency in R6/2-CB₁(MSN) (from n=4 mice) is greater than in R6/2 mice and identical to sEPSC frequency in WT mice. (B) sEPSC amplitude shows no difference between genotypes. * $p < 0.05$ one-way ANOVA with Bonferroni post-hoc.

Figure 8. Intrinsic properties of MSN with genetic restoration of CB₁ receptors

(A) At 12 weeks of age, whole-cell capacitance in MSNs of R6/2 mice (black bar, number of cells indicated within bar, from $n=9$ mice) was significantly reduced compared with WT mice (white bar, from $n=6$ mice) and restored in R6/2-CB₁(MSN) mice (grey bar, from $n=5$ mice). Data was (B) Membrane resistance measured in R6/2 MSN is significantly higher than in WT MSNs, but membrane resistance in R6/2-CB₁(MSN) mice is WT levels. (C) Resting membrane potential of MSN is more depolarised in R6/2 mice compared with WT and R6/2-CB₁(MSN) mice. (D) Summary of current-firing curves indicating that the number of evoked action potentials in response to somatic current steps in MSNs of R6/2 and R6/2-CB₁(MSN) mice are significantly different than WT mice. Typical membrane responses to somatic current steps of dorsal striatal MSNs from WT (open circle), R6/2-CB₁(MSN) (grey circle) or R6/2 (black circle) mice are shown to the left. (E) Summary current-voltage (I-V) curves from somatic current steps show a difference between WT and both R6/2 and R6/2-CB₁(MSN) mice. (A-C) $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ by Bonferroni post-hoc from one-way ANOVA (D,E) Two-way ANOVA for repeated measures with Bonferroni post-hoc. Comparison between WT and R6/2 mice denoted by $*$; WT and R6/2-CB₁(MSN) mice denoted by $\#$.