Focal expression of mutant huntingtin in the songbird basal ganglia disrupts cortico-basal ganglia networks and vocal sequences

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Edited by Solomon H. Snyder, The Johns Hopkins University School of Medicine, Baltimore, MD, and approved February 2, 2016 (received for review December 4, 2015)

The basal ganglia (BG) promote complex sequential movements by helping to select elementary motor gestures appropriate to a given behavioral context. Indeed, Huntington's disease (HD), which causes striatal atrophy in the BG, is characterized by hyperkinesia and chorea. How striatal cell loss alters activity in the BG and downstream motor cortical regions to cause these disorganized movements remains unknown. Here, we show that expressing the genetic mutation that causes HD in a song-related region of the songbird BG destabilizes syllable sequences and increases overall vocal activity, but leave the structure of individual syllables intact. These behavioral changes are paralleled by the selective loss of striatal neurons and reduction of inhibitory synapses on pallidal neurons that serve as the BG output. Chronic recordings in singing birds revealed disrupted temporal patterns of activity in pallidal neurons and downstream cortical neurons. Moreover, reversible inactivation of the cortical neurons rescued the disorganized vocal sequences in transfected birds. These findings shed light on a key role of temporal patterns of cortico-BG activity in the regulation of complex motor sequences and show how a genetic mutation alters cortico-BG networks to cause disorganized movements.

Huntington's disease | basal ganglia | songbird | motor sequence | vocalization

The execution of complex behaviors, such as speech or musicianship, depends on the ability to organize elementary motor "gestures" into precisely timed sequences of movements. In the vertebrate brain, the basal ganglia (BG) play a key role in regulating complex motor sequences, as revealed by the behavioral disruptions characteristic of neurological diseases that affect BG function (1–3). A particularly striking example is Huntington's disease (HD), which is characterized anatomically by extensive cell death in the striatum, the primary input layer of the BG network (4), and behaviorally by hyperkinesia and chorea, which can interfere with the ability to produce coordinated, sequential movements (5, 6). Despite numerous animal models of HD, the detailed neural mechanisms by which striatal dysfunction leads to disorganized movements have remained elusive. One challenge is that HD also affects brain regions other than the BG, making it difficult to identify causal links between BG circuit dysfunction and behavioral symptoms. Another challenge, especially in rodent models of HD, is the limited understanding of whether and how BG circuitry contributes to specific behaviors.

The zebra finch is a small Australian songbird that presents several advantages for addressing these challenges. Adult male zebra finches sing a learned song comprising a reproducible sequence of stereotyped syllables (i.e., a motif), and they sing both spontaneously and abundantly, allowing for extensive characterization of syllables and syllable sequences. Moreover, an anatomically differentiated network of interconnected brain nuclei important to singing distinguishes the zebra finch brain (7, 8). An anterior forebrain pathway within this song system shares many similarities to mammalian cortico-BG networks (Fig. 1*A*), including a BG nucleus (area X) that contains both medium spiny neurons (MSNs) and pallidal neurons, which closely resemble their mammalian counterparts in their intrinsic, synaptic, and circuit properties (9–13). Pallidal neurons in area X communicate via the thalamus to a cortical premotor nucleus [lateral magnocellular nucleus of the anterior nidopallium (LMAN)] that plays a critical role in modulating song variability (14–18). The dedicated nature of this pathway for singing provides a rare opportunity to explore a detailed mechanism by which deficits in the BG act across broader cortico-BG networks to cause specific behavioral symptoms. Furthermore, the spatially distributed organization of the component song nuclei is well suited to the use of viral methods for selectively expressing genetic constructs within a single node in this network (19–22).

Intriguingly, a transgenic zebra finch model of HD was recently generated that shares several similarities with human HD (23), lending support to the idea that songbirds can afford useful models in which to identify how genetic mutations that cause HD alter BG circuitry to affect behavior. Indeed, these transgenic HD songbirds showed deficits in song learning; produced songs with abnormal syllable and sequence variability as adults; and displayed other symptoms, including body rigidity, tremor, and cervical dystonia. However, as with transgenic rodent models of HD and patients with HD, the brain-wide expression of the genetic mutation that causes HD in these transgenic songbirds makes it challenging to understand the link between genotype, circuit pathology, and behavioral phenotype. Here, we overcame this challenge by virally expressing a mutant gene fragment that

Significance

Genetic mutations that impair basal ganglia (BG) function affect sequential movements, but the link between genotype, BG circuit dysfunction, and altered behavior remains unclear. Here, we found that viral expression of a genetic mutation that causes Huntington's disease (HD) in a vocalization-related region of the songbird BG leads to the selective loss of striatal medium spiny neurons, abnormal temporal patterns of cortico-BG activity during vocalization, and highly unstable vocal sequences. Moreover, silencing activity in the cortical component of this circuit stabilized vocalizations, supporting the idea that the genetic mutation that causes HD affects complex motor sequences by altering temporal patterns of cortico-BG activity.

Author contributions: M.T. and R.M. designed research; M.T., J.S.A., and M.M. performed research; M.T. contributed new reagents/analytic tools; M.T. and J.S.A. analyzed data; and M.T. and R.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1523754113/-/DCSupplemental.

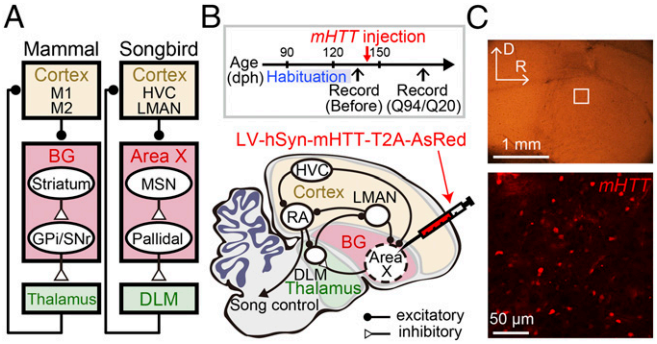

Fig. 1. Expression of *mHTT* in the songbird BG. (*A*) Simplified schematic showing some of the common features of mammalian and songbird cortico-BG networks. (*B*) Time line of experiments (*Top*) and a schematic showing a part of the neural circuitry involved in singing behaviors in the songbird (*Bottom*; sagittal view with rostral to the right and dorsal upward). LV containing a mutant form of *mHTT* and a fluorescent reporter, AsRed, with insertion of a self-cleaving T2A sequence was injected into the songbird BG (area X) of adult male zebra finches. The songs before injection (habituation) were highly stable (Fig. S1). Behavioral or neural changes were assessed at >30 d after injection. dph, days posthatch. (*C*) Square region in area X in a sagittal brain slice (*Top*; D, dorsal; R, rostral) was imaged 34 d after injection with a confocal microscope (*Bottom*) to confirm expression of *mHTT*-Q94, as revealed by coexpressed AsRed (see also Fig. S1).

causes HD [exon 1 of the huntingtin gene (*mHTT*)] in area X of adult male zebra finches. This focal genetic perturbation caused selective deficits in vocalization: The transfected birds vocalized more abundantly and sang aberrant songs in which syllable sequences, but not individual syllables, were selectively destabilized. These vocal changes were accompanied by the selective loss of MSNs in area X, a marked reduction of inhibitory synapses on pallidal neurons, and disrupted temporal patterns of singing-related activity in pallidal and LMAN neurons. Moreover, reversibly inactivating LMAN was sufficient to restore normal vocal behavior. These findings indicate that focal expression of *mHTT* in the BG disrupts motor sequences by altering temporal patterns of cortico-BG activity, which may afford a novel target for ameliorating disorganized movements in BG diseases.

Results

Expression of *mHTT* in the Songbird BG. Human HD is caused by expanded (>34) Gln (CAG) repeats in exon 1 of the huntingtin gene (*mHTT*). We transfected area X neurons in adult zebra finches (139 ± 19 d posthatch, mean ± SD; $n = 9$ birds) with lentivirus (LV) containing a mutant form of *mHTT* with either 94 or 20 CAG repeats (*mHTT*-Q94 or *HTT*-Q20) and a cleavable fluorescent reporter, AsRed, under the control of the human synapsin 1 promoter (Fig. 1*B*). Between 20 and 30 d following LV injection into area X, a time course similar to other studies that have used LV to express transgenes in songbirds (19–22), a subset of neurons expressed the AsRed reporter (Fig. 1*C*). These experiments indicate that LV-mediated gene transfer can be used to express *mHTT* in the songbird BG region area X.

Selective Disruption of Syllable Sequence Organization in *mHTT* Birds. We used LV-mediated gene transfer to address how expression of *mHTT* affects previously learned songs in adult zebra finches. Adult male zebra finches typically sing stable stereotyped motifs, with one or more motifs sung in quick succession to form a song bout (Fig. 2 *A* and *C*). Before viral injections, we confirmed that all of the birds used in our study sang a stable sequence of stereotyped syllables (Fig. S1; all songs were recorded in social isolation). Thirty to sixty days following bilateral injection of *mHTT*-Q94 in area X, the singing behavior of all birds underwent marked changes (Fig. 2 and Fig. S2; all songs were recorded in social isolation). The most notable change was that syllable sequences became highly unstable from one motif to the next, an effect that could be attributed to an increase in previously rare syllable transitions as well as the emergence of novel syllable transitions, some of which involved newly incorporated vocalizations (Fig. 2 *A–D*). In some instances, this sequence instability made comparison of motifs with those motifs produced before viral injection difficult (Fig. S2 *A* and *B*). We quantified these sequence-level effects using a variety of metrics, including sequence entropy, linearity, consistency, and stereotypy, all of which revealed significant changes (Fig. 2*E* and Fig. S2*C*). Long-term recordings revealed that changes in sequence stability could persist for many months after viral injection (Fig. S2*D*). These sequence-level effects remained even if song bouts that included newly incorporated syllables were excluded from the analysis (Fig. S2*E*), indicating that the original syllable sequences also became unstable. Moreover, calculating sequence entropy separately for each syllable revealed that transitions from most (~72%) syllables became more variable, suggestive of a global sequencing defect. We also noted that some newly integrated syllables closely resembled calls (i.e., vocalizations produced outside song bouts) that the bird had produced before viral injections, whereas others had spectral features distinct from the bird's calls (Fig. S2 *F* and *G*). Therefore, expression of *mHTT*-Q94 in area X can lead to the inappropriate incorporation of calls and novel vocalizations into the song motif, and this newly integrated vocal material can also contribute to sequence instability.

In addition to increased instability of syllable sequences, we noted increases in the daily amount of vocalization and in the duration of individual song bouts (Fig. 2 *F* and *G*), the latter of which could be attributed to an increase in the number of syllables produced during each bout [mean syllables per bout; before: 15.2 ± 1.9, after: 18.1 ± 2.7; $t(8) = 2.70$, $P = 0.030$]. There was also an increased occurrence of song bouts containing repeated syllables [repetitions occurred in 8.4 ± 4.1% of bouts before injection and in 34.1 ± 8.8% of bouts after injection; $t(7) = 4.205$, $P = 0.003$] and a trend toward increased numbers of times that the affected syllable was repeated [before: 2.13 ± 0.10 syllables; after: 2.48 ± 0.22 syllables; $t(6) = 2.045$, $P = 0.087$]. In contrast, we did not see changes in repetition of introductory notes, which are unlearned vocalizations produced before a motif [before: 2.10 ± 0.20 introductory notes, after: 2.11 ± 0.23 introductory notes; $t(8) = 1.816$, $P = 0.923$]. Transfecting area X neurons with *HTT*-Q20 did not produce sequence- or bout-level changes in song behavior (Fig. S3), indicating that these vocal changes were caused by expanded CAG repeats in *mHTT*-Q94 rather than by nonspecific effects of viral injection or damage resulting from stereotaxic surgery.

In contrast to these sequence- and bout-level changes, syllable features in *mHTT*-Q94 birds, such as duration, Wiener entropy, and variability [coefficient of variance (CV) entropy], remained unaffected (Fig. 2 *H–J*). Moreover, in the presence of a female, *mHTT*-Q94 birds reduced the variability in the pitch of individual syllables (Fig. S2*H*) and increased the number of introductory notes [no female: 4.89 ± 1.20, female: 8.31 ± 1.48; $t(7) = 6.719$, $P = 0.001$], two context-dependent features of vocal performance important to effective courtship in normal males (24, 25). We also noted that *mHTT*-Q94 birds exhibited reduced sequence entropy in the presence of a female [no female: 3.34 ± 0.11, female: 3.13 ± 0.15; $t(7) = 2.710$, $P = 0.030$]. No other gross behavioral changes, such as tremor or cervical dystonia, were detected in *mHTT*-Q94 birds. Taken together, these results indicate that expressing *mHTT*-Q94 in area X selectively impairs the bird's ability to organize syllable sequences.

Preferential Loss of Striatal Neurons and Reduction of Inhibitory Synapses on Pallidal Neurons in *mHTT* Birds. A major feature of HD is striatal atrophy resulting from the death of striatal MSNs (4), raising the possibility that the behavioral changes we detected

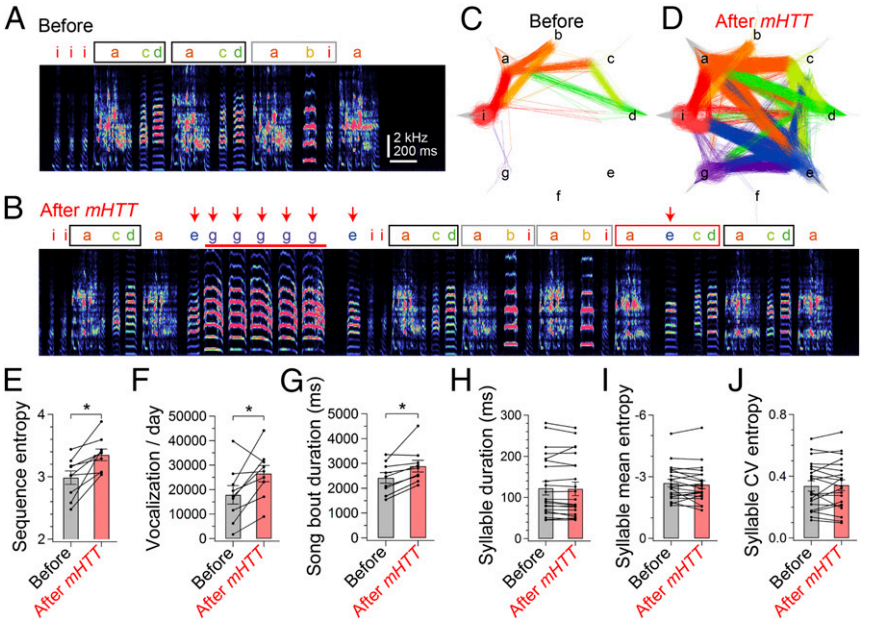

Fig. 2. Selective disruption of syllable sequences in *mHTT*-Q94 birds. Spectrogram of a representative song bout from a male adult zebra finch at 8 d before (*A*) and 47 d after (*B*) bilateral injection of *mHTT* in area X (see also Fig. S2 *A* and *B*). Characters denote different syllables. Stereotyped song motifs are demarcated with black and gray squares. Integration of new vocalizations (red arrows) accompanied a new syllable sequence (red square) and increased occurrence of syllable repetition (red horizontal bar). A syllable transition diagram shows all syllable trajectories on the day in *A* (*C*) and *B* (*D*). Each song bout starts with a gray inward line, and lines were then drawn between consecutive syllables to show a syllable trajectory of the song bout, which ends with an outward line. Colors denote preceding syllables. Repetitions of syllables are shown as curved lines with brighter colors. After injection of *mHTT*, sequence entropy (*E*), total vocalizations on the day (*F*), and mean song bout duration (*G*) were increased, indicating disrupted syllable sequencing [sequence entropy: $t(8) = 4.059$, $P = 0.010$; vocalization: $t(8) = 2.785$, $P = 0.024$; bout duration: $t(8) = 3.46$, $P = 0.011$]. $^*P < 0.050$. Spectral features such as duration (*H*), mean entropy (*I*), and CV of entropy (*J*) were not significantly changed by injection of *mHTT* [duration: $t(20) = 0.833$, $P = 0.415$; mean entropy: $t(20) = 1.122$, $P = 0.275$; CV entropy: $t(20) = 0.418$, $P = 0.681$] (see also Figs. S2 and S3).

in *mHTT*-Q94 birds resulted from the loss of striatal neurons. However, because smaller (i.e., soma area of <100 μm^2) striatal MSNs and larger pallidal neurons are intermingled in area X (10), injecting LV-*mHTT*-Q94 could potentially exert nonspecific cytotoxic effects on both striatal and pallidal neurons. Consistent with the idea that *mHTT*-Q94 expression in area X selectively kills

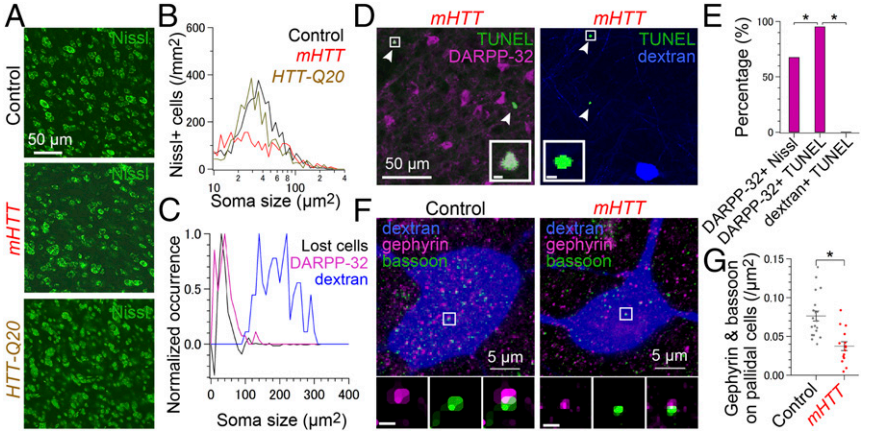

Fig. 3. Cellular and synaptic deficits in area X of *mHTT* birds. Nissl-stained area X neurons (*A*) and their soma size histograms averaged across control birds (black, $n = 4$), *mHTT* birds (red, $n = 4$), and *HTT*-Q20 birds (orange, $n = 2$) (*B*). (*C*) Peak-normalized soma size histogram of the difference between black and red traces in *B* (black, indicating lost cells), DARPP-32–positive MSNs (magenta, $n = 2$ birds) and dextran-labeled pallidal neurons (blue, $n = 2$ birds). (*D*) TUNEL-positive dying neurons (green, arrowhead) in *mHTT* birds were DARPP-32–positive MSNs (*Left*, pseudocolored magenta) but not dextran-labeled pallidal neurons (*Right*, pseudocolored blue). (*Insets*) Squared region. (Scale bars, 2 μm.) (*E*) Fraction of DARPP-32–positive MSNs was greater in TUNEL-positive neurons than in Nissl-positive neurons [$\chi^2(3) = 25.7$, $P < 0.001$], suggesting the preferential degeneration of MSNs. The fraction of DARPP-32–positive MSNs was greater than dextran-labeled pallidal neurons in TUNEL-positive neurons [$\chi^2(3) = 101.0$, $P < 0.001$] in area X of *mHTT* birds. (*F*) Maximum-projected images showing inhibitory synapses detected as colocalization of gephyrin (pseudocolored magenta) and bassoon puncta (green) on a dextran-labeled pallidal neuron (pseudocolored blue) in a control bird (*Left*) and *mHTT* bird (*Right*). (*Insets*) Median-filtered images of gephyrin (*Left*), bassoon (*Center*), and merged (*Right*) in the squared region. (Scale bars, 0.5 μm.) (*G*) Reduced inhibitory synapses on pallidal neurons in *mHTT*-Q94 birds [control: $n = 19$ cells from two birds, *mHTT*-Q94: $n = 15$ cells from two birds; $t(32) = 4.517$, $P < 0.001$]. $^*P < 0.050$ (see also Fig. S4).

MSNs, Nissl staining of fixed tissue sections revealed that the density of smaller neurons (i.e., soma area of <100 μm^2) in area X was reduced in *mHTT*-Q94 birds [Fig. 3 *A* and *B*; ∼42% loss; control: 4,147 ± 269 cells per square millimeter, *mHTT*-Q94: 2,389 ± 343 cells per square milliliter; $t(6) = 3.932$, $P = 0.008$], but not in *HTT*-Q20 birds [*HTT*-Q20: 3,595 ± 331 cells per square millimeter; $t(6) = 1.288$, $P = 0.245$]. Notably, the size distribution of cells that were lost following LV-*mHTT*-Q94 injection in area X was similar to the size distribution of MSN cell bodies, as measured by 32 kDa dopamine- and cAMP-regulated phosphoprotein (DARPP-32) immunofluorescence (Fig. 3*C*), and did not overlap with the size distribution of pallidal cell bodies, which were labeled by injecting retrograde tracer into their axonal terminal field in the song motor thalamus [i.e., the medial nucleus of the dorsolateral thalamus (DLM)]. These findings support the idea that expressing *mHTT*-Q94 in area X results in the loss of striatal MSNs, but spares the pallidal cell population.

To explore cell death triggered in area X by expressing *mHTT*-Q94, we labeled area X cells using the TUNEL system, which detects cell death by labeling cleaved DNA (26). We detected more TUNEL-positive cells in area X of birds injected with *mHTT*-Q94 [control: 1.2 ± 1.2 cells per square millimeter, *mHTT*-Q94: 36.3 ± 11.8 cells per square millimeter; $t(9) = 2.688$, $P = 0.025$]. Moreover, almost all (105 of 110 cells, $n = 3$ birds) of the TUNEL-positive cells in *mHTT*-Q94 birds were also positive for the MSN marker DARPP-32, whereas none (0 of 21 cells, $n = 2$ birds) of the TUNEL-positive cells were pallidal neurons, as identified via retrograde label from DLM (Fig. 3 *D* and *E*). In area X of birds injected with LV-*mHTT*-Q94, DARPP-32–positive cells constituted a higher fraction in the TUNEL-positive cell populations (105 of 110 cells) than in the Nissl-stained neuron populations (53 of 78 neurons, $n = 2$ birds) (Fig. 3*E*). This result indicates that expressing *mHTT*-Q94 in area X preferentially induces cell death in striatal MSNs, because nonspecific cell death would result in a similar fraction of DARPP-32–positive cells in the TUNEL-positive and Nissl-stained neuron populations.

Similar to mammalian striatopallidal organization, MSNs make inhibitory synapses onto pallidal neurons in area X (12). The loss of MSNs following *mHTT*-Q94 expression in area X raises the possibility that inhibitory synapses on pallidal neurons are also reduced in number. Consistent with this idea, the cell bodies and proximal dendrites of DLM-projecting pallidal neurons in *mHTT*-Q94 birds had half the number of inhibitory synapses, as measured by the immunocytochemical colocalization of inhibitory postsynaptic markers (gephyrin) and nonspecific presynaptic markers (bassoon) (Fig. 3 *F* and *G* and Fig. S4). Therefore, expressing *mHTT*-Q94 in area X reduces perisomatic inhibitory synapses on DLM-projecting pallidal neurons in parallel with reducing the numbers of MSNs, which are a major source of these inhibitory synapses. Because DLM-projecting pallidal neurons serve as the sole output of area X, MSN cell death followed by inhibitory synaptic reorganization on pallidal neurons could affect singing by altering the output of the BG network.

Temporally Disrupted Cortico-BG Network Activity in *mHTT* Birds. Pallidal neurons in area X influence song indirectly through LMAN, a premotor cortical structure that serves as an output of the cortico-BG network (17, 27). To examine how expressing *mHTT*-Q94 in area X affects cortico-BG network activity, we used a multielectrode array and spike-sorting methods to record pallidal and LMAN neuron activity simultaneously in freely behaving adult male zebra finches (Fig. S5*A*; all recordings were made from adult male zebra finches in social isolation). Both pallidal and LMAN neurons in *mHTT*-Q94 birds exhibited increased firing rates with singing, as was also observed in normal birds (Fig. S5 *B*–*E*).

However, abnormal activity patterns in both cell types became evident when they were aligned to a specific vocal sequence (the dominant motif: the most frequently produced motif) (*Materials and Methods* and Figs. 4 and 5). Whereas pallidal neurons in normal birds exhibited substantial modulation of firing rates at specific times during the motif (Fig. 4*A*), this temporal modulation was greatly reduced in *mHTT*-Q94 birds (Fig. 4 *B* and *C*). We also noted a reduction of the within-cell, trial-to-trial correlation of pallidal activity in *mHTT*-Q94 birds (Fig. 4*D*), suggesting that expressing *mHTT*-Q94 in area X disrupts the temporal precision of pallidal activity during singing. To estimate how changes in the temporal patterning of pallidal activity relate to syllable sequences, we calculated the mutual information between pallidal activity and consecutive time sequences in the motif. Indeed, this "timing information" was significantly decreased in *mHTT*-Q94 birds (Fig. 4*E*), indicating that pallidal activity conveys less information of moment-by-moment sequences in the song motif. Because pallidal neurons in area X can be divided into two distinct anatomical classes, only one of which projects to the thalamic nucleus DLM (28), a remaining issue is whether the firing properties of DLM-projecting pallidal cells are affected. We applied a Fano factor analysis, which has previously been used to distinguish the two pallidal cell types (29), to establish that the singing-related activity of putative DLM-projecting pallidal neurons was temporally disrupted in *mHTT*-Q94 birds (Fig. S6). These results show that expressing *mHTT*-Q94 in area X disrupts the temporal precision of

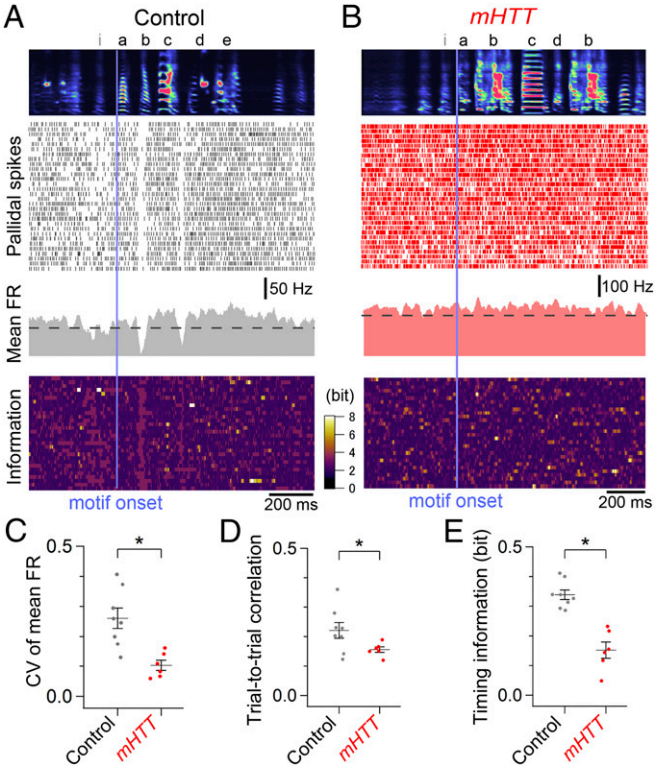

Fig. 4. Reduction of precise temporal modulation of pallidal activity in *mHTT* birds. Pallidal activity was aligned to the onset of the dominant song motif (vertical bar) in a control bird (*A*) and *mHTT* bird (*B*), showing averaged spectrogram of the first 30 renditions of songs that include the dominant song motif (*Top*), a spike raster of the corresponding pallidal neuron activity (*Upper Middle*), the cell's mean firing rate (FR) (*Lower Middle*), and information on the cell's activity calculated as -$\log_2 p(a)$, where $p(a)$ is the probability of observing the spike number a in a 20-ms bin during the motif (*Bottom*). Compared with control birds, pallidal activity in *mHTT* birds exhibited a reduction in the CV of the mean FR (*C*), trial-to-trial correlation (*D*), and timing information, calculated as mutual information between pallidal activity and motif timing (*E*) [CV of mean FR: $t(12) = 3.877$, $P = 0.002$; correlation: $t(12) = 2.525$, $P = 0.049$; timing information: $t(12) = 5.646$, $P < 0.001$]. *$P < 0.050$ (see also Figs. S5 and S6).

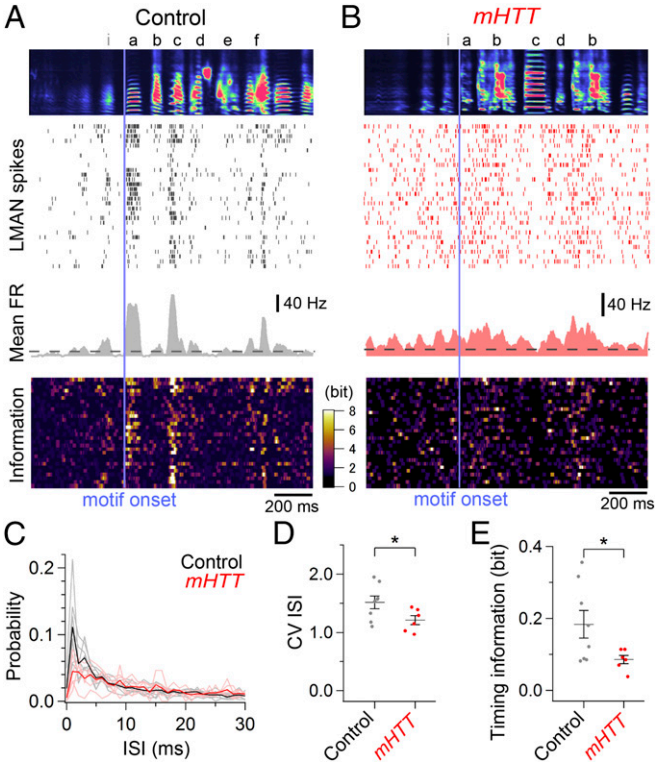

Fig. 5. Abnormally regular LMAN activity with less timing information in *mHTT* birds. LMAN activity was aligned to the onset of the dominant song motif (vertical bar) in a control bird (*A*) and *mHTT* bird (*B*), showing averaged spectrogram of the first 30 renditions of songs that includes the dominant song motif (*Top*), corresponding spike rasters of LMAN activity (*Upper Middle*), mean FR across renditions (*Lower Middle*), and information on the cell's activity as in Fig. 4 *A* and *B* (*Bottom*). (*C*) Mean ISI histogram of LMAN activity in control (black, $n = 8$ cells from three birds) and *mHTT* (red, $n = 6$ cells from three birds) birds. Bright lines indicate individual data. Compared with control birds, LMAN activity in *mHTT* birds exhibited a reduction in the CV of the ISI (*D*) and timing information (*E*) [CV ISI: $t(12) = 2.286$, $P = 0.041$; timing information: $t(12) = 2.304$, $P = 0.040$]. *$P < 0.050$ (see also Figs. S5 and S7).

pallidothalamic activity, which may degrade timing information about specific vocal sequences.

We also detected changes in the temporal patterns of singing-related LMAN activity following expression of *mHTT* in area X. We found that singing-related burst firing of LMAN neurons, which is known to be regulated by BG circuitry (22, 30), was less frequent in *mHTT*-Q94 birds and that bursts were of shorter duration when they did occur (Fig. 5 *A*–*C* and Fig. S7 *A* and *B*). Because the mean firing rate in LMAN was not altered in *mHTT*-Q94 birds [Fig. S5*D*; $t(12) = 1.027$, $P = 0.325$], LMAN firing was more evenly distributed throughout different syllables in the motif, as reflected in the reduced CV in interspike intervals (ISIs; Fig. 5*D*). Moreover, we also noted reduced timing information in LMAN neurons of *mHTT*-Q94 birds (Fig. 5*E*), indicating that the abnormally regular LMAN activity has less selectivity to specific syllables, as we observed with pallidal neuron activity in *mHTT*-Q94 birds. However, unlike for pallidal neurons, the within-cell, trial-to-trial correlation of LMAN activity was preserved in *mHTT*-Q94 birds (Fig. S7*C*), and LMAN firing still exhibited substantial modulation during singing (Fig. S7*D*). Therefore, LMAN neurons in *mHTT*-Q94 birds transmit abnormal timing information to downstream song motor structures on a background of normal trial-to-trial firing rate correlations and substantial firing rate modulations.

Inactivating LMAN Activity Reversibly Rescues Disrupted Song Behavior in *mHTT* Birds. To test directly the idea that the temporally disrupted LMAN activity is responsible for disorganized song in *mHTT*-Q94 birds, we reversibly inactivated LMAN by muscimol application through bilaterally implanted microdialysis probes (Fig. 6*A*). Notably, LMAN inactivation reduced sequence entropy to nearly normal levels (Fig. 6 *B*–*D* and Fig. S8*A*) and also reduced overall song bout duration in *mHTT*-Q94 birds (Fig. 6*E* and Fig. S8*B*). The shorter song bout duration was attributed to reduced syllable numbers in each song bout [before muscimol: 20.0 ± 2.7 syllables per bout, after muscimol: 16.7 ± 2.6 syllables per bout; $t(3) = 3.590$, $P = 0.037$]. We also noted that there was a tendency toward less frequent syllable repetitions, but this difference was not significant [before muscimol: 35.1 ± 15.8%, after muscimol: 7.3 ± 2.7%; $t(3) = 1.961$, $P = 0.145$]. Inactivating LMAN also reduced the variability in the fundamental frequency of individual syllables [CV of pitch; pre: 0.013 ± 0.003, post: 0.006 ± 0.001; $t(5) = 2.571$, $P = 0.050$], consistent with the known function of LMAN in modulating syllable variability (15, 31, 32). Taken together, these findings indicate that temporally disrupted LMAN activity is critical for driving disorganized song behavior in *mHTT*-Q94 birds.

Discussion

Here, we show that expressing *mHTT* in area X, a region of the songbird BG specialized for singing behavior, destabilizes syllable sequences and increases overall vocal activity, but leaves the structure of individual syllables intact. A cellular correlate of these behavioral changes includes the selective loss of striatal MSNs in area X paralleled by a marked reduction in inhibitory synapses on pallidal neurons that serve as the BG output. These cellular and synaptic changes were sufficient to alter the activity across a larger expanse of the BG-cortical network, because pallidal neurons and downstream LMAN neurons in *mHTT* birds exhibited abnormal patterns of singing-related activity. Moreover, reversibly inactivating LMAN rescued aberrant vocal behavior in *mHTT* birds, indicating that changes to the timing of activity in the cortico-BG network drive changes in vocal sequencing. The present findings that aberrant temporal patterns of

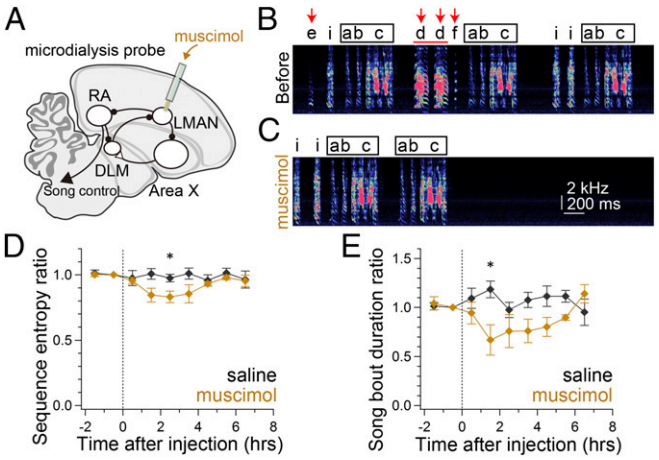

Fig. 6. Inactivating LMAN rescues aberrant song behavior in *mHTT* birds. (*A*) Schematic showing infusion of 5 mM muscimol with a microdialysis probe implanted into LMAN to inactivate LMAN activity. A representative song bout sung 1 h before (*B*) and 2 h after (*C*) infusion of muscimol in a *mHTT* bird is shown. Characters denote song syllables, and the square indicates the dominant motif. Increased sequence variability with irregular syllable insertion (red arrows in *B*) and repetition (red horizontal bar in *B*) decreased after muscimol infusion (*C*). Sequence entropy (*D*) and song bout duration (*E*) are shown as a function of time after infusion of saline (black) or muscimol (orange) in *mHTT* birds ($n = 4$ birds). Asterisks show significance (*$P < 0.050$), with a paired *t* test, after the significant main effect of condition was confirmed with two-way ANOVA [sequence entropy [$F(1,58) = 10.41$, $P = 0.002$; bout duration: $F(1,58) = 11.05$, $P = 0.002$] (see also Fig. S8).

cortico-BG activity disrupt complex motor sequences may inform strategies for ameliorating disorganized movements in BG diseases.

Parallels to HD and Selective Impairment of Sequential Movements in *mHTT* Birds. BG circuits are implicated in the organization of motor sequences, in part, by selecting for elementary gestures appropriate to a given behavioral context (i.e., action selection) (1–3). Here, we found that focally expressing *mHTT* in area X of the zebra finch affects syllable sequence organization by destabilizing syllable sequences and decreasing song termination, and also by increasing the tendency to repeat syllables. Sequence destabilization could be attributed to an increase in previously rare syllable transitions as well as the expression of novel syllable transitions, some of which involved the incorporation of new syllables into a motif. These behavioral changes are reminiscent of disorganized movements in humans with HD, including increased spontaneous movements, disorganized sequences of elementary gestures, and insertion of inappropriate gestures into sequential movements (6, 33).

These results also extend the recent finding that a transgenic zebra finch model of HD exhibits various HD-like symptoms (23) by demonstrating that focal expression of *mHTT* in area X selectively affects motor sequences related to singing behavior, thus providing a more specific link between the genetic insult, circuit defects, and aberrant behavior. Here, we observed only syllable sequence destabilization following expression of *mHTT* in area X, whereas the transgenic HD songbird displays sequence and syllable instability, as well as body rigidity, tremor, and cervical dystonia. Presumably, the additional behavioral symptoms that manifest in the transgenic finch result from the expression of *mHTT* in other song and motor regions of the brain outside of area X, many of which also express the *mHTT* gene at high levels (23). Another distinction is that the viral approach allowed for temporally restricted expression of *mHTT* in adult finches after they had already successfully learned and stabilized their songs, thus avoiding the potential confound in the transgenic finch that some of its song abnormalities are secondary consequences of impaired learning. Consequently, a useful aspect of the viral approach is that it establishes the sufficiency of spatially and developmentally restricted expression of *mHTT* in area X of the adult finch in driving sequence-specific changes to song.

Previous studies indicate that the ability to organize multiple movements temporally can be disrupted in humans with HD, leaving elementary movements relatively unaffected (5, 6, 34). Similarly, birds expressing *mHTT* in area X exhibited sequence-level changes to song, but the structure and variability of individual syllables remained unaffected. The lack of changes to previously learned syllables and the continued ability of *mHTT* birds to modulate variability as a function of social context further support a model in which the variability of individual syllables and of the sequences in which they occur are at least partly under independent control (16, 35). A possible clinical parallel is that humans with HD often exhibit grammatical errors and can struggle to produce complex sentences, even though simple phonemic generation can be preserved (33). In summary, these various observations strengthen the notion that the BG play a critical role in organizing and terminating motor sequences and show that focally expressing *mHTT* in the BG results in elevated levels of aberrant sequential behavior, reminiscent of the hyperkinetic, disorganized movements characteristic of HD.

Circuit Basis for Impaired Motor Sequences in *mHTT* Birds. The song-related effects of expressing *mHTT* in area X exert selective behavioral and anatomical effects that contrast with the behavioral and anatomical effects elicited by area X lesions, thus helping to illuminate how different components of the BG circuitry regulate sequential movements. First, sequence variability, vocal activity, and song bout duration increase in *mHTT* finches but are often unaffected following area X lesions in adult birds (36, 37; but see 38, 39). Moreover, variability and duration of individual syllables are unaffected in *mHTT* birds but are altered following area X lesions (30, 38). The song behavior of *mHTT* birds could not simply be attributed to nonspecific cytotoxic effects of viral injection, infection, or overexpression of *mHTT*, because expressing exon 1 with 20 CAG repeats [i.e., a quantity fivefold greater than the avian repeat number (23)] was without any discernible behavioral effect. More fundamentally, chemical or electrolytic lesions placed in area X destroy both MSNs and pallidal neurons, whereas expressing *mHTT* in area X selectively kills MSNs without reducing the numbers of pallidal neurons. Interestingly, chemical inactivation of the globus pallidus internus does not disrupt learned motor sequences in monkeys, suggesting that pallidal neuron activity may be unnecessary for the maintenance of previously learned behaviors (40). In *mHTT* birds, the intact population of pallidal neurons may exacerbate aberrant vocal behavior by transmitting temporally disrupted patterns of activity to downstream thalamic and cortical premotor neurons.

Although pallidal neurons are preserved in *mHTT* birds, inhibitory cells and synapses in area X undergo extensive reconfiguration. An analysis of cell death- and cell type-specific markers indicates that expressing *mHTT* in area X selectively triggers cell death in MSNs, similar to the selective loss of striatal MSNs in HD (4, 41). One consequence of MSN cell death is the marked reduction of inhibitory puncta on DLM-projecting pallidal neuron cell bodies and proximal dendrites, which presumably reflects the loss of inhibitory input from MSNs, and perhaps from other inhibitory neurons in area X. Although syllable repetitions in zebra finches with area X lesions have been speculated to arise from the replacement of MSNs through ongoing neurogenesis (38), the persistent reduction in inhibitory synapses on the DLM-projecting pallidal neurons we observed suggests that the primary factor driving abnormal vocal behavior is the massive loss of MSNs, which is a hallmark of HD. Ultimately, because pallidal neurons fire action potentials spontaneously and at high rates, and because MSN neurons generate precisely timed action potential bursts during singing (13), this loss of MSN input is likely to diminish or otherwise alter precisely timed singing-related activity in pallidal neurons.

Indeed, extracellular recordings made from pallidal neurons in *mHTT* finches revealed reduced temporal modulation during singing, consistent with the idea that diminished inhibition results in less precisely controlled pallidal neuron activity. More specifically, although pallidal neuron firing rates increased during song, the normal patterns of sharp and transient decreases in firing rate were absent, which could be accounted for by diminished inhibitory input from MSN cells. Notably, transient decreases in pallidal neuron firing rate are sufficient to generate action potential bursts in their postsynaptic targets in the song motor thalamus (i.e., DLM) (42), which, in turn, excite the LMAN neurons that are an important source of vocal variability (14, 16, 43). Therefore, expressing *mHTT* in area X has the potential to alter activity not only in the BG but also in the cortical neurons that are indirect targets of pallidal neurons and that more directly affect vocalization. In support of this view, LMAN neurons in *mHTT* birds exhibited reduced bursting activity and less variable spike timing, and ultimately conveyed less timing information during singing. Furthermore, inactivating LMAN stabilized syllable sequences, but sharply reduced song bout duration and overall vocal activity, suggesting that abnormal patterns of LMAN activity drive abnormal vocal behavior in *mHTT* birds. These findings provide insights into how a spatially restricted genetic insult can ramify through the local BG circuitry and across a broader cortico-BG network to affect a complex sequential behavior and raise the possibility that precisely manipulating this network activity may help to restore normal motor function in HD.

Possible Mechanisms by Which Cortico-BG Networks Influence Sequential Movements. A remaining question is how abnormal LMAN activity patterns affect sequential vocal behavior. Individual LMAN axons bifurcate as they exit the caudal margin of the nucleus, with separate branches terminating in the song motor cortical region [the robust nucleus of arcopallium (RA)] and in area X (18, 44, 45). The LMAN-RA pathway is known to be an important source of syllable variability (15) and could also potentially affect sequence variability through recurrent pathways involving the thalamus and the song premotor nucleus HVC (46), which lies upstream of RA and is thought to regulate song's temporal features, including intersyllable transitions (47–51). However, syllable variability was unaffected in *mHTT* birds, raising the question of how the propagation of altered LMAN activity through RA could affect sequence variability without also increasing the variability of individual syllables. One possibility is that distinct features of LMAN activity differently affect syllable sequences and variability through the LMAN-RA pathway: Disrupted temporal patterns could destabilize syllable sequences without affecting syllable variability, whereas unaltered trial-to-trial correlations and persistent firing rate modulation may support normal levels of syllable variability regardless of reduced bursting activity. However, another intriguing possibility is that abnormal activity patterns in LMAN disrupt syllable sequences by interfering with HVC activity. Indeed, a recent study revealed an indirect pathway from LMAN to HVC that includes area X, the ventral pallidum, and a midbrain cell group (A11) (16). In this view, temporally abnormal activity patterns in LMAN can promote the production of certain syllables at wrong times in the sequence by acutely altering premotor commands at the level of HVC (Fig. 7). Furthermore, altered LMAN activity patterns may also indirectly affect vocal sequences by inducing plastic changes in HVC. Indeed, LMAN lesions can prevent synaptic changes in HVC normally triggered by deafening (52), suggesting that LMAN activity can modulate synaptic organization in HVC through the BG and midbrain pathway. In mammals, striatal plasticity is known to induce dynamic changes in cortical signaling (53) and is required for the emergence of timing signals in the BG and midbrain during motor sequence learning (54). Thus, the *mHTT*-dependent loss of MSNs could engage both acute and long-term mechanisms that disrupt the precisely timed signals that normally flow through cortico-BG networks, resulting in disorganized motor sequences.

Materials and Methods

Detailed procedures are provided in *SI Materials and Methods*. All experimental procedures were performed in accordance with the NIH guidelines and approved by the Duke University Medical Center Animal Care and Use Committee.

Song Analysis. For song analysis, days before and >30 d after injection were randomly chosen [*mHTT*-Q94: 7.2 ± 3.9 d before and 61.7 ± 45.8 d after injection; *HTT*-Q20: 5.8 ± 2.9 d before and 70.8 ± 23.6 d after injection (mean ± SD); $n = 9$], and all of the recorded files on the day were processed with a custom MATLAB program (MathWorks) to extract and classify song syllables as described previously (52). To compare syllable features before and after *mHTT* injection, shared syllables (excluding introductory notes) from 100 randomly chosen renditions were analyzed. To extract song bouts, we first determined the most frequently observed syllable sequence on that day as the dominant motif. Then, song bouts were defined as successive vocalizations with intersyllable intervals of <400 ms and that included at least three syllables from the dominant motif, allowing us to exclude call bouts. To compare sequence features before and after *mHTT* injection, 100 randomly chosen song bouts were analyzed. Sequence entropy S was calculated as follows based on the empirically estimated transition probability matrix:

$$S = -\sum_i p_i \log_2 p_i,$$

where p_i is the probability of observing each syllable transition type. Linearity, consistency, and stereotypy were calculated with syllables, including introductory notes in individual song bouts, and averaged across the 100 randomly chosen bouts.

Data Analysis. To examine spike activity aligned to the dominant motif, the first 30 recordings after implantation containing the dominant motif were identified, and analyses were confined to the first 500 ms after the motif onset. Instantaneous firing rate was calculated as the reciprocal of the ISI and smoothed with a band-pass filter (25–75 Hz). Mean firing rate was the average of binned firing rates in a 20-ms sliding bin with a sliding step of 1 ms. The trial-to-trial correlation was the average of the peak between a ±10-ms time shift in cross-correlation values calculated between all of the possible combinations of the 30 iterations of smoothed instantaneous firing rate after mean subtraction. Timing information $I(T;A)$ was calculated as mutual information between timing T in the motif and spike activity A based on binned firing rates:

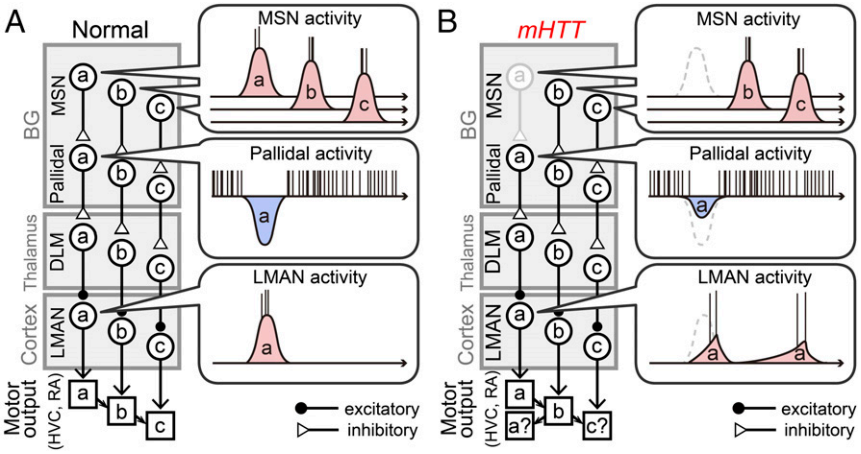

Fig. 7. Model of how MSN loss can acutely disrupt motor sequences. A simplified schematic depicts a cortico-BG module that conveys information about a motor sequence in a normal bird (*A*) and *mHTT* bird (*B*). In a normal bird, each MSN fires a short burst of action potentials that is hypothesized to encode information about a specific vocal motor gesture (*a*, *b*, or *c*) that is conveyed to pallidal, DLM, and LMAN neurons. In the *mHTT* bird, loss of the MSN that encodes *a* (gray) reduces the inhibitory modulation of related pallidal neuron activity, which subsequently disrupts the temporal pattern of the associated LMAN neuron's activity. The mistimed signals of the LMAN neuron may disturb the motor signals in the downstream song premotor region HVC, or possibly through RA (*Discussion*), promoting an insertion of motor output *a* at a wrong time when these pathways are about to induce motor output of *c*, disrupting the normal pattern of syllable sequences. Note that this model does not necessarily disrupt the spectral features of syllable *a* by LMAN activity and can be compatible with the unaltered trial-to-trial correlation of LMAN activity in *mHTT* birds (Fig. 5), which may account for the retained trial-to-trial variability of syllable *a* regardless of the reduced burst firing.

$$I(T;A) = -\sum_{t\in T} p(t)\log_2 p(t) + \sum_{a\in A} p(a)\sum_{t\in T} p(t|a)\log_2 p(t|a),$$

where $p(t)$ is the probability of observing a specific timing t in the motif, $p(a)$ is the probability of observing the spike number a in the motif, and $p(t|a)$ is the probability of observing the activity in a specific timing t in the motif given the spike number of a. Because $p(t)$ is a constant of 1/500, the first term on the right side is 8.966. Qualitatively, low timing information means syllable timings cannot be decoded from the observed spike activity.

Statistics. Error bars and values in the text indicate mean ± SEM unless otherwise noted. Paired or unpaired t tests were used to assess the effects of *mHTT*. To compare the proportion of TUNEL-positive MSNs, a χ^2 test was used. Repeated two-way ANOVA was performed in MATLAB to examine the effects of *mHTT* on social modulation of CV pitch and the effects of LMAN inactivation.

ACKNOWLEDGMENTS. We thank James McNamara, Kevin Franks, Nicole Calakos, Cagla Eroglu, Jeff Beck, and David Schneider for reading and commenting on the manuscript; Marguerita Klein in the Duke Viral Vector Core for preparing viruses; and Chia-Hui Chen for helping with viral injection. This study was supported by research grants from the Hereditary Disease Foundation (to R.M.), by NIH Grant DC02524 (to R.M.), by a fellowship from the Ruth K. Broad Foundation (to M.T.), and by a fellowship from the Astellas Foundation for Research on Metabolic Disorders (to M.T.).

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