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Msh2 Acts in Medium-Spiny Striatal Neurons as an Enhancer of CAG Instability and Mutant Huntingtin Phenotypes in Huntington’s Disease Knock-In Mice

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Abstract

The CAG trinucleotide repeat mutation in the Huntington’s disease gene (HTT) exhibits age-dependent tissue-specific expansion that correlates with disease onset in patients, implicating somatic expansion as a disease modifier and potential therapeutic target. Somatic HTT CAG expansion is critically dependent on proteins in the mismatch repair (MMR) pathway. To gain further insight into mechanisms of somatic expansion and the relationship of somatic expansion to the disease process in selectively vulnerable MSNs we have crossed HTT CAG knock-in mice (HdhQ111) with mice carrying a conditional (floxed) Msh2 allele and D9-Cre transgenic mice, in which Cre recombinase is expressed specifically in MSNs within the striatum. Deletion of Msh2 in MSNs eliminated Msh2 protein in those neurons. We demonstrate that Msh2-specific deletion of Msh2 was sufficient to eliminate the vast majority of striatal HTT CAG expansions in HdhQ111 mice. Furthermore, Msh2-specific deletion of Msh2 modified two mutant huntingtin phenotypes: the early nuclear localization of diffusely immunostaining mutant huntingtin was slowed; and the later development of intranuclear huntingtin inclusions was dramatically inhibited. Therefore, Msh2 acts within MSNs as a genetic enhancer both of somatic HTT CAG expansions and of HTT CAG-dependent phenotypes in mice. These data suggest that the selective vulnerability of MSNs may be at least in part contributed by the propensity for somatic expansion in these neurons, and imply that intervening in the expansion process is likely to have therapeutic benefit.

Introduction

Huntington’s disease (HD) is a dominantly inherited neurodegenerative disorder characterized by motor, cognitive and psychiatric symptoms [1]. The underlying cause is the expansion >35 repeats of a polymorphic CAG repeat within HTT gene that lengthens a glutamine tract in the huntingtin protein [2]. Stringent statistical analyses in a large HD patient data set indicate that the CAG expansion determines onset age in a fully dominant fashion with no evidence for a major role of either the wild-type allele or a second mutant allele [3]. While mutant huntingtin exerts its toxic effects in many brain regions as well as peripheral tissues over the course of the disease, medium-spiny GABA-ergic projection neurons (MSNs) in the striatum are the most vulnerable [4–6]. Therefore, the factors that contribute to this neuronal susceptibility are likely to provide clues to pathogenesis. Despite being caused by a single gene defect the disease is clearly complex, with a multitude of cellular pathways disrupted in response to mutant huntingtin [7]. Discerning those events that are critical to pathogenesis in order to design rational therapeutics remains a challenge.

An alternative to targeting downstream pathways that are disrupted during the course of disease is to target the CAG repeat mutation itself. Given that onset age and disease severity are highly correlated with the length of the expanded CAG repeat [3,8], one would predict that reducing CAG length, even within the disease range, would have a beneficial effect. Notably, the mutant HTT CAG repeat exhibits both intergenerational and somatic instability [8–17]. The latter is highly biased towards expansions and is tissue-specific, with the greatest expansions seen in the striatum [13]. The striatum appears to be particularly susceptible to expansion in several trinucleotide repeat diseases [18–20], consistent with findings that expansion reflects an intrinsic property of this tissue rather than being a consequence of ongoing pathogenesis [21]. However, the further expansion of the mutant HTT CAG repeat in the striatum as well in other tissues susceptible to the effects of mutant huntingtin, is predicted to contribute to the pathogenic process. Indeed, longer somatic...
expansions in HD postmortem brain correlate with an earlier age of disease onset [17]. Therefore, the factors that modify repeat instability are predicted to modify disease and may lead to novel therapeutic targets.

To study the mechanisms underlying HTT CAG instability we have developed a series of HTT homologue (Hti or Hdh) CAG knock-in mice that replicate the genetic mutation in HD patients [22,23]. Notably, these mice exhibit CAG length-dependent tissue-specific somatic expansion, with amongst the highest levels of instability seen in the striatum [21,23,24]. We have previously shown that striatal instability in HdhQ111 mice is critically dependent on mismatch repair genes Msh2 and Msh3 [25,26]. Significantly, constitutional knockout of either of these two genes delayed an early, dominant, CAG length-dependent phenotype in the striatum, the nuclear localization/epitope accessibility of mutant huntingtin, supporting the hypothesis that somatic expansions in target tissues contributes to the pathogenic process [25,26].

Here, we sought to explore further the role of Msh2 as a genetic modifier of HTT CAG repeat instability and pathogenesis. Given the particular susceptibility of MSNs to the disease process we have used a conditional knockout strategy to specifically delete the Msh2 gene in this neuronal subtype of HdhQ111 mice. This neuronal subtype-specific deletion of Msh2, allowed us to ask the following questions: 1. Is Msh2 required in MSNs to mediate HTT CAG expansion? 2. Is Msh2 required in MSNs as a modifier of CAG repeat length-dependent mutant huntingtin localization and intranuclear inclusion phenotypes?

Results

Conditional Deletion of Msh2 in Medium-spiny Striatal Neurons

To delete the Msh2 gene we used a conditional Msh2 knockout mouse line in which exon 12 that encodes part of Msh2’s essential ATPase domain is flanked by loxP sites (Msh2flox) [27]. To achieve specific deletion in MSNs we used a transgenic line (D9-Cre) in which Cre recombinase is expressed under the control of regulatory elements of the mouse Ppp1r1b gene encoding DARPP-32 [28]. Within the striatum, D9-Cre mice have been shown to express Cre specifically in MSNs from 5–6 weeks of age [28]. Crossing the Msh2flox and D9-Cre mice together demonstrated deletion of exon 12 of the Msh2 gene in striatal DNA only in Msh2flox mice that also harbored the D9-Cre transgene (Figure 1A).

Note that the undeleted (floxed) allele is still present in D9-Cre mice, which likely reflects contributions both from non-MSNs (interneurons and glia) that do not express the Cre transgene, and a small number of MSNs in which Cre-mediated recombination does not occur [28]. Comparison of genomic DNA isolated from different tissues (striatum, cerebellum, cortex, liver, tail) from Msh2flox/+ D9-Cre mice showed that deletion of Msh2 exon 12 was specific to the striatum (Figure 1B). The absence of Cre-mediated recombination in other tissues is consistent with the very low number of cortical neurons and cerebellar purkinje cells in which Cre activity was previously observed and the general absence of recombination in peripheral tissues [29]. We will refer henceforth to the conditionally MSN-deleted allele, present in Msh2flox D9-Cre mice as Msh2A.

Msh2 Protein is Eliminated in Medium-spiny Striatal Neurons

To examine the effect of MSN-specific deletion of Msh2 on phenotypes elicited by an expanded HTT CAG repeat we established crosses with HdhQ111 mice, Msh2flox mice, D9-Cre mice and Msh2 null mice (Figure S1) to generate HdhQ111/+ mice with the following five genotypes: Msh2+/+, Msh2+/-, Msh2A+/A, Msh2A+/- and Msh2+/-, allowing us to compare directly the effect of MSN-specific deletion of Msh2 and constitutional Msh2 deletion. Note that while 50% of Msh2+/-/+ mice die by 6 months of age due to tumor burden [largely in lymphatic tissue and the gut] [29] we did not observe any reduced viability or brain tumors in Msh2A+/A or Msh2A+/- mice that were aged as far as 10 months.

We first determined the expression levels of Msh2 protein in striatal extracts from mice with Msh2+/-, Msh2+/-, Msh2A+/A, Msh2A+/- and Msh2+/- genotypes. Msh2 protein levels were reduced by ~60% in Msh2A+/A and Msh2A+/- striatal extracts compared those in Msh2+/-+ and Msh2+/- striatal extracts (Figure 2A,B). To assess more precisely the level of knockdown of Msh2 in MSNs we co-immunostained striatal sections with an antibody against DARPP-32 that specifically labels MSNs and an antibody against Msh2. This revealed the specific loss of Msh2 in MSNs in Msh2A+/A and Msh2A+/- mice (Figure 2C).

Quantification of the immunofluorescent images (Figure 2D) showed significantly reduced levels of Msh2 in DARPP-32-positive MSNs in Msh2A+/A compared to Msh2+/-+ mice (p<0.01) and in Msh2A+/- compared to Msh2+/- mice (p<0.01). There was no significant difference in levels of Msh2 in MSNs between Msh2A+/A or Msh2A+/- and Msh2+/- mice, indicating the complete loss of Msh2 in Msh2A+/A and Msh2A+/- MSNs. Note that the Msh2 protein level in Msh2+/- striata (Figure 2A, B) or MSNs (Figure 2C, D) is greater than 50% of that in Msh2+/- striata, suggesting possible compensatory mechanisms, at least in striatal tissues/cells, by which the cell is able to regulate Msh2 levels to some degree.

Msh2 Acts in Medium-spiny Striatal Neurons to Effect HTT CAG Expansion

To investigate the effect of MSN-specific Msh2 deletion on the somatic instability of the HTT CAG repeat HdhQ111/+ mice with Msh2+/-, Msh2+/-, Msh2A+/A, Msh2A+/- and Msh2+/- genotypes were aged to five months of age, a time-point at which we have previously observed significant accumulation of somatic expansions in HdhQ111/+ striata. GeneMapper traces obtained from PCR-amplification of the HTT CAG repeat (Figure 3A) showed a bimodal distribution of CAG repeat lengths in striatal DNA from both Msh2+/-+ and Msh2+/- mice, as previously observed [26]. Also consistent with our previous studies of Msh2 null HdhQ111 mice [25], these somatic expansions were completely eliminated in striatal DNA from Msh2+/- mice. In striatal DNA from both Msh2A+/ and Msh2A+/- mice the somatically expanded repeats were dramatically reduced, although not eliminated as in Msh2+/- mice. Quantification of the GeneMapper traces (Figure 3B) revealed a significant reduction in instability in striata from Msh2A+/A compared to Msh2+/-+ mice (p=0.0001) and in striata from Msh2A+/- compared to Msh2+/- mice (p<0.0001). As assayed using our quantification method, there were no significant differences in striatal instability between Msh2A+/A and Msh2+/-+ mice (p=0.18) or between Msh2A+/- and Msh2+/- mice (p=0.33). Consistent with the tissue-specific Msh2 deletion shown in Figure 1B, Msh2A+/A and Msh2A+/- mice did not show any alteration in instability in liver or cortex, although instability in these tissues (high in liver and moderate in cortex) was eliminated in Msh2+/- mice.

To determine whether elimination of Msh2 in MSNs was sufficient to suppress somatic expansion over a more extensive time-period, we examined instability in HdhQ111+/Msh2A+/A and HdhQ111+/Msh2A+/- mice at ten months of age. While ten-
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Msh2 Acts in Medium-spiny Striatal Neurons as an Enhancer of the HTT CAG Repeat Length-Dependent Phenotypes

Previous data in HD patients and HdhQ111 mice are consistent with the hypothesis that somatic expansions accelerate the HTT CAG-dependent pathogenic process [17,25]. We have identified two CAG repeat-length dependent phenotypes in knock-in mice that would be predicted to be altered as a result of the loss of somatically expanded HTT CAG repeats; early (~2.5 months) diffusely-immunostaining nuclear mutant huntingtin using the anti-huntingtin antibody EM48 and later (6–12 months) intranuclear inclusions of mutant huntingtin amino-terminal fragments [30–32]. While the direct consequences to the cell of either of these phenotypes are unclear, the observation that they are dominant, CAG repeat length-dependent and occur with a strong selectivity towards MSNs [30,31] indicates that their underlying mechanisms are likely to be relevant to the pathogenic process in HD. Given the critical role of Msh2 in mediating somatic expansion in MSNs we have tested whether Msh2 is also a modifier of these two CAG length-dependent mutant huntingtin phenotypes. We previously showed that constitutional loss of Msh2 slowed the diffuse nuclear huntingtin phenotype in the striatum [25]. We have now developed a modified, quantitative assay to measure the time-dependent increase in diffuse nuclear mutant huntingtin in HdhQ111/+ mice using the anti-huntingtin monoclonal antibody mAb5374 (Figure S2). We have used this assay to determine the role played by MSN-expressed Msh2 in determining the diffuse nuclear mutant huntingtin phenotype. In our previous study [25] we did not analyze the effect of the Msh2 null mutation on nuclear inclusions in HdhQ111/+ mice as the reduced lifespan of the Msh2 null mice precluded our ability to analyze this phenotype that was not apparent until ~12 months on the genetic background of the original HdhQ111 x Msh2 null cross (mixed CD1/129Ola/FVB). Here, afforded by the earlier appearance of intranuclear inclusions on C57BL/6 backgrounds [32] we have also asked whether MSN-expressed Msh2 is a modifier of intranuclear inclusions.

Striatal sections from the same five-month and ten-month mice in which we determined somatic instability (Figure 3) were immunostained with mAb5374 (Figure 4), mAb5374 staining in five-month mice was found to depend both on the constitutive CAG repeat length as well as the position (medial versus lateral) within the striatum, with longer CAG lengths and lateral location resulting in increased staining (p<0.05 and p<0.001, respectively). Therefore, to determine the effect of Msh2 genotype on mAb5374 staining we performed a multiple regression analysis, controlling for both constitutive CAG length and lateral versus medial position in the striatum. We found that, compared to Msh2+/+ mice, mAb5374 staining was reduced in both Msh2+/A (p<0.01) and Msh2+/− (p<0.001) mice as well as in Msh2−/− mice as predicted (p<0.05) (Figure 4A, B), although the difference in staining intensities in Msh2+/+ and Msh2+/− striata did not reach statistical significance (p = 0.18).

In ten-month striata we observed a dramatic reduction in the number of mAb5374-positive intranuclear inclusions in Msh2+/A, Msh2+/− and Msh2−/− mice (Figure 4C, D). We were not able to perform statistical analyses of inclusion number in Msh2+/A or Msh2−/− mice due to an N = 1 for each of these genotypes,
Discussion

We have demonstrated that Msh2 acts in MSNs as a genetic enhancer of both HTT CAG repeat expansion and of HTT CAG repeat length-dependent mutant huntingtin phenotypes that occur as a part of an ongoing pathogenic process. As discussed previously [25], Msh2 could, in principle, modify the disease process either indirectly via its role in modulating CAG repeat length, or directly, by some other unknown mechanism. Our findings indicate that Msh2 does not accelerate the pathogenic process via detrimental effects to supportive glial cells or via detrimental systemic effects at the level of the whole organism, but rather acts in a cell-autonomous manner to influence phenotypes in MSNs. These data, therefore, are consistent with an indirect role for Msh2 as a modifier of the...
A 5 months

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B 5 months

C 10 months

D 10 months

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pathogenic process via its effect on the somatic expansion of the HTT CAG repeat that occurs predominately in MSNs. Our data support the hypothesis that somatic HTT CAG expansion in cells susceptible to the effects of mutant huntingtin accelerates the disease process, and further indicate that preventing somatic expansion in MSNs would have therapeutic benefit.

Laser capture microdissection studies in HD postmortem cortex and striatum have demonstrated that HTT CAG expansions occur both in neurons and glia, with neurons tending to have the longest expansions and greatest repeat length heterogeneity [14,16]. Analysis of HTT CAG repeat length in microdissected neuronal and glial cells from striata of R6/2 HTT exon 1 transgenic mice supports the association of the most highly expanded repeats with neuronal cells [16]. Finer microdissection of neuronal populations within the striatum of knock-in mouse models of HD (Hdh6/Q27 and Hdh4/Q80) demonstrated greater HTT CAG repeat expansion in a pan-neuronal (NeuN-positive) population compared to a nitric oxide synthase (NOS)-positive subpopulation of interneuron’s [14]. While the NeuN-positive cells served as a good approximation for MSNs that comprise >90% of striatal neurons these studies did not unequivocally demonstrate the occurrence of somatic expansions in MSNs. The present study supports the above data, and for the first time demonstrates that the bulk of the somatic expansions that occur in striatum arises in MSNs, and further demonstrates that these expansions are dependent on Msh2 expression within these neurons. Additional studies would be needed to determine whether the small proportion of expanded alleles that are present in the striata of the conditional knockout mice (Figure 3) are present in non-MSNs (neurons or glia) or in MSNs in which the floxed Msh2 allele has failed to recombine. Regardless, it is clear that at a relatively early time-point (five to ten months of age), MSNs are the major cell type in the striatum exhibiting somatic expansions, while over time, it appears that expansions accumulate in other neurons and in glia [14,16]. The finding that the earliest somatic expansions occur in the neuronal cell type that is selectively vulnerable in the disease is significant, supporting the proposition [33] that hyper-expansion of the HTT CAG repeat in MSNs contributes to their selective demise, and further suggesting that in HTT CAG mutation carriers, this is a process that may begin within months of birth.

Why are MSNs particularly vulnerable to repeat expansion? Several lines of evidence strongly indicate that instability in these neurons does not arise downstream of the pathogenic process: firstly, the spinocerebellar ataxia type 1 (SCA1) and type 3 (SCA3) CAG repeats [19,20] and the myotonic dystrophy type 1 (DMPK) CTG repeat [18] show high levels of striatal instability despite the striatum being largely unaffected in these diseases; secondly, accelerating the pathogenic process in HD/hQ111 mice does not increase striatal HTT CAG instability [21]; thirdly, bioinformatic approaches based on correlating instability with global gene expression signatures predict the presence of a cellular environment permissive for repeat instability that is intrinsic to wild-type striata [21]. Although acute overexpression of polyglutamine-containing proteins has been found to influence repeat instability in fly models [34], the overwhelming evidence in accurate genetic HD disease models expressing mutant huntingtin at physiologically relevant levels is that factors other than the pathogenic process itself are primarily responsible for the somatic expansions that occur in MSNs.

Obvious factors that might underlie this cell-type specificity are Msh2 itself and its binding partner Msh3 that is also critical for striatal HTT CAG expansion [25,26,35,36]. However, neither Msh2 nor Msh3 mRNA or protein levels showed any clear correlation with HTT CAG instability across tissues that vary in their degree of overall instability [21], although higher Msh3 protein in striatal neurons versus glia has been suggested [16]. Further investigation of the expression level, sub-cellular localization and activities of these proteins in MSNs versus striatal interneurons and glia is warranted. The stoichiometry of base excision repair proteins differs in striatum and cerebellum [37] and may contribute to the sensitivity of MSNs to somatic HTT CAG expansion. Interestingly, Xpa, a protein critical for transcription-coupled nucleotide excision repair (TC-NER), is required for instability of the SCA1 CAG repeat in brain, but not in peripheral tissues [38]. It would be of interest to determine whether Xpa and TC-NER might play a role in contributing to the MSN-selective instability of the HTT CAG repeat. Transcription has also been shown to contribute to CAG/CTG instability [39–42]. Although steady state levels of HTT sense or antisense transcript do not clearly correlate with levels of tissue instability [43], it is possible that rates of sense, antisense or convergent transcription could play a role in determining the cell-type specificity of somatic instability in HD. Unbiased genome-wide analyses of factors associated with somatic instability, however, suggest that a combination of many factors is likely to contribute to the propensity of a particular tissue or cell-type towards somatic expansion [21].

In summary, the susceptibility of MSNs to both HTT CAG instability and HD pathogenesis strongly indicates that somatic expansion is relevant to the disease process in these neurons. Further experiments are required to unequivocally establish a role for somatic expansion as a disease modifier, to assess the spectrum of disease phenotypes that are subject to modification by somatic expansion as well as the extent to which they are modified. The prediction is that phenotypes that are CAG repeat length-dependent would be accelerated to some degree by further somatic expansion. Ongoing experiments are aimed at identifying CAG repeat length dependent phenotypes that will provide additional logical endpoints with which to test the hypothesis that somatic expansion accelerates the pathogenic process in mice.

How might somatic expansion be prevented in patients? In general, eliminating MMR proteins as a therapeutic strategy is undesirable due to the potential for tumor development, although targeting to the brain would likely reduce the tumor potential as cell-types in the periphery are the most susceptible to the somatic loss of MMR proteins. However, understanding the mechanism by which MMR proteins result in CAG expansion may provide a means to specifically intervene in this process without interfering
in the role of these proteins in global genome maintenance. Alternative approaches could include the direct targeting of nucleic acid metabolic intermediates that occur during the expansion process, as recently indicated for the DMPK CTG repeat [44]. Finally, identification of additional factors that underlie HTT CAG instability in MSNs is an important goal as this would likely lead to novel targets for reducing somatic expansion in this vulnerable cell population.

Figure 4. Deletion of Msh2 in medium-spiny neurons delays nuclear huntingtin phenotypes. A, B. Nuclear mutant huntingtin immunostaining is decreased in the striata of five-month old HdhQ111/+ mice with deletion of Msh2 in MSNs. A. Fluorescent micrographs of striata double-stained with anti-huntingtin mAb5374 and anti-histone H3 antibodies for three CAG repeat length-matched mice (Msh2+/+ CAG 113, Msh2Δ/Δ CAG 112, Msh2−/− CAG 113). B. Box plot showing upper and lower quartiles, median and range for the normalized mAb5374 immunostaining intensity (total mAb5374 staining intensity normalized to the number of H3-positive nuclei). Outlier (circle) is defined by a standard interquartile method and is included in the analysis. Multiple regression analysis was used to determine the effect of Msh2 genotype on mAb5374 staining using normalized mAb5374 intensity (continuous variable) as a dependent variable and Msh2 genotype (discrete variable), constitutive CAG length (continuous variable) and position (medial versus lateral, discrete variable) as independent variables. Both constitutive CAG length (P<0.05) and medial versus lateral position (P<0.001) were significantly associated with normalized mAb5374 intensity. Asterisks above the bars indicate a significant difference from Msh2+/+ at a p-value cut-off of p<0.05 (*), p<0.01 (**), p<0.001 (***). Msh2Δ/2 was not significantly different from Msh2+/+ (p = 0.18). The five-month mice used in the quantitative analysis are as follows: Msh2+/+ (n = 6, CAG 113, 118, 119, 121, 123, 125), Msh2Δ/Δ (n = 5, CAG 113, 121, 126, 129), Msh2−/− (n = 7, CAG 113, 121, 122, 125, 126, 133) and Msh2−/− (n = 3, CAG 112, 120, 123). Note that the relatively “weak” effect of the Msh2−/− genotype likely reflects the small number of mice of this genotype and hence the least accurate estimate of the relationship of mAb5374 intensity to CAG length in the regression analysis. C, D. Intranuclear inclusions are decreased in the striata of ten-month old HdhQ111/+ mice with deletion of Msh2 in MSNs. C. Fluorescent micrographs of striata stained with mAb5374 from mice with Msh2+/+ (CAG 121), Msh2Δ/Δ (CAG 123), Msh2Δ/Δ (CAG 132) and Msh2Δ/Δ (CAG 132) genotypes. D. Quantification of the percentage of cells containing an inclusion (more than one inclusion per cell was rarely observed). The total number of cells was determined by co-staining with histone H3 (not shown). The ten-month mice used in the quantitative analysis are as follows: Msh2+/+ (n = 6, CAG 118, 121, 123, 126, 134), Msh2Δ/Δ (n = 4, CAG 116, 118, 123, 131), Msh2Δ/Δ (n = 1, CAG 133), Msh2Δ/Δ (n = 7, CAG 115, 115, 117, 120, 121, 122, 123) and Msh2−/− (n = 1, CAG 132). Bars represent mean ±S.D. doi:10.1371/journal.pone.0044273.g004
Materials and Methods

Mice
This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health under an approved protocol of the Massachusetts General Hospital Subcommittee on Research Animal Care (protocol number 2009N000216). HdhQ111 knock-in mice used in this study were on a C57BL/6J background [24] and were maintained by breeding heterozygous males to C57BL/6J wild-type mice from The Jackson Laboratories. The actual repeat size of the mice used in this study ranged from 110 to 134 CAGs. All analyses were performed on heterozygous HdhQ111/+ mice. D9-Cre transgenic mice contain a genomic fragment, comprising ~2 kb of 5’ regulatory sequence, the endogenous ATG, and the introns and exons of the mouse Ppp1r1b gene encoding DARPP-32, driving the expression of Cre recombinase [28]. These mice were on a C57BL/6J background and were maintained as homozygotes. Msh2flox mice [27] that were used in this study had been backcrossed for nine generations to C57BL/6J. Constitutional Msh2 null mice (Msh2+/−) [45] were on a C57BL/6N background. Note that we have not observed differences in HdhQ111 instability or mAb5374 immunostaining phenotypes on C57BL/6J and C57BL/6N background (not shown), therefore the mixed C57BL/6J and C57BL/6N background is unlikely to be a confounding factor in our interpretation of the results.

To generate the mice of the appropriate genotypes for this study, HdhQ111/+, Msh2flox/+ mice were crossed together to obtain Msh2+/+ mice with two functional Msh2 alleles, Msh2+/− mice with a single functional Msh2 allele, Msh2Δ/A mice in which both alleles are deleted in MSNs, Msh2Δ/A− mice with one constitutionally deleted Msh2 allele and one Msh2 allele deleted in MSNs, and Msh2−/− mice with two constitutionally deleted Msh2 alleles. All of the mice analyzed for instability and mutant huntingtin phenotypes with these genotypes were HdhQ111/+ heterozygotes. A detailed schematic of the crosses involved is shown in Figure S1.

Genotyping and Analysis of Somatic Instability
Genomic DNA was isolated from tail biopsies at weaning for routine genotyping analysis or from adult tissues (either fresh or fixed) for somatic instability analysis, using the PureGene DNA isolation kit (Genta, Minneapolis, MN, USA). Genotyping of the HdhQ111 knock-in allele was carried out as described previously [26]. Msh2flox and D9-Cre mice were genotyped as described [27,28]. Msh2 null mice [45] were genotyped in a three-primer PCR assay using 1 μM primer MSH2A. (5' CCCTCCTGTGGACGCCATCTTA), 0.75 μM of primer MSH2B (5' GCCACGTACATTGTTGATG) and 0.5 μM of primer MSH2C (5' TTCCGGTGGTTGCCGGAAT), 200 μM dNTPs, 1 x Qagen PCR buffer with 1.5 mM MgCl2 and 0.6 units of Taq polymerase (Qagen). Cycling conditions were 95°C for 9 mins; 40 cycles of 94°C for 45 sec, 56°C for 45 sec, 72°C for 45 sec; 72°C for 5 mins, generating a 188 bp product from the wild-type allele (MSHA/MSHC primers) and a 300 bp product from the Msh2 null allele (MSH2B/MSH2C primers). HdhQ111 CAG repeat size was determined using human Htt CAG-specific primers as previously described [26] with the forward primer fluorescently labeled with 6-FAM (Perkin Elmer). Products were resolved using the ABI3730xl automated DNA analyzer (Applied Biosystems). GeneMapper v3.7 with GeneScan 500-LIZ as internal size standard was used to assign repeat size, defined as the highest peak in the GeneMapper trace. All runs included the same control DNAs of known Htt CAG repeat size. Somatic instability was quantified from the GeneMapper traces as described previously [21]. Briefly, GeneMapper peaks <10% of the height of the highest peak were excluded. For peaks exceeding the 10% background threshold, normalized peak heights were calculated by dividing the peak height by the sum of all peak heights over background. The change in CAG length of each peak from the highest peak in tail DNA (main allele) was determined, the normalized peak height was multiplied by the CAG change from the main allele, and these values were summed to generate an instability index that represents the mean CAG repeat length change in the population of cells being analyzed.

Immunohistochemistry
Primary antibodies were: mouse monoclonal anti-huntingtin mAb5374 (Chemicon), and rabbit polyclonal anti-histone H3 (Abcam ab1791), rabbit polyclonal anti-MSH2 (Abcam ab70270) and mouse monoclonal anti-DARPP-32 (D92-6a; a kind gift from Dr. Angus Nairn). Immunostaining was performed on 7 mm coronal sections of periodate-lysine-parafomaldehyde (PLP)-perfused and -post-fixed, paraffin-embedded brains. Perfusion and tissue processing/embedding methods have been described previously [30]. One hemisphere of the brain was embedded for sectioning and the other was used for dissection of striatum, cortex and cerebellum for analysis of somatic instability. Sections chosen for immunostaining were aligned with respect to their anterior/ posterior location in the brain. The sections were deparaffinized, rehydrated and subjected to heat-mediated epitope retrieval (Nacitrate buffer pH 6.0) followed by quenching of endogenous peroxidase with 0.3% H2O2/methanol for 30 min at room temperature and blocked in 5% normal horse serum (NHS) in TBS for 1 h at room temperature.

For detection of diffusely immunostaining nuclear huntingtin, incubation with mAb5374 (1:100 in 1% NHS/TBS) was carried out overnight at 4°C. mAb5374 signal was then amplified using the TSA Biotin System (Perkin Elmer) according to manufacturer’s instructions. Briefly, sections were incubated sequentially with biotinylated anti-mouse IgG (Vectorstain Elite ABC kit; Vector Laboratories) at 1:200 dilution in 1% NHS/TBS for 1 h at RT, with Streptavidin-conjugated horseradish peroxidase (Streptavidin-HRP, TSA Biotin System, Perkin Elmer) at 1:100 in 1% NHS/TBS for 30 min at RT, and with biotinylated Tyramide Amplification reagent (TSA Biotin System, Perkin Elmer) at 1:75 in the diluent provided for 20 min at RT. mAb5374 staining was detected by Streptavidin-Alexa Fluor 488 (Invitrogen) at 1:500 in 1% NHS/TBS for 1 h at RT. For double staining with mAb5374/anti-histone H3 antibody, sections were incubated with both primary antibodies overnight at 4°C, and histone H3 staining was detected with donkey anti-rabbit Alexa Fluor 546-conjugated secondary antibody (Invitrogen, 1:1000) added together with Streptavidin-Alexa Fluor 488. For detection of huntingtin inclusions, sections were incubated with mAb5374 (1:200 in 1% NHS/TBS) overnight at 4°C. Signal was detected using goat anti-mouse-HRP (TSA kit #2, Invitrogen) at 1:50 in 1% NHS/TBS for 1 h at RT, followed by Alexa Fluor 488-conjugated Tyramide (TSA kit #2, Invitrogen) at 1:100 in the diluent provided with the TSA kit #2, for 25 min at RT. Detailed conditions were according to manufacturer’s instructions (TSA kit #2, Invitrogen). For double staining with mAb5374/anti-histone H3 antibody, sections were incubated with both primary antibodies overnight at 4°C, and histone H3 staining was detected with donkey anti-rabbit Alexa Fluor 546-conjugated secondary antibody (Invitrogen, 1:1000) added after Tyramide-Alexa Fluor 488. For double
staining with anti-DARPP-32/anti-Msh2 antibodies, sections were incubated with both primary antibodies (D32-6a at 1:200 and ab70270 at 1:500 in 1% NHS/TBS) overnight at 4°C, followed by sequential amplification of Msh2 and DARPP-32 signal. First, Msh2 signal was amplified using the TSA Biotin System, and quenching of HRP activity (0.1% sodium azide/0.3% H2O2 in TBS for 30 min at RT) was performed before the final addition of streptavidin-Alexa Fluor 555. Then, DARPP-32 signal was amplified by incubating with goat antiamouse-HRP (TSA kit #2, Invitrogen) at 1:100 and TyramideAlexa Fluor-488 (TSA kit #2, Invitrogen) at 1:100 in the diluent provided with the TSA kit #2, for 25 min at RT. Detailed conditions were according to manufacturer’s instructions (TSA kit #2, Invitrogen). ‘No primary antibody’ and ‘single primary antibody’ control experiments demonstrated the specificity of the Alexa Fluor-555 and Alexa Fluor-488 signals for Msh2 and DARPP-32, respectively.

Sections were mounted in ProLong Gold antifade reagent (Invitrogen). Fluorescent microscopy was performed with a Zeiss Axioskop 2 microscope equipped with AxioCamMRm camera and AxioVision 4.6 image acquisition software, using Plan Apochromat 20x/0.8 M27 or Plan Neofluar 40x/0.75 Ph2 objectives. Images that were to be quantified and compared were taken with the same exposure times.

Quantification of Immunohistochemical Data
Cell Profiler 2.0 r10997 cell image analysis software was used for quantification of mAb5374 and Msh2 immunostaining intensity. Four micrographs were taken from each mouse (one from medial and one from lateral striatum in two consecutive sections located on the same slide), each micrograph representing an area of 220x170 μm. Total (integrated) intensity of mAb5374 staining was measured in all mAb5374-positive nuclei in each area and normalized by the total number of nuclei (as determined by the number of all histone H3-positive nuclei) in the same area. The resulting value, representing the mean intensity of mAb5374 staining per nucleus, was averaged for each mouse (for medial and lateral striatal areas separately as well as for all four striatal areas). Note that there was some variation in histone H3 intensity both within and between mice (see for example Figure 4). However, this did not vary systematically with genotype, nor did it correlate with the number of histone H3-positive nuclei. We normalized against the number of histone H3-positive nuclei in order to control for potential differences in cell density in different coronal sections. However, the number of histone H3-positive nuclei between sections analyzed did not vary considerably, indicating that our sections were reasonably well matched in terms of their anterior/posterior positions. For Msh2 staining, total (integrated) intensity of Msh2 staining was measured in DARPP-32-positive cells in each area and normalized by the number of DARPP-32-positive cells. The resulting value, representing the intensity of Msh2 staining per MSN, was averaged for each mouse (from the four striatal areas as described above). The number of nuclear huntingtin inclusions in the striatum of 10 month old mice was quantified in fluorescent micrographs of mAb5374/anti-H3-stained striatal sections (four 435×435 μm striatal areas per mouse, as described above) using ImageJ software and normalized to the number of H3-positive cells.

Immunoblot Blot Analysis
Striata were homogenized in RIPA buffer supplemented with 5 mM EDTA and protease inhibitors (Halt protease Inhibitor Cocktail, ThermoScientific) by mechanical grinding with disposable pestle (Fisher Scientific) and further sonication (Branson sonifier, power level 3.5, two 10-sec pulses on ice). The homogenates were kept on ice for 30 min and then clarified by centrifugation for 30 min at 14000 rpm. Protein concentrations were measured using Bio-Rad DC protein assay kit. Striatal extracts (30 μg per lane) were resolved by SDS-PAGE in Novex 10% Bis-Tris gels (Invitrogen). Msh2 protein was detected by immunoblotting using anti-MSH2 antibody ab70270 (Abcam) at 1:4000 in 5% non-fat milk/TBS/0.1% Tween-20. Alpha-tubulin, as detected by 1:5000 DM1A (Cell Signaling Technology) in 5% milk/TBS/0.1% Tween-20, was used as loading control. Quantification was done by measuring the density of Msh2 and α-tubulin bands with QuantityOne software with subtraction of local background.

Statistical Analyses
Pairwise comparisons of instability indices, intranuclear inclusions and Msh2 protein levels were determined using a Student’s t-test (Excel). In these comparisons, Msh2+/A mice were generally compared to Msh2+/- mice, and Msh2A/- mice were compared to Msh2+/-- mice as this latter comparison provides a more stringent test of the effect of the conditional deletion on a background of a single constitutionally deleted Msh2 allele. To control for the effects of constitutive CAG length and striatal position (medial versus lateral) in the determination of the effect of Msh2 genotype on mAb5374 immunostaining intensity multiple regression analysis was carried out using normalized mAb5374 intensity as the dependent (continuous) variable and Msh2 genotype (discrete variable), constitutive CAG length (continuous variable) and position (discrete variable) as independent variables using the R statistical package (version 2.7.2).

Supporting Information
Figure S1 Breeding Scheme. The breeding scheme shows how we generated HdhQ111/+ mice of the five Msh2 genotypes (Msh2+/+, Msh2+/-, Msh2A/A, Msh2A/-, Msh2-/-) that were used in this study using the four transgenic/knock-in alleles (HdhQ111, Msh2 null, Msh2fox, D9-Cre). Breeders in the various crosses are shaded in green and separated by a green “X”. The genotypes of the resultant mice from these crosses that were analyzed are indicated in black type. Shown in red type/boxes are the five Msh2 genotypes using the final terminology we describe throughout the manuscript. Note that mice with a single functional Msh2 allele could be generated in more ways and were pooled as heterozygotes (Msh2+/- mice in red type). Similarly, mice with two functional Msh2 alleles could be generated in more than one way and were pooled as wild-types (Msh2+/+ mice in red type).

(TIF)

Figure S2 Quantitative fluorescent assay to detect the time-dependent increase in mAb5374-positive nuclear mutant huntingtin immunostaining in the striata of HdhQ111/+ mice (C57BL/6j). Left: Fluorescent micrographs of striata of three Hdh+/+ mice (upper panel) and three CAG repeat length-matched HdhQ111/+ mice (lower panel), double-stained with anti-huntingtin mAb5374 and anti-histone H3 antibodies. CAG repeat numbers: 121 (2.3 months), 120 (3.3 months), 121 (5.0 months). Note that this antibody does not stain huntingtin in wild-type (Hdh+/-) mice. Right: Quantification of the mAb5374 nuclear immunostain in HdhQ111/+ striata, normalized to the number of H3-positive nuclei. 2.3 mo N = 4; 3.3 mo. N = 3; 5.0 mo N = 3. Bars are mean±S.D. (DOCX)
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Author Contributions

Conceived and designed the experiments: MK ED VCW. Performed the experiments: MK ED JS TG JRG JN HD. Analyzed the data: MK ED JN VCW J-ML. Contributed reagents/materials/analysis tools: RK MHK MEE. Wrote the paper: MK VCW.

References