Sp1 Is Up-regulated in Cellular and Transgenic Models of Huntington Disease, and Its Reduction Is Neuroprotective

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Huntington disease (HD) is a fatal hereditary neurodegenerative disorder characterized by progressive motor and cognitive deficits caused by neuronal dysfunction and degeneration affecting selected populations of neurons. The genetic defect responsible for HD is a CAG repeat expansion in exon 1 of the HD gene leading to the expression of a mutant huntingtin (Htt) protein containing an abnormally long polyglutamine sequence. Direct interactions between mutant Htt and a variety of transcription factors have been identified, which may significantly contribute to the pathogenesis of HD (2, 3). Gene microarray studies have indicated that selective transcriptional alterations occur early in HD (4), consistent with transcriptional dysregulation (5–7). Many of the altered genes contain binding sites for Sp1, which we recently demonstrated to interact with Htt in a polyglutamine length-dependent manner (2).

Sp1 is a zinc-finger domain (C terminus) transcriptional activator (8) that has been implicated in the expression of many genes in concert with other transcription factors, including the TAFII family (2), CREB (3, 9), nuclear factor-κB (10), and vesicular endothelial growth factor receptor-2 (VEGFR-2) (11, 12). Sp1-mediated processes include aberrant transcriptional modulation of dopamine receptor genes, neurodegeneration (2, 13, 14), and inflammatory processes (12, 15), which are important in the pathogenesis of HD. However, various in vitro studies have implicated Sp1 as being prosurvival (16, 17) or prodeath (18, 19), or involved in neurodegeneration (10, 14). Whereas interactions between Sp1 and mutant huntingtin appear to be deleterious at the molecular level (2), we have also demonstrated that blocking Sp1 using mithramycin A dramatically prolonged survival in a mouse model of HD (20).

In resolve this paradox, we examined the role of endogenous Sp1 in the neurotoxicity found in three models: 1) Htt inducible neuronal cell lines (PC12 cells) expressing either normal or mutant Htt, 2) neurodegeneration induced by mutant Htt or 3-NP in vitro, and 3) in vivo, using primary neurons and mice obtained by crossing Sp1 heterozygous knock-out mice and mithramycin A, a DNA-intercalating agent that inhibits Sp1 function. The three approaches consistently yielded reduced levels of Sp1 which ameliorated toxicity caused by either mutant Htt or 3-NP. In addition, when HD mice were crossed with Sp1 heterozygous knock-out mice, the resulting offspring did not experience the loss of dopamine D2 receptor mRNA characteristic of HD mice, and survived longer than their HD counterparts. Our data suggest that enhancement of transcription factor Sp1 contributes to the pathology of HD and demonstrates that its suppression is beneficial.

Interactions between mutant huntingtin (Htt) and a variety of transcription factors including specificity proteins (Sp) have been suggested as a central mechanism in Huntington disease (HD). However, the transcriptional activity induced by Htt in neurons that triggers neuronal death has yet to be fully elucidated. In the current study, we characterized the relationship of Sp1 to Htt protein aggregation and neuronal cell death. We found increased levels of Sp1 in neuronal-like PC12 cells expressing mutant Htt, primary striatal neurons, and brain tissue of HD transgenic mice. Sp1 levels were also elevated when 3-nitropropionate (3-NP) was used to induce cell death in PC12 cells. To assess the effects of knocking down Sp1 in HD pathology, we used Sp1 siRNA, a heterozygous Sp1 knock-out mouse, and mithramycin A, a DNA-intercalating agent that inhibits Sp1 function. The three approaches consistently yielded reduced levels of Sp1 which ameliorated toxicity caused by either mutant Htt or 3-NP. In addition, when HD mice were crossed with Sp1 heterozygous knock-out mice, the resulting offspring did not experience the loss of dopamine D2 receptor mRNA characteristic of HD mice, and survived longer than their HD counterparts. Our data suggest that enhancement of transcription factor Sp1 contributes to the pathology of HD and demonstrates that its suppression is beneficial.

EXPERIMENTAL PROCEDURES

Cell Culture—PC12 cell lines containing an ecdysone-inducible protein fused with the first 17 amino acids of Htt plus 103 or 25 glutamines tagged with GFP at the C terminus (21) were kindly provided by...
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Dr. Aleksey Kazantsev. The cells were grown in Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum, 10% horse serum, 100 µM penicillin-streptomycin, 2 mg/ml Gentecin®, and 1 mg/ml zeocin (all from Invitrogen). Polyglutamine expression was induced with 2.5 µM ponasterone.

Striatal neuronal cultures were prepared from Sp1 heterozygous knock-out mice (22) (kindly provided by Dr. Sjaak Philipsen, Erasmus University Rotterdam, The Netherlands) crossed with R6/2 or N171-82Q transgenic mice, and from wild-type littermate controls. Striatal neurons from 16-day-old embryonic mice with different genetic backgrounds were isolated by our previous enzyme treatment protocol (23). Briefly, striata were dissociated in calcium-free saline and plated on poly-D-lysine (Sigma)-coated tissue culture dishes at a density of 10⁶ cell/ml. The neurons were grown in Neurobasal Media (Invitrogen) plus 10% fetal bovine serum with 25 µM penicillin-streptomycin. One hour after plating, medium with serum was replaced with medium containing B-27 supplement (Invitrogen). All cells were grown in humidified 5% CO₂ incubators for less than 2 weeks.

Animal Studies—Sp1 heterozygous knock-out mice (Sp1+/−) (22) were cross-bred with two distinct transgenic mouse models of HD, N171-82Q and R6/2 lines. The N171-82Q HD transgenic mice (line 81) express 82 CAG repeats within exon 1 and 2 of the HD gene driven by the mouse prion promoter (24). The R6/2 mice express exon 1 of the human HD gene with ~150 CAG repeats (25). All of the mice are maintained on a B6CBA background. DNA from each mouse or embryo (for neuronal cultures) was analyzed by PCR to determine its genotype. Survival analysis was performed in wild-type, N171-82Q, R6/2, and in both transgenic lines cross-bred with Sp1 heterozygous knock-out mice. For the survival study in R6/2 line, there were 5 male WT, 5 female WT, 5 male Sp1+/−, 5 female Sp1+/−, 3 male HD+/−, 5 female HD+/−, 5 male Sp1+/− HD+/−, and 4 female Sp1+/− HD+/−. For the N171-82Q there were 3 male WT, 4 female WT, 9 male Sp1+/−, 12 female Sp1+/−, 12 male HD+/−, 10 female HD+/−, 14 male Sp1+/− HD/−, and 9 female Sp1+/− HD/−. All experiments were carried out in accordance with the NIH Guide for the Care and the Use of Laboratory Animals and the Massachusetts General Hospital IACUC. The mice were weighed weekly starting from 28 days of age and continued for the duration of their life span. Mouse survival was assessed for mortality twice daily, early morning and late afternoon, as previously reported (26). Mice were euthanized upon reaching criteria for morbidity. Deaths occurring overnight were recorded the next morning.

Neurotoxicity Assay—A standard lactate dehydrogenase assay (LDH kit, Roche Applied Science) (27, 28) and the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma) (29) were adapted in this study. The LDH assay measures lysed cells, and the MTT assay estimates viable cells. Neurotoxicity was concurrently examined from supernatant for LDH and cells for MTT. Two independent assays in each culture set were performed for confirmation. For studies involving 3-NP (Sigma), the PC12 cells and the primary striatal neurons were treated with 3-NP for 4 h, both with and without pretreatment (i.e. mithramycin A for 24 h prior to 3-NP). For both the LDH and MTT assays, results from each culture set were normalized to the appropriate control values for the culture set and then combined.

Immunohistochemical and Htt Aggregate Analysis—PC12 cell cultures were washed, fixed, and then immunostained for 15–24 h at 4 °C using anti-Sp1 rabbit polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Sp1 antibody was detected with anti-rabbit Ig and Cy3-linked whole antibody. Htt aggregates were examined by eGFP fluorescence after 2.5 µM ponasterone induction for 24 h. For primary striatal neurons, mutant Htt (polyglutamine expansion, 37 Gln) and Sp1 antibodies were detected with fluorescein-linked anti-mouse Ig and Cy3-linked anti-rabbit Ig. Htt aggregates in HD mice brain were examined by immunocytochemistry using EM48 polyclonal antibody by the method reported previously (2). Mice were perfused with 3.0% paraformaldehyde and 0.1% glutaraldehyde. Brains were removed and postfixed overnight in 2% paraformaldehyde. Sections were stained with EM48 polyclonal antibody (1:2000) to detect the presence of aggregates. Immunoreactivity was detected after 48 h via an immunoperoxidase reaction using the Vectastain Elite Kit (Vector Laboratories, Burlingame, CA).

Starting from the most rostral segment of the neostriatum in the mouse forebrain, 50 µm coronal tissue sections were serially cut using a vibratome (Series 1500, St Louis, MO). Every tenth section was collected through the anterior commissure. Aggregate formation was characterized by stereology measuring nuclear aggregates alone or total aggregate burden, including neuropol and nuclear aggregates. First, using StereoInvestigator software (MicroBrightField, Williston, VT) and a Leica DMLB microscope, the volume of striatum was estimated by the Cavalieri principle (30). The number and volume of EM48-positive nuclear aggregates was analyzed using the Nucleator (31) in each cortex and striatum section. In a second procedure, EM48-positive Htt aggregate images from selected microscopic fields were captured with a CCD camera (Hamamatsu, Hamamatsu City, Japan) and digitized by computer with Openlab software (Heidelberg-Wiebingen, Germany). Total Htt aggregate burden from the cortex and striatum, each obtained from four R6/2 mice and four R6/2 crossed with Sp1 heterozygous knock-out mice, was quantified using NIH Image (particle analysis) via unbiased measurement of the number and size (area) after selecting threshold (>1 µm). All computer-identified cell profiles in each cross-sectional area were normalized to the related brain region of R6/2 mice and then pooled for statistic analysis.

Western Blot—Neuronal cultures and mouse brain tissue were lysed or homogenized in 50 mM Tris-HCl, pH 8.0, containing 0.05 M NaCl, 4 µM leupeptin, 2 µM pepstatin, 1.5 µM aprotonin, 400 µM phenylmethylsulfonyl fluoride, and 0.5% Triton-X. Equal amounts of protein were analyzed for Sp1 and mutant Htt by immunoblot analysis. Briefly, proteins were denatured, reduced, and separated by SDS-polyacrylamide gel electrophoresis. After transfer to nitrocellulose membrane, proteins were probed with polyclonal antibodies against Sp1 (Santa Cruz Biotechnology), GFP (Invitrogen), or polyglutamine expansion (37 Gln) (Chemicon Int., Inc., Temecula, CA). From the same blots, actin (AC-40; Sigma) was detected by a specific monoclonal antibody to assure that equal protein was present in each lane. Immunoreactivity was detected using a horseradish peroxidase-linked secondary antibody and chemiluminescence (PerkinElmer Life Sciences). Analyses, with a Bio-Rad GS-700 Imaging densitometer, were recorded as percentages of the sister cultures or control mouse brains.

Sp1 RNA Interference—Rat Sp1 small interfering RNA (siRNA) (sense/antisense), 5’-agccuaggauggauccau-3’/5’-auagcuacacuucaagucgu-3’, was used to target the Sp1 sequence aaacguagguauccau. The sequences of Sp1 target-specific siRNA duplexes were designed according to the manufacturer’s recommendation and subjected to a BLAST search against the rat genome sequence to ensure that no endogenous genes of the genome were targeted. Lipofectamine-mediated transient transfections of Sp1 siRNA were performed. After 48 h, the transfected cells were washed twice with phosphate-buffered saline, lysed, and analyzed by Western blot to probe the Sp1 gene expression. Moreover, cell viability in the transfected cells was concurrently assessed in order to confirm the inhibition of Sp1 function by siRNA.
Quantitative PCR—RNA isolated from primary striatal neurons, and mouse brains were extracted according to the manufacturer's instruction (RNeasy from Qiagen, Valencia, CA). Reverse transcription of RNA was conducted with a SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Quantitative real-time PCR studies used a Bio-Rad iCycler to follow the amplification of cDNA products through PCR cycles. Cycle parameters were 1 min at 95 °C, then 50 cycles of 95 °C for 30 s, 57 °C for 1 min, and 72 °C for 1 min. Primers used (upper/lower) were Sp1 (5'-H11032-aatttgccctgccctgagtgc-3') (5'-H11032-attggacccatgctaccttgc-3'), human Htt (L34020) (5'-H11032-agatggacggccgctcaggtt-3') (5'-H11032-tcatcagcttttccagggtcgccat-3'), and murine /H9252-actin (X03672) (5'-H11032-aggtatcctgaccctgaag-3') (5'-H11032-gctcattgtagaaggtgtgg-3'). Relative expression levels for each target were normalized to /H9252-actin using the 2^-ACT method (32).

Data Analysis—Data from several cultures or brains were pooled for statistical analyses. Values are expressed as mean ± S.E. One-way analysis of variance (ANOVA) followed by the Fisher post hoc test for multiple comparisons determined statistical significance. Animal survival data were analyzed using the Mantel-Cox log-rank test (33) and Kaplan-Meier survival curves. The survival curves were adjusted for sex since there were a slightly different number of males and females in each group. p < 0.05 was considered indicative of a statistically significant difference.

RESULTS

Mutant Htt Protein Increases Sp1 Levels—Immunohistochemical analysis indicated that Htt aggregates and Sp1 levels were substantially enhanced with ponasterone induction of Htt expression in PC12 cells, particularly in the nucleus (Fig. 1). Western blot analysis further demonstrated increased levels of Sp1 in HttQ103 cells (by 45% p < 0.05, Fig. 1B), suggesting that HttQ103 may be directly or indirectly involved in...
the alterations of Sp1 levels. 3-NP treatment in HttQ25 and HttQ103 cells substantially increased Sp1 levels; PC12/HttQ103 cells showed a 79% increase and PC12/HttQ25 cells showed a 34% increase, p < 0.05, Fig. 1B. Such enhancement was also obtained from WT mouse brains administered 3-NP at the dose of 55 mg/kg, twice a day for 10 days (by 68%, p < 0.05, Fig. 1C), indicating that the Sp1 levels are both mutant Htt and 3-NP inducible in CNS.

Additional results showed increased Sp1 levels in primary striatal neuronal cultures isolated from R6/2 (by 152%) and N181-82Q (by 122%) transgenic mice (Fig. 1D). Co-localization of mutant Htt (polyglutamine expansion, 37 Gln) with Sp1 was also observed in the nucleus of primary striatal neurons isolated from R6/2 mice (Fig. 1D). The DNA binding capacity of Sp1, detected by electrophoretic mobility shift assays, was also enhanced in R6/2 mouse brains (Fig. S1 in supplemental data) corresponding with the increased Sp1 protein levels (by 89%, Fig. 1E) and indicating enhanced Sp1 activity. It has also been reported that Sp1 levels are increased in human brain in the early stages of HD (2). Thus, the presence of mutant Htt corresponds both in vitro and in vivo with increased Sp1 levels and DNA binding.

Sp1 Helps Mediate Mutant Htt-associated Neuronal Cell Death—3-NP, an irreversible inhibitor of succinate dehydrogenase, causes pathology resembling HD in vivo (34). Under baseline conditions neither HttQ25 nor HttQ103 cells exhibited detectable cell death, represented as normalized LDH release. Although 3-NP significantly increased cell death in both groups, PC12 cells expressing HttQ103 were more sensitive to 3-NP treatment (Fig. 2A), corresponding to HttQ expression (data not shown). When the dose-dependent effects of 3-NP were examined in both HttQ25 and HttQ103 cells, the dose response curve significantly shifted upward for the HttQ103-expressing cells (Fig. 2B), which suggests that HttQ103 sensitizes neuronal cells to 3-NP toxicity.

To examine Sp1 function in the presence of mutant Htt, we tested the effects of pharmacological and genetic inhibition of Sp1 using mithramycin A and Sp1 RNAi, respectively. Mithramycin A is an aureolic antibiotic that selectively inhibits gene expression by blocking Sp1 binding to G-C-rich regions of promoters. We tested the effects of mithramycin A on neuronal cell death in both HttQ25 and HttQ103 cells in the presence of 3-NP treatment. 3-NP induced cell toxicity was completely abolished by mithramycin A (100 nM) in both HttQ25 (Fig. 2C) and HttQ103 (Fig. 2D). Mithramycin A decreased 3-NP toxicity in a dose-dependent manner in both HttQ25 and HttQ103 cells. Significantly greater protective effects of mithramycin A from 3-NP (20) were obtained in HttQ103 cells (data not shown). In fact, no detectable changes in Sp1 levels were observed in mithramycin A-treated HttQ103 cells (Fig. 2E), consistent with the role of mithramycin A as a functional blocker of Sp1.

Given that mithramycin A might affect other pathways in addition to Sp1, we also assessed Sp1 function using Sp1 RNAi. Because HttQ103 cells are stressed by the transfection procedures, considerable neuronal cell death was present in mock siRNA transfection. No detectable differences in cell death were obtained between blank transfection and scrambled sequence siRNA transfection (mock siRNA control). Sp1 RNAi eliminated this neuronal cell death induced by HttQ103 (Fig. 2F). In the presence of siRNA, Sp1 levels were significantly decreased (Fig. 2G) as measured by Western analysis (by 36% in HttQ25 cells and by 51% in HttQ103 cells), and the increase in Sp1 levels caused by 3-NP treatment (Fig. 1B) was eliminated (data not shown). These results demonstrate that the alterations in cellular toxicity observed in Fig. 2A corresponded with Sp1 gene expression. Sp1 RNAi also significantly decreased the expression of HttQ103 (Fig. 2H) detected by Western blot using antibody against GFP (by 74%, p < 0.05). There was no significant potentiation when mithramycin was applied to the PC12/HttQ25 cell transfected with Sp1 siRNA (data not shown), suggesting that Sp1 RNAi and mithramycin affect a common action site. Taken together, our data suggest that either blocking Sp1-driven gene expression by mithramycin A or inhibiting Sp1 gene expression by Sp1 RNAi both diminish HttQ103 and 3-NP-associated neuronal cell death.

Sp1 Gene Knock-down Ameliorates Neurodegeneration in Striatal Neurons Isolated from HD Transgenic Mice—To determine whether Sp1 plays an important role in neurodegeneration in HD, primary stria-
atal neurons from WT, Sp1 heterozygous knock-out, HD transgenic, and Sp1 heterozygous knock-out mice crossed with HD transgenic mice were examined as shown in Fig. 3. Neurons obtained from 16-day-old WT mice had distinctive morphological changes during the culture period. The phase contrast micrographs in A are WT striatal neuronal cultures at ages: 0, 3, 7, and 10 day in vitro (DIV). The time course for detecting of neurodegeneration in primary neurons was examined (B). Results were normalized to the extent of cell death observed in WT neurons. Cell death was examined in both WT and R6/2 neurons. C and D summarized the neurodegeneration (represented as normalized LDH releases) of primary striatal neurons from different genetic backgrounds, WT, HD transgenic, Sp1 heterozygous knock-out, and Sp1 heterozygous knock-out cross-bred with HD transgenic mice under baseline condition (C) and 500 μM 3-NP treatment for 4 h (D). Sp1 in this and the following figures represents Sp1 heterozygous knock-out group. The neurodegeneration was examined in neurons with different genotypes, and the data are normalized to WT. Results are pooled from four culture sets (mean ± S.E.), each set containing at least two embryos for individual genotyping group, and triplicate for each treatment. *p < 0.05.

FIGURE 3. Sp1 gene knock-down diminished neurodegeneration in striatal neurons isolated from HD transgenic mice. Striatal neurons obtained from 16-day-old mouse had distinctive morphological changes during the culture period. The phase contrast micrographs in A are WT striatal neuronal cultures at ages: 0, 3, 7, and 10 day in vitro (DIV). The time course for detecting of neurodegeneration in primary neurons was examined (B). Results were normalized to the extent of cell death observed in WT neurons. Cell death was examined in both WT and R6/2 neurons. C and D summarized the neurodegeneration (represented as normalized LDH releases) of primary striatal neurons from different genetic backgrounds, WT, HD transgenic, Sp1 heterozygous knock-out, and Sp1 heterozygous knock-out cross-bred with HD transgenic mice under baseline condition (C) and 500 μM 3-NP treatment for 4 h (D). Sp1 in this and the following figures represents Sp1 heterozygous knock-out group. The neurodegeneration was examined in neurons with different genotypes, and the data are normalized to WT. Results are pooled from four culture sets (mean ± S.E.), each set containing at least two embryos for individual genotyping group, and triplicate for each treatment. *p < 0.05.

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atal neurons from WT, Sp1 heterozygous knock-out, HD transgenic, and Sp1 heterozygous knock-out mice crossed with HD transgenic mice were examined as shown in Fig. 3. Neurons obtained from 16-day-old WT mice had distinctive morphological changes during the culture period (Fig. 3A). During the first day, cells have a rounded shape in culture, are attached to the substrate, and initiate process outgrowth. Prominent neurite outgrowth and synapse formation are observed by 7 DIV with the length and numbers of neurites increasing with culture age; by 10 DIV, they are relative stable. Neurons isolated from HD transgenic (R6/2 and N171-82Q), Sp1 heterozygous knock-out, and HD transgenic cross-bred with Sp1 heterozygous knock-out mice displayed similar morphologic developmental pattern as WT neurons during the culture period. The time course of neurodegeneration in primary neurons was examined (Fig. 3B). Considerable differences in neuronal death between WT and R6/2 neurons were observed at 5 DIV and persisted until 8 DIV. For subsequent experiments, neurodegeneration was examined at 8 DIV, because at this time point the difference in neuronal death between WT and R6/2 neurons is substantial and maintained.

The neurodegeneration occurring in primary striatal neurons from each genetic background was examined under baseline (Fig. 3C) and 3-NP-treated conditions (Fig. 3D). Under baseline conditions, neuronal death was significantly increased in R6/2 neurons (Fig. 3C), but not in Sp1 or Sp1 crossed with R6/2 neurons. Similar alterations were obtained when the neurons were treated with 3-NP (500 μM for 4 h). Neurons from all the groups are sensitive to 3-NP, but the effects of 3-NP are much more prominent in R6/2 neurons. Sp1 gene knock-down substantially protected neurons from 3-NP toxicity (Fig. 3D), especially from mutant Htt-associated toxicity (Fig. 3C and D). No significant reductions of mutant Htt, detected by Western blot with antibody against polyglutamine expansion (IC2), were observed in neurons from R6/2 mouse crossed with Sp1 heterozygous knock-out mouse (insets in Fig. 3C by 27%, p > 0.05). Our data demonstrate that the significant neurodegeneration observed in primary striatal neurons isolated from HD transgenic mouse is partially reversed by the Sp1 gene knock-down, suggesting that Sp1 may be deleterious in HD.

Characterization of Sp1 Heterozygous Knock-out Mouse Model—Sp1 gene expression and transcriptional activity was investigated in the Sp1 heterozygous knock-out mouse model. First, we examined Sp1 mRNA levels in neurons and mouse brains using quantitative PCR analysis with Sp1 primer. Sp1 mRNA levels significantly decreased in primary neurons and mouse brains by Sp1 gene knock-down (by 52% in neurons and 17% in brains, Fig. 4A). Whereas Sp1 mRNA levels substantially increased in R6/2 mice compared with WT mouse brains, the increase in Sp1 expression of the crossed mice was significantly less. Next, we examined the expression of Sp1 protein in primary neurons and mouse brains using Western blot analysis (Fig. 4B). Sp1 protein levels were substantially decreased in all neuron from the crossed groups, including those cross-bred with R6/2 (by 63%) or N171-82Q (by 79%), as well as in
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Sp1 heterozygous knock-out groups (by 84%). Similar reductions were obtained from the brains: 66% in knock-outs crossed with the R6/2 group, 52% in knock-outs crossed with N171-82Q, and 88% from the knock-outs alone (bar graphs in panel B). The alterations observed in Sp1 protein levels in both Sp1 heterozygous knock-out and crossed mice were consistent between neuronal cultures and mouse brains. Higher Sp1 levels were observed in mouse brains but not in neurons in the crossed mice compared with Sp1 heterozygous knock-out mice.

As expected, when Sp1 heterozygous knock-out mice were crossed with HD transgenic mice, a 65-kDa band corresponding to the inactive transcribed Sp1 fragment appeared (Fig. S2A, supplemental data) (22), suggesting at least a partial deletion in the Sp1 exon encoding for the zinc finger domains and the C terminus. We confirmed that the N-terminal fragment of Sp1 is not functional, consistent with previous reports by other groups (35, 36). First, transcriptional activity was decreased by 62% (p < 0.05) in neurons isolated from Sp1 heterozygous knock-out mouse as detected by a luciferase transcriptional reporter assay (Fig. S2B, supplemental data), when neurons were transfected with vector containing six Sp1 zinc finger DNA binding domains. Second, increases in the levels of the 65-kDa fragment were not detected in the crossed groups compared with Sp1 heterozygous knock-out mice (Fig. S2A, supplemental data), suggesting that this 65-kDa N-terminal fragment is unlikely to cause a gain of function effect. The increased sensitivity of Sp1 antibody for this fragment resulting in the very large band (Fig. S2A, supplemental data) may be caused by conformational differences between the fragment and the holoprotein. In addition, we confirmed that Sp1 gene knock-down caused no compensatory changes in Sp3 mRNA, and protein levels in both R6/2 neurons and brains (Fig. S3 in supplemental data). Together, these data verify the Sp1 knock-down at the mRNA, protein, and functional levels.

Sp1 Gene Knock-down Extends the Lifespan of HD Transgenic Mice—

Cohorts of Sp1 heterozygous knock-out mice crossed with N171-82Q and R6/2 mice were followed systematically from weaning. The Sp1 heterozygous knock-out crossed with R6/2 and N171-82Q mice were analyzed for changes in survival (Fig. S5A). None of the WT or Sp1 heterozygous knock-out mice died during the course of the experiment. The Sp1 heterozygous knock-out crossed with R6/2 mice lived significantly longer than the HD transgenic mice (105.3 ± 2.3 versus 97.1 ± 2.4 days, p < 0.05) (Fig. 5A, left panel) with R6/2 mice, and (151.8 ± 4 versus 140.9 ± 3.7 days, p < 0.05) (Fig. 5A, right panel) with the N171-82Q mice. Body weight in crossed mice declined with time but the difference was not significant compared with HD transgenic mice (data not shown). The increased lifespan provided by Sp1 gene knock-down in the HD transgenic mice confirms the neuroprotective effects of inhibition of Sp1 obtained in vitro.

Htt aggregate formation and mutant Htt gene expression were investigated in the Sp1 heterozygous knock-out mice crossed with R6/2 mice. Robust Htt immunoreactive aggregates could be detected by EM48 in the brains of HD transgenic and crossed mice in both cortex and striatum at the age of three months. We determined the average numbers and sizes of Htt aggregates with NIH Image and Stereoinvestigator software by analyzing the total aggregate burden or separately characterizing nuclear aggregates. We found no change in the volume of striatum between R6/2 mice and Sp1 heterozygous knock-out mice crossed with R6/2 mice (5.2 μm³ ± 0.51 versus 5.0 μm³ ± 0.22, p < 0.05). No significant reduction was detected in the numbers (total aggregate burden) and size of nuclear Htt aggregates (Fig. 5B) by Sp1 knock-down in both cortex and striatum, consistent with the neuropathology seen in mice treated with mithramycin A (20).

Mutant Htt gene expression was detected by Western blot using antibody MAB1574 against polyglutamine expansion (37 Gln), and by real time PCR using a primers specific against the transgene in exon 1 (see “Experimental Procedures”). Examining Htt transgene expression by Western blot and real time PCR showed that Sp1 gene knock-down tended to reduce mutant Htt by 20% and human Htt mRNA by 15% (Fig. 5C), though these reductions were not statistically significant. There was no change in WT Htt gene expression (data not shown). A similar trend was also obtained from in situ hybridization (decreased by 18%) by oligodeoxynucleotides labeled with 35S-dATP, specifically probing the Htt transgene (data not shown). The reduction in Htt transgene expression was not statistically significant in the crossed mice, although Sp1 RNAi significantly decreased the expression of mutant Htt in the PC12 model (Fig. 2H). Taken together, Sp1 gene knock-down in HD transgenic mice may have a small effect on Htt transgene expression, which could contribute to the observed increases in the lifespan of crossed mice.
In this study we have examined the potential role of Sp1 in HD using complementary cellular and transgenic models, demonstrating that Sp1 is up-regulated in the presence of mutant Htt and its reduction is neuroprotective. Our approach was to determine the effects of mutant Htt and the mitochondrial toxin 3-NP on Sp1 levels in PC12 cells, assess Sp1 levels in primary neurons and transgenic mice, and modulate Sp1 by siRNA, mithramycin A, and genetically using Sp1 heterozygous knock-out mice crossed with HD transgenic mice. At the same time we accounted for effects on the expression of Sp1-regulated genes as well as of Htt and Sp3. We found that mutant Htt or 3-NP treatment enhanced Sp1 levels and that knock-down of Sp1 improved the survival of cellular and animal models. Sp1 knockdown was associated with modest alterations in Htt gene expression, restored down-regulated D2 receptors, and did not significantly affect Sp3 expression.

In previous studies, evidence was provided that molecular interactions between Sp1 and mutant huntingtin may be deleterious (2). However, specific blockade of Sp1 binding sites on DNA by mithramycin A is the most potent neuroprotective treatment that has so far been administered to the R6/2 model of HD (20). Our studies help resolve this paradox by examining how endogenous Sp1 modulation affects cellular and mouse models of HD. All of our experiments consistently demonstrate that reducing or blocking Sp1 is neuroprotective in the presence of pathogenic mutant huntingtin. Thus, while Sp1 function may be compromised by aberrant interactions with mutant huntingtin, our data indicate that this compromise is outweighed functionally by the benefits of Sp1 inhibition. Our data further validate Sp1 as a therapeutic target in HD.

The precise contributions of transcriptional dysfunction to neurodegeneration occurring in HD remain unclear. Ubiquitous gene expression and intricate functional relationships have precluded a simple interpretation of the gross actions of Sp1-responsive genes in vitro (37). Our experiments addressed the possible interaction of mutant huntingtin, and Sp1 and provide evidence for a role of Sp1 in HD pathogenesis. Sp1 gene knock-down achieved in cell culture by RNAi (Fig. 2) revealed an association between Sp1 and HttQ103 levels, which result from Sp1 binding to G-C-rich regions on the promoter (21). Sp1-mediated down-regulating of HttQ103 (Fig. 2H) could partially contribute to the neuroprotection (Fig. 2F). In addition, Sp1 RNAi (data not shown) and knocking out the Sp1 in mice (Fig. 3) both resulted in similar protective effects on mitochondrial complex II inhibitor, 3-NP, toxicity. Our cell culture
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