Huntington's disease is a neurodegenerative disease resulting from a CAG (glutamine) trinucleotide expansion in exon 1 of the Huntingtin (Htt) gene. The role of the striatum-enriched A2A adenosine receptor (A2A-R) in Huntington's disease has attracted much attention lately. In the present study, we found that expression of mutant Htt with expanded poly(Q) significantly reduced the transcript levels of the endogenous A2A-R in PC12 cells and primary striatal neurons. Cotransfection of a poly(Q)-expanded Htt revealed that the Htt mutant suppressed the core promoter activity of the A2A-R gene. Stimulation of the A2A-R using CGS21680, forskolin, and a constitutively active cAMP-response element-binding protein (CREB) mutant elevated the reduced promoter activity of the A2A-R gene by mutant Htt. Moreover, the effect of CGS was blocked by an A2A-R-selective antagonist (CSC), two inhibitors of protein kinase A, and two dominant negative mutants of CREB. The protein kinase A/CREB pathway therefore is involved in regulating A2A-R promoter activity. Consistently, an atypical CRE site (TCCAGG) is located in the core promoter region of the A2A-R gene. Electrophoretic gel mobility shift assay and mutational inactivation further demonstrated the functional binding of CREB to the core promoter region and showed that expression of poly(Q)-expanded Htt abolished the binding of CREB to this site. Stimulation of the A2A-R restored the reduced CREB binding caused by the mutant and concurrently reduced mutant Htt aggregation. Collectively, the poly(Q)-expanded mutant Htt suppressed expression of the A2A-R by inhibiting its core promoter at least partially by preventing CREB binding.

Huntington's disease (HD) is an autosomal dominant neurodegenerative disease characterized by chorea, dementia, and psychiatric symptoms. The causative mutation is a CAG trinucleotide expansion in exon 1 of the Huntingtin (Htt) gene. Normal chromosomes have 35 or fewer CAG repeats in the N-terminal region, whereas HD is associated with 36 or more repeats (1). The CAG repeats are translated into polyglutamine residues (poly(Q)) in the Htt protein. When the number of CAG repeats exceeds 36, specific degeneration of several brain areas (e.g. the striatum and cortex) occurs. The reason for such regional susceptibility to mutant Htt with poly(Q) expansion remains unclear. Accumulating evidence from different laboratories suggests that mutant Htt forms aggregates and causes aberrant protein-protein interactions with a number of important transcription factors or coactivators, such as p53, CREB binding protein (CBP), SP1, and TAFI1130 (2–6). Alteration of the transcription profiles of cells expressing mutant Htt aggregates is therefore expected (7–12). Sequestration of and/or interference with important transcriptional regulatory proteins have been proposed to be critical mechanisms in HD progression. For example, mutant Htt is known to be associated with the Sp1-TAFI1130 complex and causes improper location of RNA polymerase II on the promoter region of the dopamine D2 receptor gene, which subsequently hinders transcription of the gene (5). Moreover, mutant Htt has been shown to act as a transcriptional coactivator of the nuclear hormone receptor (13). Note that the effect of mutant Htt on the overall gene expression profiles appeared to be specific because no changes in the expression of cytoskeletal proteins (including MAP2, glial fibrillary acidic protein, myelin basic proteins, and neurofilaments), enzymes of intermediary metabolism (such as glycerol-3-phosphate dehydrogenase and glucose-6-phosphate isomerase), caspases, mitochondrial proteins, or proteolytic enzymes were observed in a transgenic HD mouse model (14).

The A2A adenosine receptor (A2A-R) is enriched in Htt/poly(Q)ₙ-sensitive, enkephalin-containing striatal neurons (15–17). We previously cloned cDNA and the gene of the A2A-R, which contains seven transmembrane domains and belongs to the G protein-coupled receptor family (18–22). The A2A-R gene contains two independent TATA-less promoters (P1 and P2), which produce transcripts whose 5′-untranslated regions are of different lengths but that encode the same protein (18, 19). Our previous study demonstrated that P2 is the major promoter of the A2A-R gene, which is active in various tissues including the brain (19, 21). One of the physiological functions of A2A-Rs is to protect cells against various assaults. By using PC12 as a

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Inhibition of A2A-R Promoter by Poly(Q)-expanded Huntingtin

Cell Culture and Transfection—PC12 cells were originally obtained from the American Type Culture Collection (Manassas, VA) and were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 5% fetal bovine serum (FBS, Invitrogen) plus 10% horse serum (Invitrogen) in an incubation chamber gassed with 10% CO2, 90% air at 37 °C. A123 is a PKA-deficient variant of PC12 cells (36), kindly provided by Dr. J. A. Wagner (Cornell University Medical College, New York, NY). A123 cells were maintained in DMEM supplemented with 5% (v/v) horse serum and 10% (v/v) FBS. Cells were grown on tissue culture plates coated with poly-l-lysine (Sigma). The day before transfection, cells were seeded onto a 35-mm dish at a density of 2 × 10⁴ cells per well. For transfection, cells were grown in wells for 24 h and then exposed to a mixture of 3 μl of Lipofectamine 2000 (Invitrogen), and 2 μg of the desired DNAs for 5 h in serum-free DMEM. Next, cells were washed with PBS, followed by one more wash with normal growth medium, and were cultured as described above for another 48 h before the indicated drug treatment(s) for an additional 24 h.

Primary striatal neuron cultures were prepared from brains of E19 SD rats. Briefly, tissues were minced and treated with trypsin (0.1%, Invitrogen) for 10 min at 37 °C. Cells were mechanically dissociated by gentle pipetting in minimum Eagle’s medium (Invitrogen) and seeded onto poly-l-lysine-coated 24-well plates (Nunc) at a density of 2 × 10⁶ cells per well. After a 3-h incubation, the culture medium was replaced with B27 Neurobasal medium (Invitrogen) supplemented with 200 μM glutamate, 10 mM glutamate, and penicillin/streptomycin. Cells were cultured in an incubation chamber gassed with 5% CO₂, 95% air at 37 °C. For transfection, cells 7 days in vitro were transfected with LF2000 (5 μl) and 2 μg of DNAs for 5 h in Opti-MEM (Invitrogen). Cells were washed with PBS once and fresh B27 medium once and were incubated for another 72 h. Immunostaining of an astrocyte marker (glial fibrillary acidic protein) indicated that the neuronal cultures were free of astrocytes (data not shown).

Immunofluorescence—Seventy two hours after transfection, cells grown on glass coverslips were fixed with 4% paraformaldehyde, 4% sucrase in PBS for 30 min at room temperature and permeabilized by 0.05% Nonidet P-40 in PBS at room temperature three times for 20 min each. After a 1-h blocking in 2% PBS, bovine serum albumin (2%), and normal goat serum, cells were stained with the desired primary antibody reconstituted in PBS, 2% goat serum at 4 °C for 14–16 h. Dilution of the anti-MAP2 antibody (Chemicon International, Temecula, CA) was 1:500. The anti-Htt antibody was raised against the first 17 amino acids of human Htt plus two glutamine residues and was utilized at a dilution of 1:500. After extensive washing, the slides were incubated with the corresponding secondary antibody conjugated with Alexa Red (1:500; Molecular Probes, Leiden, The Netherlands) for 1 h at room temperature, followed by washing, and analysis with the aid of a laser confocal microscope (Bio-Rad, MRC-1000).

Measurement of Mutant Htt Aggregate Formation—For measuring aggregate formation, cells were transfected with the indicated expression constructs and incubated for 3 days. Cells with green fluorescence were identified and counted under a confocal laser fluorescence microscope (MRC-1000, Bio-Rad). Percentages of cells containing visible fluorescent aggregates were quantified. Approximately 100 transfected cells were counted. Data points represent the mean ± S.E. of at least three independent experiments.

 Luciferase Assays—Seventy two hours post-transfection, cells were harvested and lysed in the Cell Culture Lysis Buffer of the Dual-luciferase Reporter Assay System (Promega). Lysates were then centrifuged at 1500 rpm for 5 min to remove insoluble materials. Luciferase activities of 10 μl of lysate were determined following the manufacturer’s protocol using a TD-20/20 Luminometer (Promega). Results were obtained by using at least two different preparations of plasmids. The protein concentrations of lysates were determined by the Bradford analysis (37), and the luciferase activities were normalized to the amount of proteins in the lysate. At least three independent transfections were performed for each experiment.

 Galactosidase Detection—To determine the expression of β-galactosidase in the transfected cells, cells were first lysed in the Cell Culture Lysis Buffer (Promega). After low speed centrifugation, the supernatant was collected and subjected to a luminometric assay using o-nitrophenyl-β-D-galactopyranoside as the substrate by measuring the absorbance of the product at 420 nm with a spectrophotometer. The β-galactosidase activity was linear with time for A₄₂₀ values up to 1.

 Western Blot Analysis—Equal amounts of protein were separated by SDS-PAGE by using 10% polyacrylamide gels according to the method of Laemmli (38). The resolved proteins were electroblotted onto Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA). Mem-
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PC12 cells and primary striatal neurons were transfected with the mutant Htt construct with the indicated number of poly(Q) (Htt-Qn-hrGFP). Forty eight hours post-transfection, PC12 cells were treated with or without an A2A-R agonist (CGS, 1 μM) for 24 h as indicated. Percentages of cells containing Htt aggregates in the cytoplasm and/or in nuclei were quantified using fluorescence microscopy. For each experiment, approximately 100 transfected cells were counted. Data points represent the mean ± S.E. of at least three independent experiments.

| Cell types        | (Q)n | CGS | Aggregates at cytoplasm | Aggregates at nucleus |
|-------------------|------|-----|-------------------------|-----------------------|
| Striatal neuron   | 25   | -   | 0                       | 0                     |
| Striatal neuron   | 109  | -   | 43.07 ± 4.16            | 56.93 ± 4.16          |
| PC12 cells        | 25   | -   | 0                       | 0                     |
| PC12 cells        | 25+  | 0   | 0                       | 0                     |
| PC12 cells        | 48   | -   | 15.10 ± 0.97            | 0                     |
| PC12 cells        | 73   | -   | 15.37 ± 0.32            | 0                     |
| PC12 cells        | 109  | -   | 70.50 ± 4.20            | 12.13 ± 2.19          |
| PC12 cells        | 109+ | 34.00 ± 3.67             | 0º                    |

* Specific comparison between cells treated with or without CGS (p < 0.001; one-way analysis of variance).

Flow Cytometry and Cell Sorting—Cells were harvested by centrifugation, resuspended in PBS to a final density of 5 × 10⁶ cells/ml, and filtered through a nylon membrane to remove cell aggregates. Flow cytometry and sorting of hrGFP-positive cells were performed using a FACSVantage (BD Biosciences) with a 530 ± 15 nm bandpass filter as they traversed the beam of an argon ion laser (488 nm, 100 milliwatts). Data acquisition and analysis were performed with CellQuest software (BD Biosciences). Sorted cells were harvested into tubes for further isolation of total RNA or protein.

RNA Isolation and Quantitative Real Time PCR—Total RNA was isolated using the TriReagent kit (Molecular Research Center, Cincinnati, OH), treated with RNase-free DNase (RQI; Promega) to remove the potential contamination of genomic DNA, and transcribed into cDNA using Superscript II reverse transcriptase. Real time quantitative PCR was performed using the TaqMan kit (PE Applied Biosystems, Foster City, CA) on a TaqMan ABI 7700 Sequence Detection System (PE Applied Biosystems) using a heat-activated TaqDNA polymerase (AmpliTaq Gold; PE Applied Biosystems). The PCR mixtures were incubated at 50 °C for 2 min and 95 °C for 10 min, and then 40 PCR cycles were conducted (95 °C for 15 s and 65 °C for 1 min). The sequences of primers are listed below: for A2A-R (the target gene), 5'-TGCGCTTGT-TGTCTCTGTGCTA-3' and 5'-TTGTCCTGCT-3'; for Bcl-2 (the target gene), 5'-CATGTGATGCGTGTTGACAG-3' and 5'-GAGGCGCAGCTGGACAC-3'; and for GAPDH (the reference gene), 5'-TATTGGTGTGGATCAGCATGT-3' and 5'-ACAACCTGCCTCCTCGTAGTA-3'. Independent reverse transcription-PCRs were performed using the same cDNA for both the target gene and reference gene. A melting curve was created at the end of the PCR cycle to confirm that a single product was amplified. Data were analyzed by the ABI 7700 operating software to determine the threshold cycle (CT) above the background for each reaction. The relative transcript amount of the target gene, which was calculated using standard curves of serial RNA dilutions, was normalized to that of GAPDH of the same RNA.

Nuclear Extract Preparation—Cells (5 × 10⁶) were washed with 1× PBS (137 mM NaCl, 2.6 mM KCl, 9.5 mM Na₂HPO₄, and 1.4 mM KH₂PO₄ (pH 7.4)), suspended in 500 μl of buffer A (10 mM Heps (pH 8), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 1 mM Na₂VO₄, 20 mM NaF, 100 mM okadaic acid, and 25% glycerol) and incubated for 60 min on ice. Nuclear extracts were next collected by centrifugation at 14,500 rpm for 5 min at 4 °C. The protein concentration of the nuclear extracts in the supernatant was measured with the Bio-Rad protein assay reagent.

Electrophoretic Gel Mobility Shift Assay (EMSA)—The double-stranded DNA fragment comprising the core promoter region of the A2A-R gene (−250 to −145) was generated by excising the indicated DNA fragment from the core-promoter construct (pGL2−[−1997−145]) into PC12 cells or primary striatal neurons for 72 h. Luciferase activity of each construct was measured and normalized to the protein content. Values are expressed as percentages of the promoter activity in the presence of pcDNA3.1-(Q)₁₀₀-Htt-hrGFP in the indicated cell type and represent the mean ± S.E. of at least three independent experiments. **, specific comparison between cells transfected with pcDNA3.1-(Q)₁₀₀-Htt-hrGFP and cells transfected with pcDNA3.1-(Q)₅₀-hrGFP (p < 0.001; one-way analysis of variance).
min, 25 °C for 25 min, and 4 °C for 15 min. The sequences of oligonucleotides are listed below: for wild type, 5'-ACTCTCTCTTTCGAGTAGTCGCTCTGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA
cells transfected with Htt-(Q)109-hrGFP contained neuronal intranuclear inclusions and cytoplasmic Htt aggregations (CGS21680, CGS) significantly reduced the formation of neuronal intranuclear inclusions, whereas those transfected with Htt-(Q)25-hrGFP showed only dispersed hrGFP in the cytoplasm.

Similarly, transient expression of mutant Htt with expanded poly(Q) led to the formation of aggregates in PC12 cells (Table I; Fig. S-2 of the Supplemental Material). With 48 or 73 copies of poly(Q), ~15% of the transfected PC12 cells harbored Htt aggregates in the cytoplasm, whereas no neuronal intranuclear inclusions were detected. Further expansion of poly(Q) to as many as 109 copies led to a more severe aggregation of mutant Htt in both the cytoplasm and nucleus (Fig. S-2, E and F). The aggregation of mutant Htt led to verilization by colocalization of hrGFP and Htt as shown in Fig. S-2H. Most intriguingly, stimulation of the A2A-R using an A2A-R-selective agonist (CGS21680, CGS) significantly reduced the formation of neuronal intranuclear inclusions and cytoplasmic Htt aggregations (Table I). Because a reduction in the size or number of Htt aggregates has been observed concurrent with improvements in major symptoms of HD and/or reduced toxicity caused by poly(Q)-associated Htt (7, 40–42), suppression of Htt aggregations in primary striatal neurons, more than half (56.9% ± 14.2%) of the cells transfected with Htt-(Q)109-hrGFP contained neuronal intranuclear inclusions, whereas those transfected with Htt-(Q)25-hrGFP showed only dispersed hrGFP in the cytoplasm.

FIG. 4. Stimulation of the A2A-R selectively reversed the suppression of the A2A-R promoter by poly(Q)-expanded Htt in PC12 cells. A, indicated Htt construct was cotransfected with an A2A-R promoter construct (pGL2 (~1997−145)) into PC12 cells for 48 h. Cells were then treated with the indicated inhibitor (10 μM CSC, 10 μM H89, or 1 μM KT5720) for 30 min, followed by a 24-h incubation with CGS (1 μM) or forskolin (10 μM). B, the indicated Htt construct was cotransfected with an A2A-R promoter construct (pGL2 (~1997−145)) into a PKA-deficient PC12 cell variant (A123) for 48 h. Cells were then treated with CGS (1 μM) or forskolin (10 μM) for 24 h. Luciferase activity of each construct was measured and normalized to the protein content. Values are expressed as percentages of the promoter activity of pGL2 (~1997−145) in the presence of pcDNA3.1-(Q)25-Htt-hrGFP under control conditions (no treatment) and represent the mean ± S.E. of at least three determinations. a, specific comparison between the indicated group and that transfected with pcDNA3.1-(Q)109-Htt-hrGFP with no treatment (p < 0.001; one-way analysis of variance). b, specific comparison between control and inhibitor-treated cells in each group (p < 0.001; one-way analysis of variance).

underlying the reduction of A2A-R expression in HD. To characterize gene regulation of the A2A-R by mutant Htt, expression constructs were created of mutant Htt (pcDNA3-Htt-(Q)n-hrGFP) that encode chimeras (Htt-(Q)n-hrGFP) containing hrGFP at the C terminus fused to a fragment of Htt encoded by its exon-1 and the indicated number of poly(Q) repeats. Transient expression of Htt-(Q)109-hrGFP in primary striatal neuronal culture for 3 days produced Htt aggregates that could readily be observed and quantified by using confocal microscopy (Table I; Fig. S-1 of the Supplemental Material). In primary striatal neurons, more than half (56.9% ± 4.2%) of the cells transfected with Htt-(Q)109-hrGFP contained neuronal intranuclear inclusions, whereas those transfected with Htt-(Q)25-hrGFP showed only dispersed hrGFP in the cytoplasm.

Similarly, transient expression of mutant Htt with expanded poly(Q) led to the formation of aggregates in PC12 cells (Table I; Fig. S-2 of the Supplemental Material). With 48 or 73 copies of poly(Q), ~15% of the transfected PC12 cells harbored Htt aggregates in the cytoplasm, whereas no neuronal intranuclear inclusions were detected. Further expansion of poly(Q) to as many as 109 copies led to a more severe aggregation of mutant Htt in both the cytoplasm and nucleus (Fig. S-2, E and F). The aggregation of mutant Htt was verified by colocalization of hrGFP and Htt as shown in Fig. S-2H. Most intriguingly, stimulation of the A2A-R using an A2A-R-selective agonist (CGS21680, CGS) significantly reduced the formation of neuronal intranuclear inclusions and cytoplasmic Htt aggregations (Table I). Because a reduction in the size or number of Htt aggregates has been observed concurrent with improvements in major symptoms of HD and/or reduced toxicity caused by poly(Q)-associated Htt (7, 40–42), suppression of Htt aggregation by the A2A-R might lead to ameliorated symptoms of HD, such as abnormal gene regulation.

We first determined whether mutant Htt with 109Q affected the expression of the endogenous A2A-R in PC12 cells and in primary striatal neurons. Cells were transiently transfected with Htt-(Q)25-hrGFP or Htt-(Q)109-hrGFP as indicated. The hrGFP-positive cells were identified and harvested using a flow cytometer to collect RNA. Expression levels of the endogenous A2A-R in the presence of mutant Htt with either 25Q or 109Q were determined using a quantitative PCR method. As shown

FIG. 5. CREB played a critical role in mediating the effect of A2A-R on suppression of the A2A-R promoter by poly(Q)-expanded Htt. A, PC12 cells were transiently transfected with the indicated mutant Htt construct, an A2A-R promoter construct (pGL2 (~1997−145)), and a constitutive expression construct (pGL2 (~1997−145)), and a constitutive expression construct (pGL2 (~1997−145)), and a construct encoding a negative CREB mutant (CREBm1 or CREBR287), or an empty vector in the molar amounts of 7, 7, and 1, respectively, for 48 h. Cells were then treated with a 24-h incubation with or without CGS (1 μM) or forskolin (10 μM). B, PC12 cells were transiently transfected with the indicated mutant Htt construct, an A2A-R promoter construct (pGL2 (~1997−145)), and a construct encoding a constitutively active CREB mutant (VP16-CREB) or a control vector (VP16) in the molar amounts of 7, 7, and 1, respectively, for 72 h. Luciferase activity of each construct was measured and normalized to the protein content. Values are expressed as percentages of the promoter activity of pGL2 (~1997−145) in the presence of pcDNA3.1-(Q)25-Htt-hrGFP under control conditions (no treatment) and represent the mean ± S.E. of at least three determinations. a, specific comparison between the indicated group and that transfected with pcDNA3.1-(Q)109-Htt-hrGFP with no treatment (p < 0.001; one-way analysis of variance). b, specific comparison between control and inhibitor-treated cells in each group (p < 0.001; one-way analysis of variance).
In Fig. 1A, expression of mutant Htt with expanded poly(Q) markedly reduced the expression of the endogenous A2A-R in both primary striatal neurons and PC12 cells. The suppressive effect of mutant Htt was selective because no detectable effect on endogenous bcl2 expression was found (Fig. 1B).

To determine whether mutant Htt affects expression of the A2A-R gene by regulating its promoter, the pGL2-(−1997/−145) construct, which contains two promoters of the rat A2A-R gene (18), and the expression construct encoding mutant Htt with the indicated number of CAG repeats (Q) were cotransfected into PC12 and primary striatal neurons. As shown in Fig. 2, marked repression of the A2A-R promoter was observed in the presence of 109Q-Htt in both cell types tested. To locate the DNA region responsible for the above suppressive effect, a series of 5′-deleted A2A-R promoter constructs was transfected into PC12 cells along with mutant Htt harboring 25Q or 109Q. The suppressive effect of 109Q-Htt persisted when the A2A-R gene fragment was 5′-deleted to −250 bp where the major core promoter region resides (Fig. 3A) (18). Thus, poly(Q)-expanded Htt inhibited A2A-R expression by suppressing its core promoter region (18). Consistently, the proteins-A2A-R promoter complex from cells expressing 109Q-Htt migrated faster than those from cells containing 25Q-Htt, indicating that specific binding of nuclear proteins to the core promoter region of the A2A-R gene was altered in the presence of 109Q-Htt when compared with 25Q-Htt (Fig. 3B). Note that the proteins-A2A-R promoter complex from nontransfected PC12, which expressed no mutant Htt, migrated to the same position as that from PC12 cells expressing Htt with 25Q (Fig. S-3; Supplemental Material). Overexpression of the Htt fragment by itself therefore did not alter formation of the proteins-A2A-R promoter complex. Most importantly, such a suppressive effect of poly(Q)-expanded Htt was also observed on three other promoters (p21, SV40, and TK) but not on the cytomegalovirus promoter (Table II), further supporting the hypothesis that alteration of gene regulation by mutant Htt is specific.

Another very interesting observation was that stimulation of the A2A-R in the presence of 109Q-Htt by CGS restored the proper protein-binding pattern in the EMSA to that with 25Q-Htt (Fig. 3B). Activation of the A2A-R thus might protect inhibition of the A2A-R promoter from poly(Q)-expanded Htt. In agreement with the above observation, CGS treatment elevated the reduced promoter activity by 109Q-Htt (Fig. 4A). Moreover, pretreating cells with an A2A-R-selective antagonist, 8-(3-chlorostyryl) caffeine (CSC), markedly reduced the effect of CGS (Fig. 4A). The elevating effect of CGS therefore appeared to be specifically mediated by the A2A-R. Because stimulation of the A2A-R has been shown to cause a transient increase in cAMP in PC12 cells (43), we next examined whether the cAMP/PKA pathway plays an important role in the A2A-R reversing effect. As shown in Fig. 4A, direct stimulation of adenyl cyclase using forskolin (FK), which subsequently activates the cAMP/PKA pathway, also significantly reversed the reduction in A2A-R promoter activity by 109Q-Htt. Treatment with two selective PKA inhibitors (H-89 and KT5720) effectively blocked the reversing effect of CGS and FK. In addition, CGS exerted no reversing effect on the suppressed A2A-R promoter activity by 109Q-Htt in a PKA-deficient PC12 variant (A123, Fig. 4B). PKA therefore is essential for the rescuing effect of A2A-Rs.

Because CREB has been implicated in the action of mutant Htt (2, 44), and because we earlier demonstrated that stimulation of the A2A-R led to activation of CREB via PKA (10), we next investigated the involvement of CREB in the promoter activity of the A2A-R-reversing effect. In PC12 cells, expression of two dominant negative CREB mutants (CREBm1 and CREB-R287L) was shown previously to significantly reduce the activity of CREB upon A2A-R stimulation (6). Expression of either CREBm1 or CREB-R287L effectively reduced the A2A-R-reversed suppression of the A2A-R promoter caused by 109Q-Htt (Fig. 5A). Consistently, overexpression of a constitutively active CREB mutant (VP16-CREB, Fig. 5B), but not VP16 alone, markedly elevated A2A-R promoter activity. CREB thus appears to play a critical role in the action of A2A-R. The

**Table III**

| Name | CRE site sequence (5′→3′) |
|------|--------------------------|
| Wild type | TTCAGG |
| M2 | GCCCGG |
| M4 | GACCTG |
| M6 | GAACCT |

FIG. 6. CREB directly bound to the core promoter of the A2A-R gene. A, atypical CRE site (underlined) is located at the core promoter region of the A2A-R. B, a combination of EMSA and Western blot analyses was performed as follows: 10 μg of the unlabeled A2A-R (−250/−145) probes containing the wild type (WT) or the mutated CRE site (M2, M4, or M6) as indicated was first incubated with 0.5 μg of nuclear extract of PC12 cells and subjected to EMSA electrophoresis. Gels were stained with Coomassie Blue to identify the location of the proteins-DNA complex. The complex was then excised and separated by SDS-PAGE, followed by Western blot analyses using an anti-CREB and an anti-CBP antibody. C, supershift analyses of the A2A-R core promoter complex. NE (2.5 μg) was prepared from PC12 cells transfected with pcDNA3.1-(Q)25-Htt/ hrGFP or pcDNA3.1-(Q)109-Htt/ hrGFP as described in the legend of Fig. 3B, and NE was preincubated for 1 h with anti-CREB or an irrelevant anti-AC6D (39) antibody, followed by incubation with double-stranded, 32P-labeled 105-bp DNA containing the core promoter region of the A2A-R gene (A2AR−250/−145) for EMSA. Reaction products were separated in 8% polyacrylamide gels by electrophoresis. This is one representative result of three independent experiments performed. Arrowhead indicates the supershifted bands. Arrows mark the protein-A2A-R promoter complexes.
Inhibition of A2A-R Promoter by Poly(Q)-expanded Huntingtin

The functional CRE site was critical for A2A-R promoter activity and contributed to suppression of the A2A-R gene by Htt with expanded poly(Q). A. Nuclear extract (2.5 μg) prepared from cells expressing (Q)25-Htt-hrGFP or (Q)109-Htt-hrGFP as described in Fig. 6 was reacted with a [32P]-labeled A2A-R(250-145) oligonucleotide probe containing the wild type (WT) or the mutated CRE site (M2, M4, or M6) as indicated. Reaction products were separated in 8% polyacrylamide gels by electrophoresis. This is one representative result of three independent experiments performed. B, the indicated Htt construct with the desired number of poly(Q) was cotransfected with these four A2A-R core promoter constructs (−250/−145) that contain the WT or the mutated CRE site (M2, M4, or M6) as indicated into PC12 cells for 72 h. Forty eight hours post-transfection, cells were treated with or without an the A2A-R agonist (CGS, 1 μM) or forskolin (FK, 10 μM) for 24 h. Values are expressed as percentages of the promoter activity of wild type pGL2(−250/−145) in the presence of pcDNA3.1-(Q)25-Htt-hrGFP and represent the mean ± S.E. of at least three determinations. a, specific comparison between the indicated CRE mutant and the WT in the presence of 25Q-Htt. b, specific comparison between control, nontreated cells transfected with pcDNA3.1-(Q)25-Htt-hrGFP and those with pcDNA3.1-(Q)109-Htt-hrGFP in the corresponding group. c, specific comparison between cells treated with or without the indicated drug in the presence of 109Q-Htt in each corresponding group. d, specific comparison of the suppression of A2A-R promoter activity by109Q-expanded Htt mediated between WT and the indicated CRE mutant. e, specific comparison of the CGS or forskolin-rescuing effect between WT and the indicated CRE mutant (p < 0.05; one-way analysis of variance).

Observation that VP16-CREB exhibited a much more significant effect on elevating the activity of the A2A-R promoter than that of A2A-R stimulation was because of the latter only causing transient elevation in cAMP/CREB (43).

Most interestingly, an atypical CRE site (TCCAGG, −246 to −241) (45) is located in the core promoter region of the A2A-R gene (Fig. 6A). To determine whether CREB binds to the core promoter region of the A2A-R gene, we carried out combined EMSA and Western analyses. We first performed EMSA using four A2A-R core promoter probes that contain wild type or distinct mutations in the CRE region (Table III). Mutations at only two residues (designated M2) was shown previously to retain the binding activity of CREB, whereas the M6 mutant completely lost its CREB binding activity (46). The proteins-DNA complexes were first isolated from non-denaturing gels in the EMSA and then were subjected to separation by SDS-PAGE, followed by Western blot analyses. The presence of CREB and a CREB-binding protein (CBP) in the protein complex bound to the wild type and M2 core promoters of the A2A-R gene is clearly demonstrated in Fig. 6B. In contrast, CREB and CBP were not detected in the proteins-M4 and proteins-M6 complexes. To verify the direct involvement of CREB in regulating the A2A-R promoter, an anti-CREB antibody or irrelevant serum (39) was incubated with nuclear extract collected from the PC12 cells expressing Htt-(Q)25-hrGFP or Htt-(Q)109-hrGFP prior to incubation with a labeled A2A-R core promoter probe (A2A-R−250/−145) for the EMSA. As shown in Fig. 6C, adding the anti-CREB antibody, but not an irrelevant antibody (AC6D), created a supershift in the EMSA of control cells expressing Htt-(Q)25-hrGFP. In contrast, no supershift in the EMSA complex was found in cells expressing Htt-(Q)109-hrGFP, suggesting that CREB did not bind to the core promoter of the A2A-R gene in the presence of poly(Q)-expanded Htt. These results support the hypothesis that a functional CRE site is located in the core promoter region of the rat A2A-R gene and is involved in mutant Htt-evoked suppression of the A2A-R gene. Moreover, the anti-CREB antibody caused a supershift in the EMSA of cells expressing Htt-(Q)109-hrGFP treated with CGS (Fig. 6C), demonstrating that A2A-R stimulation restored CREB binding to the core promoter of the A2A-R gene, as well as by elevating the suppressed A2A-R promoter activity by poly(Q)-expanded Htt (Fig. 4A).

To further verify that the CRE site is important for A2A-R core promoter activity, we first performed EMSA using three A2A-R core promoter probes that contain distinct mutations in the CRE region (Table III). Mutations at only two residues (M2), which were shown not to affect the binding activity of CREB (46), did not change the EMSA profile (Fig. 7A). In contrast, the M6 mutant that lacks CREB binding activity (46) caused a downshift of the bands in the presence of 25Q-Htt (Fig. 7A). The EMSA pattern of the M4 mutant that harbors mutations at four residues was very similar to that of M6, suggesting that M4 is also a null CRE site. These findings are in line with the combined EMSA/Western analyses that showed that CREB and CBP did not bind to the M4 or M6 probes (Fig. 6B). Downshifts of the proteins-M4 and proteins-M6 complexes in the presence of 25Q-Htt reflect the loss of CREB/CBP binding to the core promoter of the A2A-R gene. Consistently, in the presence of 109Q-Htt, none of those CRE mutants exhibited altered EMSA profiles.
CREB did not bind the core promoter of the A2A-R gene in the presence of poly(Q)-expanded Htt.

Consistent with the observation based on the EMSAs of cells expressing Htt with the normal repeat number of poly(Q) (25), promoter constructs containing inactivated CRE sites (M4 or M6) exhibited markedly reduced promoter activities compared with those containing a functional CRE site (WT or M2; Fig. 7B). Binding of CREB therefore plays an important role in the basal promoter activity of the A2A-R gene. Although expression of 109Q-Htt caused a suppression of all A2A-R promoters tested, the inhibition was much less severe for the M4 and M6 promoters. The rescue effect of A2A-R stimulation on inhibition of the A2A-R promoter by 109Q-Htt was also significantly reduced in promoters containing null CRE sites (M4 or M6). Likewise, the marked enhancing effect of forskolin on wild type and M2 promoters in the presence of expanded poly(Q) was greatly reduced in the CRE-null mutant promoters (M4 and M6). The CRE site therefore was significantly involved in regulation of the A2A-R promoter by mutant Htt with expanded poly(Q). Note that in the presence of the null CRE site, 109Q-Htt caused a suppression of all A2A-R promoters tested, the inhibition was much less severe for the M4 and M6 promoters. The rescue effect of A2A-R stimulation on inhibition of the A2A-R promoter by 109Q-Htt was also significantly reduced in promoters containing null CRE sites (M4 or M6).

Because the A2A-R core promoter probe (105 bp, A2A-R-256/237) that harbors the wild type CRE site was 32P-labeled and incubated with nuclear extract (5 µg) prepared from nontransfected PC12 cells (PC) or transfected PC12 cells expressing (Q)25-Htt-hrGFP or (Q)109-Htt-hrGFP as indicated. Formation of DNA-protein complexes competed with the indicated double-stranded A2A-R-256-237 or the control 20-bp DNA (B-20) in a 50-fold molar excess. For the supershift analyses, NE (5 µg) prepared from the indicated cells was preincubated for 1 h with an anti-CREB or an irrelevant anti-AC6D (39) antibody, followed by incubation with the 32P-labeled 20-bp DNA probe for EMSA. PC, nontransfected PC12 cells. B, NE (5 µg) prepared from cells expressing (Q)25-Htt-hrGFP or (Q)109-Htt-hrGFP was reacted with a 20-bp 32P-labeled A2A-R-256/237 oligonucleotide probe containing the wild type (WT) or the mutated CRE site (M4 or M6) as indicated. Reaction products were separated in 6% polyacrylamide gels by electrophoresis. This is one representative result of three independent experiments performed. The arrowhead indicates the supershifted bands. Arrows mark the protein/A2A-R promoter complexes.

Figure 8. Expression of poly(Q)-expanded Htt interfered with the binding of CREB to the atypical CRE site of the A2A-R gene. PC12 cells were transfected with pcDNA3.1-(Q)25-Htt-hrGFP or pcDNA3.1-(Q)109-Htt-hrGFP. Seventy two hours post-transfection, GFP-positive cells were harvested by fluorescence-activated cell sorter to prepare the corresponding NE. A, double-stranded oligonucleotide probe (20 bp, A2A-R-256/237) that harbors the wild type CRE site was 32P-labeled and incubated with nuclear extract (5 µg) prepared from nontransfected PC12 cells (PC) or transfected PC12 cells expressing (Q)25-Htt-hrGFP or (Q)109-Htt-hrGFP as indicated. Formation of DNA-protein complexes competed with the indicated double-stranded A2A-R-256-237 or the control 20-bp DNA (B-20) in a 50-fold molar excess. For the supershift analyses, NE (5 µg) prepared from the indicated cells was preincubated for 1 h with an anti-CREB or an irrelevant anti-AC6D (39) antibody, followed by incubation with the 32P-labeled 20-bp DNA probe for EMSA. PC, nontransfected PC12 cells. B, NE (5 µg) prepared from cells expressing (Q)25-Htt-hrGFP or (Q)109-Htt-hrGFP was reacted with a 20-bp 32P-labeled A2A-R-256/237 oligonucleotide probe containing the wild type (WT) or the mutated CRE site (M4 or M6) as indicated. Reaction products were separated in 6% polyacrylamide gels by electrophoresis. This is one representative result of three independent experiments performed. The arrowhead indicates the supershifted bands. Arrows mark the protein/A2A-R promoter complexes.

Figure 9. Mutant Htt with expanded poly(Q) reduced the activation of CREB and the expression of CBP. PC12 cells were transfected with pcDNA3.1-(Q)25-Htt-hrGFP or pcDNA3.1-(Q)109-Htt-hrGFP for 72 h. GFP-positive cells were then harvested by fluorescence-activated cell sorter to prepare the corresponding nuclear extract. A, equal amounts of nuclear protein (15 µg) were loaded into each lane and analyzed by Western blotting using the corresponding antibody. B, an illustration is given of arbitrary values for the protein of interest normalized with the level of lamin from three independent experiments. **, specific comparison between cells transfected with pcDNA3.1-(Q)109-Htt-hrGFP and those with pcDNA3.1-(Q)25-Htt-hrGFP in the corresponding group. (p < 0.001; Student’s t test).

M6 mutants) to verify the importance of CREB. When the radiolabeled probe harboring the wild type CRE site was incubated with nuclear extracts from nontransfected PC12 cells or cells expressing Htt with 25Q, a prominent DNA-protein complex was observed. Such a complex disappeared in the presence
of an excess amount of the unlabeled wild type probe (A2A-R, 256–237), but not an irrelevant 20-bp probe (B–20; Fig. 8A). Adding the anti-CREB antibody, but not an irrelevant antibody (AC6D), created a supershift in the EMSA of cells expressing Htt-(Q)25-hrGFP. These data further support the hypothesis that CREB recognizes the atypical CRE site of the A2A-R core promoter. No DNA-protein complex was detected from cells expressing 109Q-Htt, demonstrating that CREB did not bind to the atypical CRE site in the presence of poly(Q)-expanded Htt (Fig. 8A). Moreover, no DNA-protein complex was found when the DNA probe contained a mutated CRE site (M4 or M6; Fig. 8B). Collectively, the above EMSA analyses using oligonucleotide probes containing only the atypical CRE site strengthen our hypothesis that binding of CREB to this atypical CRE site was altered by the expression of poly(Q)-expanded Htt and might be involved in mutant Htt-evoked suppression of the A2A-R gene.

We next determined whether the lack of CREB binding and the suppression of A2A-R promoter activity by poly(Q)-expanded Htt were because of lower amounts of soluble CREB and its cofactor (CBP) using Western blot analyses. As shown in Fig. 9, the levels of soluble CREB in cells expressing 25Q-Htt or 109Q-Htt were very similar. The lack of CREB binding to the A2A-R promoter therefore might not have been due to the sequestration of CREB by Htt aggregates but rather might have been caused by conformational inactivation as reported for other transcription factors by toxic Htt (47). Most interestingly, the amounts of activated CREB, determined by assessing the phosphorylation level at Ser133, and the levels of CBP were reduced in cells expressing poly(Q)-expanded Htt. Both deficiencies should contribute to suppression of the A2A-R gene by poly(Q)-expanded Htt.

Alterations in the overall gene expression profiles have been reported in several poly(Q) disease models, including those of HD. Previous studies have suggested that poly(Q)-expanded mutant Htt might disrupt the transcription machinery through sequestration or direct inactivation of important transcription factors (3, 6, 12, 47, 48). Among them, the role of CREB has been suggested to be responsible for one of the earliest dysfunctional events at the transcription level in HD (44). Moreover, disruption of CREB has been shown to cause progressive striatal degeneration similar to that found in HD (49). A reduced level of CBP because of enhanced ubiquitination and degradation of CBP has also been implicated in the suppression of CREB-mediated transcription in poly(Q)-associated neurodegenerative diseases (47). Our findings that the levels of activated/phosphorylated CREB and CBP were reduced in the presence of poly(Q)-expanded Htt are consistent with those of previous studies, which support a critical role of CREB in HD. The most important observation was that stimulation of the PKA pathway, which activates CREB by phosphorylation, effectively restored the ability of CREB to bind to the A2A-R core promoter and to mediate the proper promoter activity of the A2A-R gene in the presence of toxic poly(Q)-expanded Htt. Phosphorylation of CREB might reactivate its conformation to allow the proper binding to an atypical CRE site on the A2A-R promoter. Our finding that mutant Htt did not affect the level of CREB is in agreement with a recent report using PC12 cells (44). Nevertheless, in contrast to our findings (Figs. 7 and 9), those authors reported that mutant Htt does not alter the binding activity of CREB. Such a discrepancy appears to have resulted from the distinct CRE sites involved. According to Sugars et al. (44), the binding ability of CREB was assessed based on a consensus CRE site (5′-TGACGTCA-3′), whereas the CRE site (5′-TCCAGT-3′) at the A2A-R core promoter studied here was atypical. Ionescu and colleagues (46) demonstrated that this atypical CRE site binds CREB less strongly than does the consensus CRE site. Mutant Htt might functionally destabilize CREB, as has been reported for the TATA box-binding protein (47), thus causing reduced binding affinity toward atypical CRE sites but not to the consensus CRE site. Note that this atypical CRE site varies slightly from another atypical CRE site (5′-TCCAGT-3′) previously found to be functional in at least four other promoters (50–52), suggesting that expression of these genes might also be affected in HD.

The A2A-R has been shown to exert a protective effect upon a wide variety of traumas (23–25, 27), and both agonists and antagonists of the A2A-R have been implicated in neuronal degenerative diseases (27, 29, 53). In the present study, we identify an atypical CRE site located in the core promoter of the A2A-R gene that mediates the suppression of the A2A-R gene by poly(Q)-expanded Htt. Several lines of evidence suggest that Htt with expanded poly(Q) prevents the binding of CREB to this atypical CRE site and markedly reduces the expression of the A2A-R in HD. Concurrently, stimulation of the A2A-R reduced Htt aggregation (Table I) and restored its own gene expression by a PKA/CREB-mediated pathway. It is possible that stimulation of various receptors (particularly those in the G protein-coupled receptor superfamily) that enhance the PKA/CREB pathway might normalize the transcriptional dysfunction of the CREB-mediated pathway evoked by toxic Htt. Results of the present study provide important insights into both the understanding of gene regulation of the A2A-R and the potential usage of cAMP-elevating drugs in HD.

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