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CREB-binding protein sequestration by expanded polyglutamine

Alexander McCampbell+, J. Paul Taylor, Addis A. Taye, Jon Robitschek, Mei Li[1], Jessica Walcott[2], Diane Merry[2], Yaohui Chai[3], Henry Paulson[3], Gen Sobue[1] and Kenneth H. Fischbeck

Neurogenetics Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, 10 Center Drive, Building 10, Room 3B11, Bethesda, MD 20892-1250, USA, [1]Department of Neurology, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi, Japan, [2]208 Blumle Life Science Building, Thomas Jefferson University, 233 South 10th Street, Philadelphia, PA 19107, USA and [3]Department of Neurology, University of Iowa College of Medicine, 2007 RCP, Iowa City, IA 52242, USA

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Spinal and bulbar muscular atrophy (SBMA) is one of eight inherited neurodegenerative diseases known to be caused by CAG repeat expansion. The expansion results in an expanded polyglutamine tract, which likely confers a novel, toxic function to the affected protein. Cell culture and transgenic mouse studies have implicated the nucleus as a site for pathogenesis, suggesting that a critical nuclear factor or process is disrupted by the polyglutamine expansion. In this report we present evidence that CREB-binding protein (CBP), a transcriptional co-activator that orchestrates nuclear response to a variety of cell signaling cascades, is incorporated into nuclear inclusions formed by polyglutamine-containing proteins in cultured cells, transgenic mice and tissue from patients with SBMA. We also show CBP incorporation into nuclear inclusions formed in a cell culture model of another polyglutamine disease, spinocerebellar ataxia type 3. We present evidence that soluble levels of CBP are reduced in cells expressing expanded polyglutamine despite increased levels of CBP mRNA. Finally, we demonstrate that over-expression of CBP rescues cells from polyglutamine-mediated toxicity in neuronal cell culture. These data support a CBP-sequestration model of polyglutamine expansion disease.

INTRODUCTION

The polyglutamine diseases are progressive neurodegenerative disorders caused by expansion of trinucleotide (CAG) repeats, encoding polyglutamine tracts, which confer a novel toxic function to the mutant protein. There are at least eight polyglutamine diseases, including spinal and bulbar muscular atrophy (SBMA), which is caused by a CAG expansion in exon 1 of the androgen receptor (AR) gene (1). Expression of truncated AR constructs coding for the expanded polyglutamine tract recapitulates the toxicity in transgenic mice and in cultured cells (2–4). Nuclear inclusions, a pathologic feature of the polyglutamine diseases, can be detected in these models. The role of the nuclear inclusions in the mechanism of the polyglutamine diseases is controversial, but evidence from cellular and transgenic animal models indicates that nuclear localization of the mutant protein is important in toxicity (5,6).

A possible consequence of nuclear accumulation of mutant protein is the sequestration of an important low-abundance nuclear factor such as CREB-binding protein (CBP). CBP and the related protein, p300, are transcriptional co-activators that interact with multiple cell signaling factors, including AP-1 and the nuclear hormone receptors as well as CREB (7, reviewed in ref. 8). These pathways compete for functionally limiting quantities of CBP and, whereas p300 and CBP have many overlapping functions, heterozygous loss of either gene is sufficient to cause severe developmental deficits (9,10).

Biochemical and functional tests show that CBP interacts with the normal AR (11,12). Glutathione-*S*-transferase pull-down experiments mapped the interaction to both the N- and C-terminal regions of AR (11). Immunoprecipitation of CBP also precipitates AR, and expression of CBP enhances AR-mediated transcription (12). Furthermore, CBP itself contains a polyglutamine tract, which may enhance its interaction with AR and other expanded polyglutamine-containing proteins (13,14). Given the critical role of CBP in cell function, its relatively low abundance, and interaction with the polyglutamine-containing region of AR, we hypothesized that CBP is sequestered to AR-positive nuclear inclusions, and that this sequestration is toxic to the cells. We present evidence that CBP is recruited to polyglutamine inclusions, resulting in a decrease of soluble CBP and CBP-dependent transcription. Furthermore, polyglutamine-induced toxicity is blocked by over-expression of CBP.

+To whom correspondence should be addressed. Tel: +1 301 435 9288; Fax: +1 301 480 3365; Email: mccampba@ninds.nih.gov

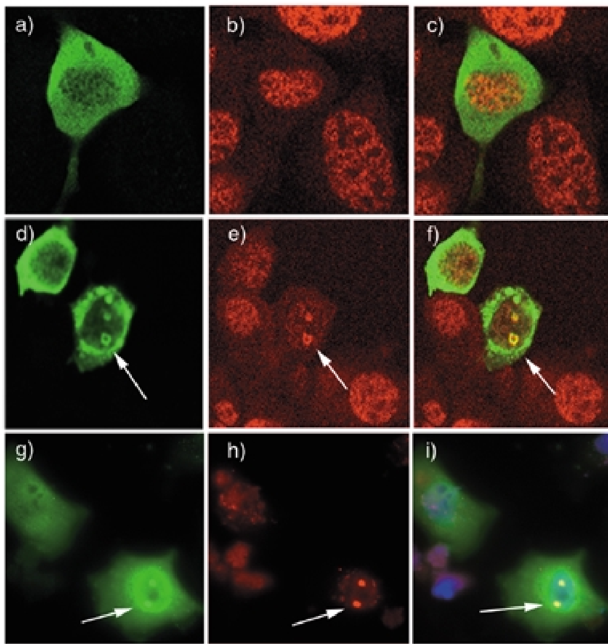

Figure 1. AR, ataxin-3 and CBP co-localization in nuclear inclusions in transfected cells (arrows). (**a**–**c**) MN-1 cells were transfected with a truncated normal repeat-length construct (AR16Δ) and labeled with both anti-AR (FITC) (a) and anti-CBP (Cy5) antibodies (b). Normal repeat AR is seen throughout the cytoplasm, and CBP is nuclear. (c) The stains superimposed. (**d**–**f**) MN-1 cells transfected with truncated expanded repeat AR (AR112Δ) and stained for AR and CBP. Arrows indicate nuclear inclusions with signal for both AR (d) and CBP (e). The redistribution of CBP depletes the background Cy5 signal in the inclusion-bearing nuclei, consistent with CBP sequestration. The lack of cytoplasmic CBP stain indicates that the antibodies do not cross-react. (**g**–**i**) HeLa cells expressing full-length ataxin-3 with 78 glutamines stained for ataxin-3 and CBP. CBP distribution is pan-nuclear in HeLa cells that do not express ataxin-3. CBP signal (h) redistributes to ataxin-3-positive inclusions (g). In each case, the cells were cultured for 48 h after transfection.

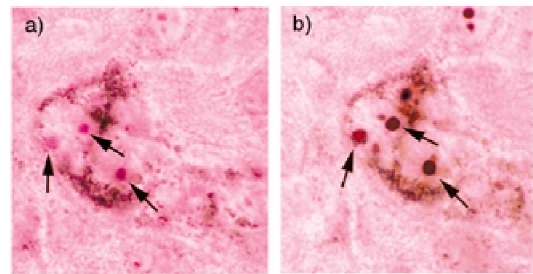

Figure 2. CBP localization in nuclear inclusions in patient tissue (arrows). Frozen sections of human scrotal tissue were labeled first with CBP-fast red (**a**) and then with AR-DAB (**b**) to demonstrate the co-localization of AR and CBP. Scrotal tissue from SBMA patients has been previously shown to have abundant nuclear inclusions (15). In control tissue, CBP staining was not observed, suggesting that it is too diffuse to be detected normally. CBP-positive, AR-negative inclusions were also found, confirming that there is no antibody cross-reaction.

RESULTS

CBP co-localization with polyglutamine inclusions

To examine the effect of polyglutamine on endogenous CBP distribution, we transfected truncated forms of AR with normal and expanded polyglutamine tracts into motor neuron–neuroblastoma hybrid (MN-1) cells. These cells have been found to form nuclear inclusions when expressing mutant AR, and they exhibit repeat length-dependent cell death (3). In this study, AR and CBP distribution was examined by co-immunofluorescence. Transfection with a truncated normal repeat length construct (AR16Δ) yielded diffuse cytoplasmic AR expression, leaving endogenous CBP evenly distributed in the nucleus, as in non-transfected cells (Fig. 1a–c). In contrast, the product of a truncated expanded repeat construct (AR112Δ) was found both diffusely in the cytoplasm and as inclusion bodies in the nucleus (Fig. 1d). In cells with AR-positive nuclear inclusions, CBP was redistributed to the intranuclear inclusions. The stains completely overlapped when the cells were viewed by traditional fluorescent light microscopy (data not shown). When observed by confocal microscopy, the CBP appeared as a rim around the inclusion bodies (Fig. 1f), suggesting either that CBP was coating the surface of the inclusion or that the antibody could not penetrate the interior of the inclusion. Furthermore, it appeared that much of the CBP was recruited to the inclusions, as the remaining CBP stain in the nucleus was reduced to background levels. Nearly all the AR-positive nuclear inclusions were CBP positive, although no CBP staining was observed in the cytoplasm, confirming that the stain was specific to CBP and not caused by an interaction between the CBP and AR antibodies.

CBP sequestration was not limited to inclusions formed by mutant AR. In a similar experiment, HeLa cells were transfected with either normal or mutant full-length ataxin-3, the disease gene product in spinocerebellar ataxia 3. Normal ataxin-3 was found diffusely in the cytoplasm and nucleus, whereas mutant ataxin-3 formed nuclear inclusions (Fig. 1g). In cells with nuclear inclusions, CBP was again redistributed from a pan-nuclear pattern to concentration within the ataxin-3 inclusions (Fig. 1g–i). Similar results were obtained in PC12 cells transfected with full-length ataxin-3 (data not shown).

We extended these results by looking at CBP distribution in transgenic mice expressing AR112Δ driven by the prion protein promoter (4). Mice expressing AR112Δ have intranuclear inclusions in neurons throughout the brain. CBP- and AR-positive inclusions were observed in adjacent sections (data not shown). Co-immunofluorescent staining showed that about half (120/223) of the AR-positive inclusions were also CBP positive.

CBP distribution was also examined in SBMA patient tissue. Scrotal skin samples were used because of the abundance of nuclear aggregates found in this tissue (15). CBP and AR staining in frozen sections showed that CBP co-localizes with AR-positive inclusions in the keratinocytes (Fig. 2). Again, the rate of co-localization was high, with 58 of 120 AR-positive inclusions staining positively for CBP. In addition, there were CBP-positive inclusions that did not stain for AR. This may have been due to the AR epitope being obscured by the inclusion or to low frequency detection of other CBP-containing nuclear bodies.

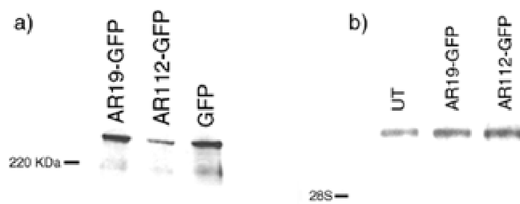

Figure 3. Soluble CBP protein levels are decreased in polyglutamine-expressing cells, whereas the transcript is modestly increased. (**a**) MN-1 cells were transfected with vector alone (GFP), normal repeat AR (AR19–GFP) or mutant AR (AR112–GFP) and cultured for 48 h. CBP in AR112–GFP-expressing cells was decreased relative to those expressing AR19–GFP or GFP alone in three of four experiments. (**b**) CBP transcript levels in cells expressing different forms of AR. HEK-293 cells were transfected with either normal (AR19–GFP) or mutant (AR112–GFP) AR. At 52 h post-transfection, CBP levels are marginally higher in cells expressing AR112–GFP than in untransfected (UT) and AR19–GFP-transfected cells. Signal strength is increased by 40%. This marginal increase indicates that the loss of soluble CBP protein is not due to decreased transcription.

Soluble CBP is decreased in AR112 cells

To confirm the observation that much of CBP is redistributed to the inclusions, we assayed soluble CBP protein levels. In order to enhance endogenous CBP signal strength, western blotting was preceded by immunoprecipitation. CBP levels were markedly lower in cells expressing a truncated, expanded repeat length construct tagged with green fluorescent protein (GFP) (AR112–GFP) (Fig. 3a). To determine whether the decrease in protein was caused by decreased production of CBP, the CBP transcript levels were examined. We found that CBP transcription was slightly up-regulated (40%) in the presence of AR112–GFP (Fig. 3b) compared with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript levels (data not shown). This mild increase indicates that the cellular response to CBP depletion was limited, and that most of the soluble CBP was recruited to the inclusions.

CBP-dependent transcription is decreased

Since CBP is an important co-activator for transcription, CBP function was assayed by measuring the ability of a Gal4–CBP fusion construct to drive transcription from a reporter construct (G5–luc) with Gal4 response elements driving expression of the firefly luciferase gene. Transfection of these constructs resulted in high levels of luciferase activity. In the presence of vector alone [cytomegalovirus (CMV)–GFP] or a truncated normal repeat length construct (AR19–GFP), luciferase was reduced to 30% of the activity found in cells with Gal4–CBP and G5–luc alone (8- to 10-fold over background). We attribute this decrease to elements in the CMV promoter. AR112–GFP further reduced luciferase activity to near background levels (Fig. 4a), indicating that the Gal4–CBP was sequestered and thus not available to drive transcription.

CBP blocks polyglutamine induced toxicity

Lastly, to determine whether the sequestration of CBP was responsible for cellular toxicity, we measured the effect of CBP over-expression in cells transfected with polyglutamine-containing AR constructs. As reported previously (3), cells expressing expanded polyglutamine died over 48–72 h (Fig. 4b). Cells over-expressing exogenous CBP showed enhanced viability, and the toxic effect of the expanded polyglutamine was blocked (Fig. 4c). CBP failed to rescue cells co-transfected with constructs for the pro-apoptotic factor bax (data not shown), indicating that the CBP was not simply having a non-specific effect on cellular toxicity.

DISCUSSION

We have found that CBP, normally present in a diffuse nuclear pattern, is sequestered in nuclear inclusions formed in cells expressing expanded polyglutamine proteins. This sequestration is found not only in cultured cells, but also in transgenic mice and patient tissue, and is common to at least two of the polyglutamine disorders. The ability of over-expressed CBP to block polyglutamine-induced toxicity in cell culture suggests that this sequestration may be an important step in the disease pathogenesis.

CBP as a general target in polyglutamine disease

CBP is a particularly good candidate gene for disruption, as it is present at functionally limiting levels in the cell. Mice lacking one allele of the CBP gene have pronounced developmental defects, despite the presence of the second allele and two copies of the gene for p300, a protein thought to be largely redundant with CBP. Furthermore, ample evidence indicates that different cell signaling pathways compete for use of CBP in driving different transcriptional programs that influence cell proliferation and survival. Our finding that over-expression of CBP blocks polyglutamine-induced toxicity in these cells suggests that the toxicity is due to CBP depletion.

Recently, CBP was shown to co-localize with mutant huntingtin in cells co-transfected with plasmids for both genes (14). The recruitment of CBP to the inclusions in this study was dependent on the polyglutamine domain of CBP. This domain is in the C-terminal region of the protein and consists of 18 glutamines in humans and 15 in mice. Furthermore, CBP has been found in nuclear inclusions formed in mice transgenic for exon 1 of huntingtin (16). Importantly, the CBP antibodies used in both studies were raised against an epitope that does not include the polyglutamine repeat. Finding that CBP binds to huntingtin-positive inclusions, together with our evidence that it binds to AR- and ataxin-3-positive inclusions, indicates that CBP sequestration may be a general phenomenon of polyglutamine expansion diseases.

CBP and nuclear inclusions

We have used nuclear inclusions as a marker for pathological changes in the interactions of CBP; however, it is not necessary that nuclear inclusions directly cause toxicity for the CBP sequestration or any other sequestration model to be valid. Instead, redistribution of CBP to inclusions, and the concomitant decrease in CBP-dependent transcription, only show that polyglutamine-containing proteins bind CBP sufficiently to suppress CBP-dependent processes. The sequestration model is dependent only on decreased turnover of polyglutamine-containing proteins in the nucleus, as could result from the formation of nuclear inclusions or from impaired proteasome-mediated degradation. In this regard, our

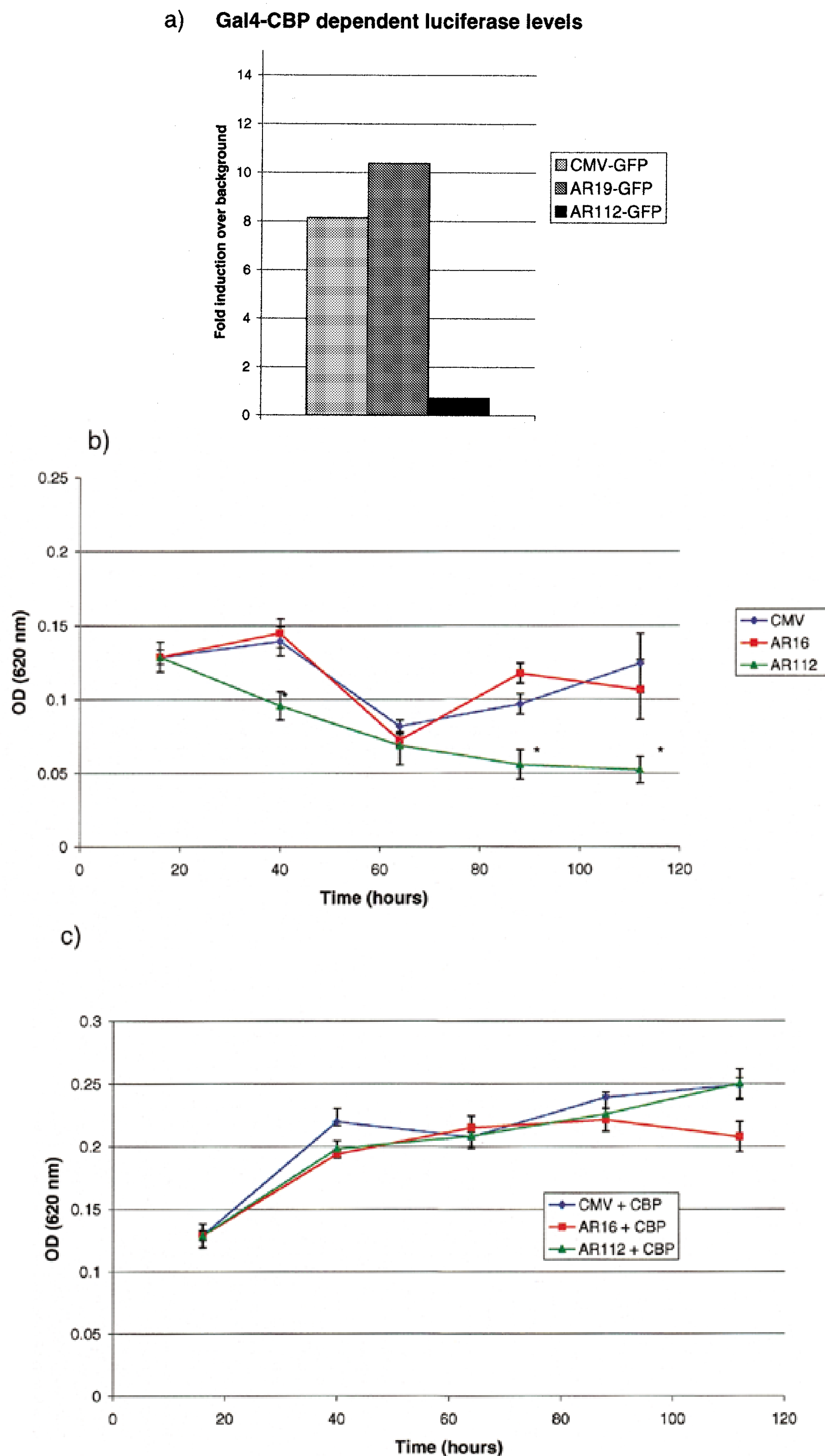

Figure 4. Functional disruption of Gal4–CBP and rescue of polyglutamine-induced toxicity. (**a**) Polyglutamine-induced decrease of Gal4–CBP-mediated transcription. MN-1 cells were co-transfected with Gal4–CBP fusion constructs and G5–luciferase reporter construct. In addition, cells were transfected with vector alone (CMV–GFP), normal repeat length AR (AR19–GFP) or mutant AR (AR112–GFP). Values are expressed as fold induction of activity over background. The results represent three separate transfections with two readings per condition. Although the CMV promoter alone suppresses CBP-mediated transcription, AR112–GFP further reduces the luciferase activity to near baseline levels. (**b**) Length-dependent polyglutamine-induced toxicity. HEK-293T cells were transfected with combinations of plasmid alone (CMV), normal repeat length AR (AR16) or mutant AR (AR112). Viability was measured by MTT reduction, so that lower absorbance corresponds to a lower number of viable cells. At 40, 88 and 112 h, AR112-transfected cells had significantly lower viability than controls ($P < 0.05$). (**c**) Co-transfection of CBP blocks polyglutamine-induced toxicity. HEK-293T cells were transfected as above, with pCDNA3.1-CBP (27) added to each transfection. All three conditions with CBP survived better than those with vector alone. Immunofluorescence of these cells showed co-localization of CBP and AR112, as in MN-1 cells. Similar results were obtained with direct counts of transfected cells. There was no significant difference among the three different CBP co-transfection experiments.

data are supported by recent evidence showing that disruption of ataxin-1 ubiquitination, a necessary step in targeting proteins for degradation, enhances toxicity in mice transgenic for mutant ataxin-1 (17).

CBP and transcription

Whereas CBP co-localizes with AR-positive inclusions in nearly 100% of the nuclear inclusions formed in cell culture, it is only found in ~50% of the nuclear inclusions in the mouse and patient tissue. The percent overlap is probably an underestimate, in particular in the patient tissue, as CBP-positive, AR-negative inclusions were also observed.

There are several possible explanations for the CBP-positive, AR-negative inclusions. The AR epitope may be masked, or sequestered in the interior of the inclusion. This is supported by our observation that the inclusions stain as ring-like structures in tissue culture. Another possibility is that the CBP-positive inclusions are altered promyelocytic (PML) oncogenic domains.

In addition to CBP, other components of the transcription apparatus interact specifically with polyglutamine-containing proteins. PML, nuclear co-repressor (NCoR), steroid receptor co-activator and glucocorticoid receptor interacting protein 1 have all been shown to differentially interact with expanded polyglutamine-containing proteins (18–20). Each of these proteins (including CBP) influences chromatin condensation by altering histone acetylation (21). Decreases in histone acetylation might result in toxicity by broadly disrupting transcriptional regulation.

In contrast to the other interacting factors, CBP has been implicated in mediating signals critical for neuronal survival. CREB, whose transcriptional activity is mediated by CBP, has been identified as a critical component of neuronal responses to neurotrophins (22,23). CBP has been directly linked to neuronal responses to nerve growth factor (NGF) in studies showing up-regulation in CBP-dependent transcriptional activity in the presence of NGF (24). One possible mechanism for polyglutamine disease is that as CBP is sequestered, neuronal response to trophic factors essential for survival is diminished. Our data showing a decrease in soluble CBP in the presence of expanded polyglutamine and the ability of exogenous CBP to rescue cells from polyglutamine-induced toxicity indicate a central role for CBP in the pathogenesis of polyglutamine-expansion diseases.

MATERIALS AND METHODS

Co-localization of CBP with AR

MN-1 cells were grown as described (25) and transfected with previously reported AR constructs (3), using the GenePorter reagent (Gene Therapy Systems, San Diego, CA). AR was visualized by immunofluorescence, using N-20 (Santa Cruz Biotechnology, Santa Cruz, CA), a rabbit polyclonal antibody directed against the first 20 amino acids, and fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA). Endogenous CBP was visualized with C-1 (Santa Cruz), a mouse monoclonal antibody directed against the last 12 amino acids, and Cy5-conjugated donkey anti-mouse secondary antibody (Jackson ImmunoResearch). Images were taken at the Confocal Imaging Facility of the National Institute of Neurological Disorders and Stroke.

Full-length ataxin-3, expressed in transiently transfected HeLa cells, was stained with an anti-ataxin-3 rabbit polyclonal antibody (26) and visualized with FITC-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch). In these experiments, the secondary antibody for C-1 was tetramethyl rhodamine isothiocyanate conjugated. Images were taken with traditional fluorescent microscopy at the University of Iowa.

The transgenic mice were generated with the prion promoter driving expression of the same AR constructs as used in the cell culture experiments (4). AR was stained with the N-20 antibody and horseradish peroxidase-conjugated secondary antibody. Adjacent sections were stained for CBP with C-1. Samples were viewed under Nomarski optics.

Immunohistochemistry of AR and CBP in human tissue followed standard procedures. C-1 antibody was applied to a 4 μm thick frozen section of the scrotal skin of an SBMA patient, incubated overnight and visualized with fast red. After photography, the slide was stripped with 0.3% H_2O_2 in methanol, then incubated overnight with the N-20 antibody and visualized with diaminobenzidene.

Gal4–CBP-mediated transcription

CBP depletion was measured in HEK-293 cells by assaying the Gal4-mediated transcriptional activity of a Gal4–CBP fusion protein. The cells were co-transfected with Gal4–CBP (a gift from Azad Bonni, Harvard University), G5-firefly luciferase reporter plasmid (Stratagene, La Jolla, CA) and a plasmid encoding sea pansy luciferase (Promega, Madison, WI). The sea pansy luciferase was used as a control for transfection efficiency and cell viability. In addition, the cells were transfected with constructs for vector alone (CMV–GFP), normal polyglutamine (AR19–GFP) or expanded polyglutamine (AR112–GFP). These constructs were derived from previously described AR cDNA clones (3), by subcloning an *Xba*I–*Eco*RI fragment into the N1–GFP vector (Clontech, Palo Alto, CA). After 48 h, cells were harvested and lysed. Addition of substrate permitted both firefly and sea pansy luciferase activity to be measured as relative light units, and the firefly luciferase activity was normalized to sea pansy activity.

CBP transcript and protein levels

CBP transcript levels were determined by northern blot. RNA was harvested from HEK-293 cells transfected with AR19–GFP or AR112–GFP using the RNeasy kit (Qiagen, Valencia, CA). The samples were run on a denaturing gel, then transferred overnight to a ZetaProbe GT blotting membrane (Bio-Rad, Hercules, CA). The probe for CBP was generated by cutting pcDNA3.1-CBP (27) with *Eco*RI–*Bam*HI and labeling with the RediPrime kit (Amersham Pharmacia, Indianapolis, IN). After washing, the membrane was exposed to a phosphoimaging plate for 12 h. The plate was scanned with a Molecular Dynamics Storm 860 imaging system, and the bands were quantified using the ImageQuant v1.2 software package (Molecular Dynamics, Sunnyvale, CA). The membrane was stripped and probed for the GAPDH transcript to confirm equal loading and transfer.

CBP protein levels were measured by immunoprecipitating CBP from MN-1 cell lysates using the anti-CBP-CT antibody from Upstate Biotechnology (Lake Placid, NY). The immunoprecipitation was performed by the manufacturer's protocol. Following immunoprecipitation, samples were run on 7.5% SDS–polyacrylamide gel. CBP was detected using anti-mouse CBP-CT, with horseradish peroxidase-conjugated donkey anti-mouse secondary. Bands were visualized by enhanced chemiluminescence (NEN Life Science Products, Boston, MA). Protein levels were normalized before immunoprecipitation by DC assay (Bio-Rad), and prominent background bands were compared after development to insure comparable loading levels.

Cell viability assay

Cell viability was measured by reduction of the tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma, St Louis, MO)]. Briefly, HEK-293 cells were transfected with truncated AR constructs, including the polyglutamine repeat, with and without full-length CBP (27), and plated in 96-well plates. MTT (5 mg/ml) was added at 24, 48 and 72 h, and the cells were incubated for 2 h. The resultant crystals were dissolved in a solution of 20% SDS in 50% dimethyl formamide. The optical density was measured at 570 nm and normalized to controls transfected with vector alone.

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