Chapter-II

Transcriptional dysregulation of genes important for neuronal function and the downstream effects in a cellular model of SCA17
2.1 Introduction

Expansion of CAG repeats in protein coding regions of genes, resulting in expanded polyglutamine stretches in proteins, is associated with nine neurodegenerative disorders (Table 1, Chapter-I). Though the mechanistic basis of trinucleotide repeat expansion has been studied extensively, the molecular effects that lead to neurodegeneration have remained elusive, in spite of common features of these disorders implying a shared mechanism (Cummings CJ et al., 2000). Considerable efforts have been made into studying the consequence of pathogenic proteins in several polyglutamine neurodegenerative diseases, by over-expressing the pathogenic allele in cell culture, creating transgenic animal models expressing the pathogenic allele or by using fly models for identifying genetic modifiers. However, limited knowledge about the normal functions of these proteins has prevented studies into the loss/gain of function due to Trinucleotide Repeat expansion in them. For instance, Huntington’s disease, the most common of the neurodegenerative disorders caused by the expansion of a CAG repeat in the coding region of the Htt protein has been studied extensively, to establish the mechanism of neurodegeneration and for developing therapeutic applications (reviewed in McMurray CT et al, 2001). However, the normal role of the ubiquitously expressed, large multi-domain huntingtin protein is not understood yet.

Spinocerebellar Ataxia 17, is caused by the expansion of a polyglutamine repeat in the poorly conserved, structurally uncharacterized N-terminal half of the human TATA binding protein (TBP). TBP is a well studied general transcription factor required for transcriptional initiation at a majority of eukaryotic promoters, irrespective of the RNA polymerase (I, II or III) that transcribes the gene. TBP, in association with other TBP associated factors (TAFs) binds to the core promoter element, TATA box (TAT/AAAT) upstream of transcription start site and initiates transcription by allowing assembly of pre-initiation complex (Hampsey M, 1998; Gill G et al., 1992). At promoters which lack the TATA element (TATA less promoters) TBP associates with other proteins which interact with DNA elements. The length of the polyglutamine stretch ranges from 29 to 42 in normal individuals whereas in patients they maybe as high as 63 (Koide R et al., 1999; Nakamura K et al., 2001; Zuhlke C et al., 2001). The disease is characterized by neurodegeneration in specific regions of
Transcriptional dysregulation of genes and downstream effects in a cellular model of SCA17

the brain presumably resulting in the clinical symptoms like morphological level and gait disturbance, tremor and dementia. Three lines of evidence have led to the proposal that TBP may play a more general role in a common mechanism for trinucleotide repeat expansion mediated neurodegeneration (van Room-Mom WM et al., 2005; Luthi-Carter R et al., 2002). Firstly, TBP has been detected in intranuclear protein aggregates in brain tissues from Huntington and Alzheimer patients (van Room-Mom WM et al., 2005) suggesting that abberant localization of TBP to protein aggregates might be a common step in many neurodegenerative diseases. Secondly, huntingtin protein and TBP have been shown to interact directly (Schaffar G et al., 2004). Lastly, recent evidence, showing the association of expanded CAG repeats in TBP, with a clinical condition called huntington’s like 4 (HDL4) in a Caucasian family with Huntington like symptoms (Stevanin G et al., 2003) also supports the hypothesis that beyond its specific and causative role in SCA17, TBP might play a more general, mechanistic role in other protein aggregation mediated neurodegenerative disorders.

Out of a number of mechanisms proposed for neurodegeneration in trinucleotide repeat expansion disorders, transcriptional dysregulation and inappropriate response to unfolded protein accumulation have gained attention in recent years. Polyglutamine stretches are known to occur commonly in transcription factors. The transcriptional dysregulation hypothesis suggests that expansion of these polyglutamine stretches results in aberrant protein-protein interaction between these proteins. Such abberant protein-protein interaction could in theory result in loss of function by preventing the normal function of the transcription factors. Alternatively, it could lead to gain of function through inappropriate expression of downstream genes. The strongest evidence for transcriptional dysregulation comes from the observed interaction between the glutamine rich regions of Sp1 with Huntingtin protein harbouring polyglutamine expansion in brain tissue from Huntington patients (Dunah AW et al., 2002). Riley and Orr have recently highlighted the importance of transcriptional regulation in polyglutamine disorders and suggested that studying the role of TBP in neurons will help understand how mutations in ubiquitous transcription factors result in disease effects in a restricted set of neurons (Riley BE et al., 2006).

Another widely held hypothesis for the mechanistic basis of polyglutamine diseases is that the aggregation of proteins in neuronal cells triggers unfolded protein response,
Transcriptional dysregulation of genes and downstream effects in a cellular model of SCA17

probably initially as a protective response. Chaperones have been found in aggregate in several polyglutamine diseases and artificial expression of chaperones has been shown to have some protective effect (Jana NR et al., 2000; reviewed in Kobayashi Y and Sobue G, 2001; Kitamura A et al., 2006). However, with the chronic accumulation of the protein aggregates, the protective, unfolded protein response fails to compensate, eventually signaling neurodegeneration. The molecular evidence and basis for these hypotheses have emerged from studies on huntington’s and other polyQ diseases, but there has been no previous attempt to study the molecular events following polyQ expansion in TBP. TBP being a general transcription factor, with a general role in polyQ mediated neurodegenerative diseases, we rationalized, could be involved in transcriptional dysregulation in such diseases.

Here, we have explored the role of transcriptional dysregulation by expanded polyglutamine stretch containing TBP in SCA17 using a mouse neuronal cell culture based model. Previous studies in our laboratory have shown that mouse neuronal cells expressing a variant of human TBP harboring 59Q repeats accumulate intranuclear aggregates whereas a variant harbouring a 16Q stretch did not show any signs of aggregation (Pandey, N, Ph.D Thesis “Effect of polymorphic triplet repeat on protein structure and its implication in neurodegenerative diseases”, 2003). We used this model to carry out microarray experiments to identify transcriptional changes caused by the presence of the expanded polyglutamine containing TBP allele, as compared to cells expressing a short non-pathogenic polyQ allele (16Q) of TBP. We found that genes involved in localized neuronal translation, cytoplasmic beta-actin (Actb), eukaryotic elongation factor2 (Eef2) and eukaryotic elongation factor 1 (Eef1alpha 1); retrograde transport, p25 subunit of dynactin (Dctn5); survival related gene, Vdac1 and ubiquitin related gene, ubiquitin B (Ubb) were induced in cells with TBP containing expanded polyQ. On the other hand, unfolded protein response seemed to be unaffected. Chromatin Immunoprecipitation studies at a TATA containing and a TATA less promoter from the differentially expressed genes suggested that TBP occupancy was elevated in vivo. Overexpression of the mitochondria and ER associated voltage gated anion channel, Vdac1, has been shown to affect mitochondrial flux and trigger apoptosis in non-neuronal cellular models. In our studies, VDAC1 overexpression lead to elevated cytochrome c release, a marker of apoptosis, in mouse neuronal cells. We found increased cytochrome c release
and apoptotic cell death from the mitochondria in mouse neuronal cells expressing a variant of human TBP harboring abnormally expanded polyQ tract. Our results identify putative link between transcriptional dysregulation and cell death in trinucleotide repeat associated neuronal dysfunction.

2.2 Results

We used Neuro-2a cells transfected with 16Q and 59Q polyQ containing TBP alleles in fusion with GFP (Fig 2.1) to study the effect of polyQ length in TBP on transcription profile from promoters in their natural genomic context. Earlier study in our laboratory has substantiated the model by cytological observation of large intranuclear aggregates only in cells transfected with TBP variants carrying expanded polyQ (Fig 1.4, Chapter-I). We used cDNA microarrays to identify changes in gene expression between 16Q and 59Q expressing cells. It is pertinent to note that the mouse neuronal cells contain the indigenous mouse TBP which contains 13 glutamines in the corresponding region. Thus the model recapitulates the dominant negative model, with the simultaneous presence of normal and pathogenic alleles in the cell.
Fig 2.1: Neuro-2a cells transfected with vector (N3), 16QhTBP-GFP and 59QhTBP-GFP. Fluorescence field view: Left panel, Bright field: right panel. N3: transfected construct contains GFP; 16Q, 59Q: transfected construct contains GFP tagged hTBP with CAG repeat length 16 and 59 respectively. (Scale bar: 100um).
2.2.1 Gene expression profiling reveals specific changes in expression level of functionally important genes

We set out to identify both global changes in gene expression as well as specific changes in genes important in neuronal function or cell death survival. The quality of the cDNA data was not suitable for reliable interpretation of global gene expression changes. We carried out a more extensive study on global changes using oligonucleotide arrays and RNA samples from different time points which is described in detail in Chapter-III. However, consistent changes in functionally important genes were identifiable from the cDNA array experiments. We further validated these specific changes in selected functionally important genes.

A comparison of the microarray based transcription profile of cells transfected with vector and 59Q showed distinct differences. Survival related gene, voltage dependent anion channel (Vdac1) and ubiquitin related gene, ubiquitin B (Ubb) and genes involved in localized neuronal translation, cytoplasmic beta-actin (Actb), eukaryotic elongation factor2 (Eef2) and eukaryotic elongation factor 1 (Eef1alpha 1); retrograde transport, p25 subunit of dynactin (Dctn5), were amongst the genes that showed significant upregulation in 59Q transfected cells as compared to internally normalized expression levels in vector transfected controls (Fig. 2.2).
Fig 2.2: Up-regulation of Detn5, Vdac1, Ubb, Actb, Eef2 and Eef1a1 in 59QTBP-GFP transfected (59Q) Neuro-2a cells compared to vector (N3) transfected controls observed by cDNA-microarray analysis. X-axis depicts transcript name. Z-ratio of each transcript was plotted on Y axis. Error bar represents mean ± SEM of at least two independent microarray experiments.
2.2.2 Validation of findings of the microarray studies

The results from microarray experiments were confirmed using real-time PCR analysis (Fig. 2.3A) and northern blotting (Fig. 2.4). In comparison with vector transfected controls, 16Q-TBP transfected cells showed marginal elevation of the expression levels of all the genes tested. But the level of induction was however significantly lower than that in 59Q TBP transfected cells in all cases barring Eef1a1. The upregulation of genes in 16Q TBP may be a consequence of higher total levels of functional TBP resulting from expression of the endogenous as well as the transfected gene. Any indirect effect arising from differential transfection or expression of the transfected TBP in 16Q and 59Q constructs was ruled out by real-time PCR analysis and northern analysis of GFP transcript expressed in fusion with the transfected TBP alleles (Fig. 2.3B, Fig. 2.4A-C).
Figure 2.3: Real time PCR analysis of gene expression. (A) Differential gene expression of Ubb, Vdac1, Dctn5, Eef2, Eef1a1 and Actb in Neuro-2a cell line transfected with human TBP (hTBP) containing different lengths of polyglutamine. Mouse 18s rRNA was used as an endogenous reference. N3 was used as a calibrator for determining relative expression. (B) Expression levels of reporter (Gfp) in transfected Neuro-2a cells determined by Real time PCR: similar levels of expression of TBPGFP in 16Q and 59Q transfected cells. N3: transfected construct contains GFP; 16Q, 59Q: transfected construct contains GFP tagged hTBP with CAG repeat length 16 and 59 respectively. Data shown are the mean ± SEM of two to four independent experiments. (*, p<0.05, Student’s t-test).
Fig 2.4: Differential expression of Vdac1 gene, Dctn5 and Ubb in Neuro-2a cells transfected with N3, 16Q TBP GFP and 59Q TBP GFP. (A & C) Northern blot using Vdac1 (A), Dctn5 and Ubb as a probe (C), GFP probe was used to show expression levels of GFP and TBPGFP in transfected cells (A, C), 18s rRNA was used as a loading control (lower panels in A & C); (B, D & E) The intensity of Vdac1 (B), Dctn5 (D) and Ubb (E) band were quantified and each band intensity was normalised to the band of 18s rRNA. N3 band intensity was used for determining relative expression. N3: transfected construct contains GFP; 16Q, 59Q: transfected construct contains GFP tagged hTBP with CAG repeat length 16 and 59 respectively.
2.2.3 Effect of polyQ expansion on promoter localization of TBP

It has been shown that many gene specific transcription factors exert their effects by directly or indirectly interacting with TBP and stabilizing TBP-TATA box interaction at the proximal promoter region (Kuras L et al., 1999; Shen WC et al., 2003). Therefore we studied the effect of polyQ length on TBP occupancy at promoters of differentially expressed genes by chromatin immunoprecipitation (ChIP) using TBP specific N12 antibody. We used anti-TBP antibody rather than the 1C2 antibody against polyQ as 1C2 antibody interacts with polyQ stretches in other transcription factors also and anti-TBP antibody recognizes TBP expressed from the endogenous gene as well as transfected gene. Total TBP localization to promoters seems to be a better index of promoter occupancy since normal and expanded polyQ TBP co-exists in the cell and may form heterodimers.

Promoter occupancy of TBP was higher in 59Q TBP transfected cells compared to N3 and 16QTBP transfected cells at the Vdac1 and Actb promoters (Fig. 2.5). The Vdac1 gene was predicted to be under the control of a TATA-less promoter (Sampson MJ et al, 1997). The predicted Sp1 site in the Vdac1 gene was of special interest since aberrant Sp1 mediated interaction has earlier been implicated in Huntington’s disease mechanism (Dunah AW et al., 2002). TBP localization could not be demonstrated in spite of repeated attempts at amplifying the region spanning the predicted Sp1 site (Fig. 2.5C, lower). On the other hand, TBP occupancy at the sterol repressor element 1 (SRE1) site, further upstream was enhanced in the presence of expanded polyQ variants (Fig. 2.5C, upper). We examined the downstream cellular effects of the upregulation of Ubb in unfolded protein response and Vdac1 in apoptosis to correlate the changes in gene expression to neurodegeneration.
Transcriptional dysregulation of genes and downstream effects in a cellular model of SCA17

Fig 2.5: Chromatin immunoprecipitation (ChIP) analysis revealed promoter localization of TBP in transfected Neuro-2a cells. Promoter occupancy of TBP was higher in 59Q TBPGFP transfected cells as compared to vector (N3) and 16Q TBPGFP transfected cells. (A & B) schematic representation of regulatory sites in Vdac1 promoter (predicted (16)) (A) and Actb promoter (B); SRE1: Sterol repressor element 1, Sp1: specificity protein 1. Cells transfected with 16QTBP and 59Q TBPGFP were treated with 1% formaldehyde and chromatin prepared as described earlier (15). Anti TBP antibody (N-12, Santa cruz) was used for immuno-precipitation. PCR was performed from immunoprecipitates, total input chromatin (Input). Samples treated without antibody (No Ab) did not show any product after PCR. PCR product for SRE1 (upper), Sp1 (lower) (C) and Actb promoter (D) were resolved on agarose gel. (E & F) intensity of band for SRE1 (E) and Actb promoter (F) were quantified and normalized to input band. Data represented relative to the N3 transfected control. Data shown are mean ± SEM of three independent experiments performed. (*, p<0.01, Student’s t-test).
2.2.4 Unfolded protein response

The cell responds to aggregated protein accumulation in two ways: initially a corrective mechanism is adopted through the unfolded protein response/ER stress pathway, which induces chaperones and provides an environment for correct folding of the proteins and finally, ubiquitinylation and destruction of the malformed proteins are attempted. Ubb was amongst the genes with increased expression in cells expressing expanded polyQ containing TBP. We analyzed the Unfolded Protein Response pathway using xbp1-splicing assay as an indicator (Yoshida H et al., 2001). The ratio of the spliced and unspliced variants of xbp1 showed no alteration in cells expressing the different polyQ TBP alleles (Fig. 2.6).
Fig 2.6: Poly Q expanded TBP transfected cells do not show ER stress. ER stress was detected by reverse transcription (RT) reaction followed by PCR amplification of spliced (S) and unspliced Xbp1 (U). PCR products were resolved in 5% polyacrylamide gel, stained with vista green. (A) Unspliced and spliced Xbp1 PCR products detected in vector (N3), 16QTBP-GFP and 59QTBP-GFP transfected Neuro-2a cells (B) Vista green stained bands were quantified and ratio (spliced/unspliced) was calculated. Higher ratio in DTT treated positive control indicates ER stress. 59QTBP-GFP and 16QTBP-GFP transfected cells were showing similar ratio as observed in vector (N3) transfected control. Data represent mean ± SEM of two independent experiments.
2.2.5 Effect of VDAC1 over expression in neuronal cells

Recently, the voltage dependent anion channel, Vdac1, has been shown to localize to both the mitochondrial outer membrane and the cytoplasmic membrane (Shoshan-Barmatz V et al., 2004). A change in the expression level of Vdac1 has recently been shown to be a critical determinant of cell death in embryonic kidney cells (bu-Hamad S et al., 2006). Vdac1 plays an important role in transport of ATP, calcium ions and other metabolites across the mitochondrial membrane (Shoshan-Barmatz V et al., 2003). Calcium induced permeability transition and cytochrome c release from the mitochondria is enhanced in cellular models expressing the mutant huntingtin protein (Choo YS et al., 2004). Further, Ruan et al., observed that mutant huntingtin was unable to release cytochrome c in the cytosol and striatal cells undergo non-apoptotic death (Ruan Q et al., 2004). We first confirmed that the transcriptional upregulation of Vdac1 was associated with a similar increase in the expression level of the protein. As shown in Fig. 2.7, a specific antibody against VDAC1 was used to monitor the level of VDAC1 protein in cells transfected with a vector control, 16Q TBP or 59Q TBP. VDAC1 protein levels were induced by 30 percent in 59Q TBP containing cells. Further, we studied cytochrome c release from the mitochondria following increase in VDAC1 expression. The scheme for subcellular fractionation is presented in Fig 2.8A.
Fig 2.7: Expression of VDAC1 protein in Neuro-2a cells transfected with vector (N3), 16QTBPGFP and 59QTBPGFP. (A) Western blotting with VDAC1 specific antibody (N-18, Santa cruz) (upper panel), TBP specific antibody (N-12, Santa cruz) (middle panel). Equal sample loading was verified by staining the blot with Ponceau S. DNA was isolated from transfected cells (see materials & methods) and equivalent amount of plasmid transfection was checked by PCR amplification using GFP specific primers (Table1) (B) Immunoreactive band intensity was quantified and plotted. Data presented relative to the vector (N3) transfected control. Data shown are mean ± SEM of three independent experiments performed. (*, p<0.05, Student’s t-test).
Transcriptional dysregulation of genes and downstream effects in a cellular model of SCA17

Fig 2.8: Quantitative analysis of cytochrome c release into the cytosol of transfected Neuro-2a cells. (A) Scheme for isolation of cytosol from cultured cells; (B) Time dependent release of cytochrome c in the cytosol was measured by using a solid phase ELISA assay. Data represents mean ± SEM (n=2). (C) Apoptosis in 16Q TBPGFP and 59Q TBPGFP transfected cells were analyzed by counting annexinV-PE positive cells in a flow cytometer. Data represents mean ± SEM of four independent experiments performed. (*, p<0.01, Student’s t-test).
Cytochrome c release in the cytosolic fraction was monitored by ELISA. The cells expressing 59Q TBP showed a gradual increase in cytochrome c release resulting in substantially higher Cyt c levels at time points beyond 36 hours compared to cells which express the vector alone (Fig. 2.8B), in agreement with increased expression of VDAC1. We further monitored apoptotic cell death using flow cytometry of AnnexinV positive cells. Neuro-2a cells harbouring polyglutamine expanded TBP showed 48% apoptotic cells while control cells showed less than 30% apoptotic cells (Fig. 2.8C).

Previous study have shown that overexpression of VDAC1 can lead to apoptosis in non-neuronal cells (Zaid H et al., 2005). We confirmed a similar effect of VDAC1 in neuronal cells by cloning and overexpressing mouse VDAC1 (Fig. 2.9A-E). Cytochrome c release, a marker of apoptosis, was three fold higher in VDAC1 overexpressing cells (Fig. 2.9F) which indicates that elevated expression of VDAC1 led to neuronal apoptosis.
Fig 2.9: Over-expression of murine Vdac1 (mVdac1) in Neuro-2a cells induces apoptosis. (A-D) Neuro-2a cells transfected with pEGFPN3 vector alone (N3) (A & C) and mVdac1GFP (B & D) (A & B, Fluorescent field; C & D, Bright field view under 10X objective in an epifluorescence microscope (Nikon)). (Scale bar, 100um). (E) Expression of mVdac1Gfp in Neuro-2a cells was confirmed by Reverse transcription followed by PCR (RT-PCR) amplification from cDNA using a Vdac1 specific forward primer and GFP specific reverse primer (Upper panel). Expression of GFP was checked in N3 and mVdac1GFP transfected cells by RT-PCR using GFP specific forward and reverse primers. RT+: with reverse transcriptase, RT-: without reverse transcriptase. (F) Release of cytochrome c into cytosol in mVdac1 transfected cells was determined by using a solid phase ELISA method 65H after transfection. Data is presented relative to vector transfected control (N3). Data represents mean ± SEM of two independent experiments each in replicate (*, p<0.01, Student’s t-test)
2.3 Discussion

In this study we show that alleles of TBP harbouring abnormally expanded polyQ tracts not only form intranuclear aggregates in cultured mouse neuronal cells, but also result in overexpression of the voltage dependent anion channel, Vdac1 at both the RNA and protein level. VDAC can associate with small ions, control the passage of key adenine nucleotides, interact with other proteins like cellular kinases and regulate mitochondrial permeability (Shoshan-Barmatz V et al., 2004, Shoshan-Barmatz V et al., 2003). VDAC localizes to the outer mitochondrial membrane and associates with the Adenine nucleotide translocator in the inner mitochondrial membrane to form the permeability transition pore through which cytochrome c is released in apoptotic cells. Pro-apoptotic members of Bcl2 family of proteins like Bax and Bak are seen to promote Cyt c release by binding to and modulating Vdac action (Shimizu S et al., 1999). It has been reported previously that Vdac1 overexpression results apoptosis in non-neuronal cells (Zaid H et al., 2005). We independently confirmed that Vdac1 overexpression in mouse neuronal cells results in higher cytochrome c release, a marker of cellular apoptosis. In our studies the cells expressing 16Q TBP showed higher constant levels of cytosolic Cyt c compared to cells which express the vector alone. 59Q TBP cells, however, showed a significant difference in the cytochrome c release pattern. At early time points, cytosolic Cyt c levels are comparable to controls but with time they steadily rise and saturate at levels 30% higher than in 16Q TBP cells by 70 hours. Concurrently, 59Q TBP expressing cells showed a 20 % increase in number of apoptotic cells.

Besides, SCA17, caused by the expansion of polyQ tract in the TATA binding protein itself, normal TBP is also found in intranuclear protein aggregates formed in other neurodegenerative diseases, notably, huntington’s disease (van Room-Mom WM et al., 2005). Degeneration of cells in specific regions of the brain is a well known feature of polyQ mediated neurodegenerative disorders. Vdac1 overexpression, independently implicated in triggering cell death was one of the effects of polyglutamine expansion in TBP, identified in our study. Our results suggest a previously unknown mechanistic basis linking transcriptional dysregulation to cell death in neurodegeneration through TBP mediated alteration of Vdac1 expression.
The promoter of the Vdac1 is predicted to be devoid of a TATA box (Sampson MJ et al., 1997). TATA less promoters depend on TBP participation in the pre-initiation complex by localizing it to the promoter through other DNA binding proteins. Even at TATA box containing promoters, TBP binds inefficiently in the presence of chromatin and requires gene specific trans-activators (Kuras L et al., 1999). We find that TBP occupancy at the promoter of upregulated genes was elevated irrespective of the presence of the TATA box. Expansion of polyQ and formation of TBP containing protein aggregates suggest that TBP can cause enhanced expression of a downstream gene by titrating away a gene specific repressor. Alternatively, polyQ containing TBP can form heterodimers in solution with the endogenous functional TBP and alter its availability at various promoters. The structural or functional role of polyQ stretch within TBP has not been studied. The yeast homolog of TBP does not have the N-terminal part harboring the polyQ stretch while a gradual increase in the length of the polyQ stretch during evolution is observed. The conserved C-terminal domain of TBP is sufficient for binding to the TATA box and can be co-crystalized with it (Kim JL et al., 1993). Using an artificially tethered TBP with promoter-reporter fusions and at the genome-wide level it has been shown that many gene specific transcription factors exert their effects by directly or indirectly interacting with TBP and stabilizing TBP-TATA box interaction at the proximal promoter region (Kuras L et al., 1999; Shen WC et al., 2003). The interaction of expanded polyQ containing TBP with other factors in the eukaryotic transcription machinery will provide a more detailed picture of the transcriptional dysregulation.

VDAC action is known to be modulated by pharmacological agents. Besides synthetic and naturally occurring polyanions like spermine which increase the voltage dependence of VDAC channel, ruthenium red can reduce the voltage dependence of VDAC channel (Zaid H et al., 2005). The potent anti-depressant, Prozac (Fluoxetine) has earlier been shown to prevent apoptosis and enhance cell proliferation in the dentate gyrus. Fluoxetine has been shown to interact with the VDAC channel and modify its voltage dependence and conductance and in certain cell types can provide protection from apoptosis (Nahon E et al., 2005). TBP, being a general transcription factor is likely to have diverse, direct and indirect effects on gene expression and consequently in cellular function. These effects are unlikely to be mediated solely through Vdac1. Further investigations using chemical inhibitors or gene knock down
strategies to negate the elevated Vdac1 transcript level can delineate the contribution of Vdac1 to the phenotypic effects of polyglutamine expansion in TATA binding protein.

Apart from Vdac1, genes found to be differentially expressed in expanded polyQ-TBP containing neuronal cells in our study namely, the products of cytoplasmic beta-actin, eukaryotic elongation factor 2 and eukaryotic elongation factor 1-alpha 1 genes are known to interact with each other (Bektas M et al., 1994; Munshi R et al., 2001; Gross S R et al., 2005). The Dctn5 gene product is a newly characterized subunit of the dynactin complex, which plays an important role in retrograde transport (Eckley DM et al., 1999). Pathway analysis using Pathway studio software (Ariadne Genomics) and further study of literature (Table 2.1) shows that the differentially expressed genes are linked to each other and moreover closely linked to Vdac1 and p53 (Fig. 2.10). TBP and p53 have been shown to interact directly (Martin DW et al., 1993, JBC). In summary, this study offers a set of functionally related pathways that may be further explored for the link between transcriptional dysregulation and neurodegeneration.
Transcriptional dysregulation of genes and downstream effects in a cellular model of SCA17

Fig 2.10: Interacting network among the genes differentially expressed (bold) in our study. Functional relation among genes was generated by using pathway studio v 4.0 software (Ariadne Genomics) and Pubmed (NCBI). p53: tumor protein 53, p73: transformation related protein 73, BRCA1: breast cancer suppressor protein 1, Mot: mortalin, Bax: Bcl-2 associated X protein, MT: Microtubule.
Table 2.1: Interaction between any two gene product and reference (s) to support this interaction

<table>
<thead>
<tr>
<th>Relating partner</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eef2 and p53</td>
<td>(1)</td>
</tr>
<tr>
<td>Eef2 and Actin</td>
<td>(2)</td>
</tr>
<tr>
<td>Actin and Vdac1</td>
<td>(3)</td>
</tr>
<tr>
<td>Actin and p53</td>
<td>(4)</td>
</tr>
<tr>
<td>P53 and Eef1a1</td>
<td>(5)</td>
</tr>
<tr>
<td>Eef1a1 and Actin</td>
<td>(6, 7)</td>
</tr>
<tr>
<td>Actb and p73</td>
<td>(8)</td>
</tr>
<tr>
<td>P73 and p53</td>
<td>(9)</td>
</tr>
<tr>
<td>P73 and Bax</td>
<td>(10)</td>
</tr>
<tr>
<td>Vdac1 and Bax</td>
<td>(11)</td>
</tr>
<tr>
<td>Bax and p53</td>
<td>(12, 13)</td>
</tr>
<tr>
<td>Vdac1 and Mot</td>
<td>(14)</td>
</tr>
<tr>
<td>Mot and p53</td>
<td>(15)</td>
</tr>
<tr>
<td>Vdac1 and MT</td>
<td>(16)</td>
</tr>
<tr>
<td>P53 and MT</td>
<td>(17)</td>
</tr>
<tr>
<td>MT and Dynein</td>
<td>(17)</td>
</tr>
<tr>
<td>P53 and Dynein</td>
<td>(18)</td>
</tr>
<tr>
<td>BRCA1 and p53</td>
<td>(19)</td>
</tr>
<tr>
<td>Vdac1 and dynein</td>
<td>(14)</td>
</tr>
<tr>
<td>Ubb and BRCA1</td>
<td>(20)</td>
</tr>
<tr>
<td></td>
<td>Bind id:1999732</td>
</tr>
</tbody>
</table>

References (for table 2.1):


2.4 Materials and methods

2.4.1 Generation of GFP fusion clones

2.4.1.1 Cloning of hTBP

hTBP-GFP clones were made previously in our laboratory and have been reported elsewhere (Pandey, N, Ph.D Thesis “Effect of polymorphic triplet repeat on protein structure and its implication in neurodegenerative diseases”, 2003). The vector maps are provided in the Appendix (B).

2.4.1.2 Cloning of mVdac1

The coding sequence of murine Vdac1 (mVdac1) (gene bank id NM_011694.3) was amplified from cDNA synthesized from N2a cells. The following primers introduced BglII and KpnI site, respectively, in the PCR product (restriction sequence underlined):

5’-AAAAGATCTATGGCCGTCGCTCCC-3’ and
5’AAAGGTACCTGCTTGAATTCCAGTCC 3’

The gel purified PCR product was cloned in BglII-KpnI site of pEGFP-N3 (Clontech) vector, in frame with green fluorescent protein (GFP) and confirmed by sequencing.

Expression of mVdac1GFP in transfected N2a cells was confirmed by reverse transcription followed by PCR (RT-PCR). The following primers were used for amplification:

5’-AATGACGGGACAGAGTTTGG-3’ (Specific for Vdac1)
5’-GGTGTTCTGCTGGTAGTGGT-3’ (Specific for GFP)

Expected product size was confirmed by resolving the PCR product on a 1% agarose gel.

2.4.2 Cell culture and transfection

Murine neuroblastoma cells (ATCC number CCL-131; Neuro-2a or N2a) were maintained in Minimum essential medium (MEM) (GIBCO-BRL) supplemented with 10% fetal calf serum (Biological Industries, Israel), 2mM L-glutamine (Sigma), 1mM sodium pyruvate (Sigma) and antibiotic-antimycotic solution (100X stock) (Sigma) at 37°C humidified incubator with 5% CO₂. Approximately 3×10⁵ cells were seeded in each well of 6 well plate (Axygen) 24 h prior to transient transfection when 50-80% confluency was reached. Cells were washed once with Opti-MEM (GIBCO-BRL) and maintained in 1 ml Opti-
MEM. 1µg of transfection quality DNA (Endo free plasmid maxi kit; Qiagen) and 3µl of fugene 6 transfection reagent (Roche) were used for preparation of transfection complex. Transfection of N3, 16Q and 59Q was performed following the protocol from the manufacturer. After 60 h of incubation cells were observed in an inverted epifluorescence microscope (TE200-U, NIKON) under 10X objective. Transfection has also been performed by using Amaxa nucleofection technology. Transfection efficiency was found to be about 45 to 50% and used for normalization.

2.4.3 RNA isolation and purification

After 60 H of transfection total RNA was isolated from the cells using TRIzol reagent (GIBCO-BRL) according to the manufacturer’s instruction. The RNA pellets were washed with 70% ethanol, centrifuged and dried. Pellets were re-suspended into 30 µl of DEPC treated water followed by the addition of 10X reaction buffer and 2U of RNAsase free DNase I (Fermentas) in a total volume of 45 µl. Samples were incubated at 37°C for 30 minutes. Then the RNA was cleaned using RNeasy Mini Kit (Qiagen) following the protocol by the manufacturer. RNA concentration and purity was determined by measuring optical density at 260 nm and 280 nm using a spectrophotometer (Eppendorf) and running the RNA samples on a 1.5 % agarose formaldehyde gel.

2.4.4 Reverse transcription (RT) and real time polymerase chain reaction (PCR)

cDNA was generated from the total RNA samples by using random hexamer (New England biolabs Inc, NEB) and M-MuLV reverse transcriptase (NEB) at 42°C for 1 h. Real time PCR was performed using SYBR green master mix (Applied Biosystems, ABI) in ABI 7500 real time PCR instrument. The forward and reverse primers are found in Table 2.2. The real time PCR program consisted of activation of uracil-N-glycosylase (UNG) at 50°C for 2 min., then 95°C for 10 min to inactivate UNG and activate ampli Taq Gold DNA polymerase, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 55°C for 30 sec and extension at 60°C for 1 min. The products were analyzed on 5% polyacrylamide gel to confirm the appropriate product size.

Data analysis: Relative quantitation of gene expression was carried out using the mathematical expression described recently (Pfaffl MW et al., 2001). PCR efficiency of target gene and endogenous control gene has been determined from the slope of the respective standard curve (Table 2.2).
Table 2.2: Mouse specific primer sequences and amplicon sizes used in real time RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’-3’)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dctn5</td>
<td>F: CGAGGAAGACTGTGGTGGTCA</td>
<td>192</td>
</tr>
<tr>
<td></td>
<td>R: AGCTCCCTGAGAAAGTCC</td>
<td></td>
</tr>
<tr>
<td>Actb</td>
<td>F: CTAAGGCGAACCGTAAAAAG</td>
<td>126</td>
</tr>
<tr>
<td></td>
<td>R: CCATCAACATTGGCTTGGTA</td>
<td></td>
</tr>
<tr>
<td>Vdac1</td>
<td>F: AATGACGGGACAGAGTTTG</td>
<td>231</td>
</tr>
<tr>
<td></td>
<td>R: AATGACGGGACAGATTGGG</td>
<td></td>
</tr>
<tr>
<td>Eef1a1</td>
<td>F: CTTCTCTGACTACCCTCCAC</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R: CCACAGCTTTGATGACACCC</td>
<td></td>
</tr>
<tr>
<td>Eef2</td>
<td>F: TGAGCAAGTGGTGGTGG</td>
<td>134</td>
</tr>
<tr>
<td></td>
<td>R: TGTTGGATCGAGATCAGCGG</td>
<td></td>
</tr>
<tr>
<td>Ubb</td>
<td>F: TCTTTCTGAGGGGTGTTTC</td>
<td>131</td>
</tr>
<tr>
<td></td>
<td>R: GTCACTGGGCTCCACCTCA</td>
<td></td>
</tr>
<tr>
<td>18s rRNA</td>
<td>F: CTTTCGAGGCCCTGTATTTG</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>R: CCTCAAATGGATCCTCGTAA</td>
<td></td>
</tr>
<tr>
<td>GFP</td>
<td>F: CTACAACAGCCACACGTC</td>
<td>127</td>
</tr>
<tr>
<td></td>
<td>R: GGTTGTCTGCTGGTAGTGGT</td>
<td></td>
</tr>
</tbody>
</table>

The table lists the sequences (5’ to 3’) of the forward and reverse primers used in real time PCR reaction to amplify various gene transcripts. The size of the amplicon is given as base pairs (bp). The designation “F” indicates forward and “R” indicates reverse.
2.4.5 Microarray studies and data analysis

Total RNA was isolated from N3 and 59Q transfected N2a cells as mentioned above. Total RNA labeled by Micromax NEN TSA labeling system (Perkin-Elmer Life Sciences, USA) according to manufacturer instruction. Labeled total RNA was hybridized onto mouse cDNA array (Microarray centre, University Health Network, Ontario, Canada). Six different replicates were performed from the cultured N2a cells in different batches. The slides were scanned using an Axon scanner and data were acquired and analysed using Genepixpro. Data from 59Q transfected (treated) and N3 transfected (control) samples were normalized using Z score transformation method described by Cheadle et al., (Cheadle C et al., 2003). Z ratio value $\pm 1.96$ was considered significant ($p<0.05$) (Cheadle C et al., 2003). Genes which are involved in localized neuronal translation, retrograde transport, ubiquitin related and related to apoptosis have been selected for a priori study in this thesis. Microarray data has been submitted to GEO (Accession no. GSE5807).

2.4.6 Northern analysis

Total RNA was transferred to the nylon membrane after separation on a 1.5% agarose formaldehyde gel. Subsequently, radioactively labeled probe prepared from purified PCR products of Vdac1, Dctn5, Ubb and Gfp using NEBlot™ kit (NEB) according to manufacturer instruction. Hybridized probe signal was detected by phosphorimager (Fujifilm FLA 2000IR) and intensity was quantified using MultiGauge software.

2.4.7 Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation reaction was performed using standard protocol (Wells J et al., 2002). Briefly, $10^6$ cells were fixed with 1% formaldehyde at room temperature for 10 min. Glycine (0.125M) was added to stop crosslinking and twice wash with 1X PBS (ice cold) was given. Cells were scrapped in 1X PBS and centrifuged to pellet down. Cells were then lysed in lysis buffer (5mM PIPES pH8.0, 85 mM KCl, 0.5% NP40, 1X Protease inhibitor cocktail) and incubate in ice for 10 min. Nuclei were pelleted at 5000 rpm for 5 min at 4°C. Nuclei were lysed with nuclei lysis buffer (50 mM Tris-Cl pH8.1, 10 mM EDTA, 1% SDS, 1X protease inhibitor cocktail) and incubate in ice for 10 min. After that sonication was
Transcriptional dysregulation of genes and downstream effects in a cellular model of SCA17

performed at the program setting (Output=3, 15sec on, 1min off, 10 times) in a Sonicator (MISONIX sonicator 3000). Supernatant was collected after centrifugation at 14,000 rpm for 10 min in room temperature. ProteinA agarose beads was used for precleaning the supernatant (15 min on a rotating platform in cold room) and then centrifuged to pellet down the beads. Precleaned supernatant was equally divided into two parts: with antibody (where antibody has to be added) and no antibody and dilution buffer (0.1% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-Cl pH8.1, 167mM NaCl) was added to make up the volume up to 500 ul. 2 µg anti TBP antibody (N-12: sc-204; Santa cruz Biotechnology Inc.) was added for immunoprecipitation overnight at 4°C. ProteinA agarose beads were added then for 15 min at room temperature, kept in a rotating platform and pelleted down after that. Supernatant for no antibody control is used as Total input chromatin (TIC). TIC samples were processed similarly, like other samples, except washing steps (mentioned below). Pelleted beads were washed once with 1X dialysis buffer (2 mM EDTA, 50 mM Tris-Cl pH8.0, 0.2% sarkosyl) and four times with wash buffer (100 mM Tris-Cl pH8.0, 500 mM LiCl, 1% NP40, 1% deoxycholic acid). Protein DNA complex was then eluted from beads by using elution buffer (50 mM NaHCO₃, 1% SDS). Eluted samples were treated with RNAase A and de-crosslinking (by adding 0.3M NaCl, final concentration and then heating at 65°C for 5 hrs) was performed. After that 2½ volume of EtOH was added and kept at -20 °C, overnight and then centrifused to pellet down at 13,000 rpm for 20 min in 4 °C. Pellet was resuspended in 200 ul of water, after 70% EtOH wash and air drying. Proteinase K (PK) treatment was performed at 45 °C for 2 hrs after addition of 25 ul 5X PK buffer (50 mM Tris-Cl pH7.5, 20 mM EDTA, 1.25% SDS) and 1.5 ul proteinase K (20 mgml⁻¹). Subsequently, phenol chloroform extraction was performed to remove proteins and DNA was precipitated by using 0.3M sodium acetate and 2½ volume of EtOH, overnight at -20°C. Yeast t-RNA (10ug) and glycogen (10ug) was added for efficient precipitation. Precipitated DNA samples were analyzed by PCR (35-38 cycles). The following forward and reverse primers were used for each gene promoter respectively:
Transcriptional dysregulation of genes and downstream effects in a cellular model of SCA17

Vdac1 SRE1:
5’-GGGAGAGTTTAATTTGCAACTGACT-3’
5’-CTGGAAGCATTTGGGAAGAG-3’

Vdac1 Sp1:
5’-GAGACTGGTCTGGGCGCTGTC-3’
5’-TGGGAGCGCAGCGAACGGGCC-3’

Actb:
5’-CCATCGCCAAAAACTCTTCAT-3’
5’-AAGGAGCTGCAAAGAAGCTG-3’

PCR products were resolved on a 2% agarose gel. Ethidium bromide stained bands were quantified using Quantity one software (BioRad). Band intensity was normalized to the input band.

2.4.8 PCR-based assay for Xbp1 splicing

cDNA synthesized from total RNA isolated from transfected cells was used as template for PCR. Primers and PCR conditions were used as described by Marciniak et al. (Marciniak SJ et al., 2004). 480 bp product obtained from unspliced Xbp1 while spliced Xbp1 gave 454 bp product. PCR products were separated on 5% polyacrylamide gel and stained with Vista green (Amersham Biosciences, UK). Fluoroimage was captured by gel doc system (BioRad Laboratories, USA) and intensity was quantified using quantity one v 4.1.1 software.

2.4.9 Western analysis

Neuro-2a cells were transfected with vector alone (N3), 16QTBP-GFP and 59QTBP-GFP using either fugene6 or Amaxa nucleofection device. Transfected cells grown on 6-well plate were scrapped after 60-65h of incubation. Cells were lysed using 1X SDS sample buffer (50mM Tris-Cl, pH 6.8, 100mM 2-mercaptoethanol, 2% (w/v) SDS, 10% glycerol) and incubated at 55°C for 1h, then boiled for 5 min. Total protein was estimated using BCA protein estimation kit (Sigma). Bromo-phenol blue was added to each sample and equal amount of protein was separated on 12% SDS-polyacrylamide gel. The separated protein was transferred to a nitrocellulose membrane and stained with Ponceau S to check equal loading. Membrane was
Transcriptional dysregulation of genes and downstream effects in a cellular model of SCA17

blocked in a solution of 137 mM NaCl, 3 mM KCl, 25 mM Tris-HCl, 0.1% Tween 20, pH 7.4 containing 5% (w/v) skimmed milk for overnight at 4°C. VDAC1 specific polyclonal antibody (N-18: sc-8828) (1:500 dilution) was added and incubated for 2h at room temperature, washed and then incubated with alkaline phosphatase (AP) conjugated secondary antibody (1:3000). For TBP specific western TBP specific polyclonal antibody (N-12:sc-204) (1:500 dilution) was used. Immunoreactive bands were detected using BCIP/NBT solution. Western blot was scanned and band intensity was quantified by using Quantity one software (BioRad). Transfected N2a cells were lysed by using a lysis buffer (50mM Tris-Cl pH8.1, 10mM EDTA, 1% SDS), boiled for 10 minutes for complete lysis, and then spun at 5000 rpm to remove debris. DNA has been precipitated from the collected supernatant by using 5M Nacl to a final concentration 0.3M and kept for overnight at -20°C. DNA was pelleted down, washed with 70% ethanol and dissolved in TE. PCR amplification from that template has been performed by using GFP specific primers (Table 2.2); run on a 2% EtBr stained agarose gel to resolve the product.

2.4.10 Measurement of cytochrome c release

Neuro-2a cells were transfected using Amaxa nucleoefection device following manufacturer protocol. Transfected cells were grown on 6 well plates in Opti-MEM (GIBCO-BRL). Cells were scraped after 24 hr, 36 hr, 48 hr and 70 hr of incubation, washed in PBS, washed in 1X extraction buffer A (10 mM HEPES, pH 7.5, 200 mM manitol, 70 mM sucrose and 1 mM EGTA)(sigma) and re-suspended in the same buffer containing 2 mg ml\(^{-1}\) bovine serum albumin (Sigma). After 1 h incubation on ice, cells were lysed by a B-type Dounce homogenizer with 30-35 strokes. Homogenates were centrifuged at 4°C in the subsequent steps to remove nuclei, debris and mitochondria (Fig. 2.8A). Supernatant was re-centrifuged at 15,000 × g at 4°C to get cytosolic fraction. To rule out mitochondrial contamination in the cytosolic fraction a colorimetric assay was performed using cytochrome c oxidase assay kit (Sigma) following manufacturer protocol. Protein estimation in the cytosolic fraction was performed by using BCA protein estimation kit (Sigma). Equal amount of protein from cytosolic fraction was used from N3, 16Q and 59Q transfected samples and cytochrome c concentration was quantitatively examined by a solid phase ELISA kit (Quantikine ® Rat/Mouse, R & D systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.
Similar method was followed for Cyt c release assay in case of mVdac1GFP transfected cells. Assay was performed 65 H after transfection.

2.4.11 Flow cytometry analysis

Untransfected and transfected N2a cells grown on 6 well plate were harvested by trypsinization/scraping. Untransfected cells were used as a negative control. Cells were stained with Annexin V-PE and 7-AAD by using Nexin™ kit (Guava technologies) following manufacturer’s protocol. Data acquisition and analysis were performed by using GuavaEasyCyte flow cytometer and Guava Nexin cytosoft software (v3.6.1). Transfection efficiency has been calculated by counting GFP positive cells in the above mentioned flow cytometer using Guava Express plus software (v3.6.1). For data analysis total annexin V-PE positive cells were normalized to transfection efficiency.