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Transcriptional and proteomic profiling in a cellular model of DYT1 dystonia

Janine N. Martin¹, Thomas B. Bair², Nicole Bode³, William T. Dauer⁴, and Pedro Gonzalez-Alegre^{1,3,5,6}

¹Graduate Program in Genetics, The University of Iowa, Iowa City, IA

²DNA Facility, Carver College of Medicine, The University of Iowa, Iowa City, IA

³Department of Neurology, Carver College of Medicine, The University of Iowa, Iowa City, IA

⁴Departments of Neurology and Pharmacology, Columbia University, New York, NY

⁵Graduate Programs in Neuroscience, The University of Iowa, Iowa City, IA

⁶Graduate Programs in Molecular & Cellular Biology, The University of Iowa, Iowa City, IA

Abstract

DYT1, the most common inherited dystonia, is caused by a common dominant mutation in the *TOR1A* gene that leads to a glutamic acid deletion in the protein torsinA. Wild type torsinA locates preferentially in the endoplasmic reticulum while the disease-linked mutant accumulates in the nuclear envelope. As a result, it has been proposed that DYT1 pathogenesis could result either from transcriptional dysregulation caused by abnormal interactions of mutant torsinA with nuclear envelope proteins, or from a loss of torsinA function in the endoplasmic reticulum that would impair specific neurobiological pathways. Aiming to determine whether one or both of these potential mechanisms are implicated in DYT1 pathogenesis, we completed unbiased transcriptional and proteomic profiling in well-characterized neural cell lines that inducibly express wild type or mutant torsinA. These experiments demonstrated that the accumulation of mutant torsinA in the nuclear envelope is not sufficient to cause transcriptional dysregulation. However, we detected expression changes at the protein level that, together with other reports, suggest a potential implication of torsinA on energy metabolism and regulation of the redox state. Furthermore, several proteins identified in this study have been previously linked to other forms of dystonia. In conclusion, our results argue against the hypothesis of transcriptional dysregulation in DYT1 dystonia, suggesting potential alternative pathogenic pathways.

Dystonia is a neurological syndrome characterized by twisting involuntary movements, frequently stereotyped and repetitive, that cause disabling abnormal postures (Fahn et al., 1998). Several inherited forms of dystonia have been described (Gonzalez-Alegre, 2007). Understanding the biological basis underlying genetic forms of dystonia could provide us with relevant information pertaining to the pathogenesis of the more common sporadic forms of the disease.

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Correspondence: Pedro Gonzalez-Alegre, MD, Department of Neurology, Roy J and Lucille Carver College of Medicine, University of Iowa, 3111A MERF, Iowa City, IA 52242-1101, USA, pedro-gonzalez-alegre@uiowa.edu.

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DYT1 is the most common inherited dystonia, caused by a dominant three base-pair, in-frame deletion in the *TOR1A* gene (Ozelius et al., 1997). *TOR1A* encodes torsinA (torA), a AAA protein (ATPases Associated with diverse cellular Activities) (Hanson and Whiteheart, 2005) of unknown function. The DYT1 mutation causes the loss of one of a pair of glutamic acid residues in torA (torA(Δ E)) (Breakefield et al., 2001). The subcellular localization of torA and the consequences of the DYT1 mutation have been explored in cell- and animal-based experiments. Whereas torA is a lumenally oriented endoplasmic reticulum (ER) and nuclear envelope (NE)-resident glycoprotein (Hewett et al., 2000, Kustedjo et al., 2000), torA(Δ E) concentrates preferentially in the nuclear envelope (NE) (Gonzalez-Alegre and Paulson, 2004, Goodchild and Dauer, 2004, Naismith et al., 2004). Upon redistribution to the NE, torA(Δ E) recruits torA(wt) from the ER (Torres et al., 2004, Gonzalez-Alegre et al., 2005). Collectively, published studies indicate that torA(Δ E) acts through a dominant negative effect by redistributing to the NE and “sequestering” torA(wt), leading to an overall loss of torA function (Goodchild and Dauer, 2004, Naismith et al., 2004, Torres et al., 2004, Gonzalez-Alegre et al., 2005, Goodchild et al., 2005, Hewett et al., 2007, Hewett et al., 2008).

Two proposed hypotheses of DYT1 pathogenesis posit that either the abnormal accumulation of torA(Δ E) in the NE causes dysregulation of gene expression triggering neuronal dysfunction or, alternatively, that the loss of torA function in the ER leads to dysfunction of specific biological pathways. The first possibility derives from experimental observations demonstrating that torA interacts with NE proteins. NE proteins function, in part, by regulating transcription through direct and indirect interactions with chromatin (Worman and Courvalin, 2000) and transcription factors (Heessen and Fornerod, 2007). Stabilization of torA interactions with NE proteins in DYT1 could cause transcriptional dysregulation leading to neuronal dysfunction. Consistent with this hypothesis, there is a growing list of diseases caused by mutations in genes encoding NE proteins (Burke et al., 2001, Burke and Stewart, 2002, Worman and Courvalin, 2002), some of them thought to result from transcriptional dysregulation (Hwang et al., 2001). These observations provide a rationale to speculate that torA(Δ E) alters the transcriptional control carried out by NE proteins, resulting in dysregulation of gene expression. However, a role for torA in other functional aspects of NE proteins, such as regulation of nuclear shape or movement, can not be excluded.

It has also been suggested that torA(Δ E) causes a loss of torA function in the ER through a dominant negative effect. TorA has been implicated in the modulation of cytoskeletal and synaptic functions and in protective responses against the accumulation of misfolded proteins and oxidative stress (Hewett et al., 2003, Kamm et al., 2004, Hewett et al., 2006, Balcioğlu et al., 2007, Hewett et al., 2007, Granata et al., 2008). A recent report from Thomas and colleagues suggests that torA acts as a redox sensor, coupling energy metabolism to the cellular redox state (Zhu et al., 2008). By recruiting torA(wt) from the ER, torA(Δ E) could impair those biological functions, independently of potential effects on transcription.

Aiming to determine whether torA(Δ E) impairs regulation of transcriptional programs from the NE, interferes with the regulation of ER-linked biological pathways, or both, we completed unbiased transcriptional and proteomics profiling in well-characterized neural cell lines that inducibly expresses either torA(wt) or torA(Δ E).

EXPERIMENTAL PROCEDURES

Cell lines

PC6-3 cells—We employed previously reported inducible PC6-3 torA(wt) (clone #28) and torA(Δ E) (clone #33) cell lines (Gonzalez-Alegre and Paulson, 2004). PC6-3 cells are a subclone derived from rat PC12 cells that differentiates into a neural phenotype upon treatment with nerve growth factor (NGF) and is used to study neuronal biology. The PC6-3 clonal line

stably expressing the tet-repressor (PC6-3/TR), initially used to generate the inducible torA lines, was used as a control. Cells were grown and maintained as described in collagen-coated tissue culture dishes in RPMI media supplemented with 10% equine serum (ES), 5% fetal bovine serum (FBS) and 1% pen/strep (P/S) containing blasticidin (5 µg/ml) and hygromycin (100 µg/ml). To achieve a neural phenotype, the media was changed to low serum media with NGF (RPMI, 2% ES, 1% FBS, 1% P/S, 100 ng/ml NGF). Transgene transcription was induced with 1.5 µg/ml of doxycycline (DOX).

HEK293 cells—To generate HEK293 cell lines stably expressing a single copy of human torsinA(ΔE) cDNA, we employed the Flp-In system developed by Invitrogen following the manufacturer's recommendations. These cell lines have a stably integrated FRT site at a transcriptionally active genomic locus. We co-transfected a Flp-In vector encoding torA(ΔE) driven from a CMV promoter and the Flp recombinase vector, pOG44, into HEK293 Flp-In cells, resulting in targeted integration of torA(ΔE) cDNA to the same locus in every cell, then selected by antibiotic resistance.

Transcriptional profiling

For each one of 3 independent experiments, a liquid nitrogen-frozen vial of each line (torA (wt), torA(ΔE) and control PC6.3/TR) was thawed and seeded in a 10cm culture dish. Once reaching confluency, cells were trypsinized, resuspended and split into two 10 cm collagen-coated culture dishes. When cells were approximately 80% confluent, the media was changed to low serum media containing NGF with or without DOX for 48 hours before harvesting. Total RNA and protein were isolated using TRIzol (Invitrogen) following the manufacturer's protocol. Isolated RNA was resuspended in 100µl of RNase-free water and concentration and purity of RNA was measured using NanoDrop ND-1000 spectrophotometer (Thermo-Fisher Scientific). RNA samples were then shipped in dry ice to the UCLA Microarray Core Facility through the NIH Neuroscience Microarray Consortium (<http://arrayconsortium.tgen.org>).

Expression profiling was performed according to the Affymetrix guidelines (GeneChip® Expression Analysis Data Analysis Fundamentals). Samples were analyzed for integrity using the Agilent Bioanalyzer 2100. One µg of total RNA was amplified and labeled using the GeneChip HT one-cycle cDNA synthesis kit (Affymetrix). Fragmented, labeled cRNA was hybridized to Affymetrix Rat Genome 230 2.0 array for 16 hrs at 45°C. The GeneChips® were washed and stained according to the manufacturer's recommendations using the GeneChips® Fluidics Station 450 (Affymetrix). Each chip was scanned using the GeneChips® Scanner 3000 (Affymetrix). The Affymetrix GeneChip® Operating Software version 1.4 (GCOS) was used to perform global scaling by bringing the overall intensity of the arrays to a target intensity value of 150 to normalize the data for inter-array comparisons. Target intensity levels and detection calls of each gene were generated using the Statistical Expression Algorithm. Results are publicly available at <http://arrayconsortium.tgen.org>. Using Partek Genomics Suite 6.2 (Partek Inc), Affymetrix data was normalized following the RMA algorithm. Before significance analysis, Partek's batch correction method, which reduces variation due to random factors, was used to enhance signal. ANOVA models along with linear contrasts were used to find genes that responded to factors (cell-line, and DOX treatment), interaction between factors and to determine p-values and fold changes between groups. To control for multiple testing, we employed false discovery rate correction. Genes with a fold change of ≥ 1.5 and a false discovery rate of 5% were considered to be differentially expressed.

Proteomic Analysis

PC6-3 cells lines were plated in collagen coated 10-cm culture dishes, differentiated with NGF as above, with or without DOX. Cells were harvested, washed to remove culture medium, frozen in liquid nitrogen, stored at -80°C and sent directly for 2-DIGE and mass spectrometry

analysis by Applied Biomix (Hayward, CA). In summary, the non-induced and induced samples for each cell line were covalently linked to Cy3 and Cy5 CyDye respectively, mixed and run on first dimension isoelectric focusing, and second dimension SDS-PAGE. Image scans were carried out following the SDS-PAGE using Typhoon TRIO (Amersham BioSciences), the scanned images analyzed by Image QuantTL software (GE-Healthcare), and subjected to in-gel analysis and cross-gel analysis using DeCyder software version 6.5 (GE-Healthcare). The ratio change of the protein differential expression was obtained from in-gel DeCyder analysis. To determine the ID of differentially expressed proteins, selected spots were picked up by Ettan Spot Picker (GE-Healthcare) following the DeCyder software analysis and spot picking design. The selected protein spots were subjected to in-gel trypsin digestion, peptides extraction, desalting and followed by MALDI-TOF/TOF (Applied Biosystems) analysis to determine the protein identity.

Western blotting

Whole cell lysates were obtained using Laemmli buffer with 200mM DTT, incubated at 95°C for 5 min and briefly sonicated. Samples were loaded and run alongside molecular weight markers in 12% PAGE, electroblotted onto PVDF membranes (BioRad) and western blot completed and quantified as described (Gonzalez-Alegre et al., 2005). The following antibodies were used: human torsinA (TA913) (Gordon and Gonzalez-Alegre, 2008), torsinB (a generous gift of Xandra Breakefield, Harvard University), α -tubulin (Sigma), tropomyosin 4 (Ab5449, Chemicon) and the following antibodies from Santa Cruz: annexin 5 (H-3), secretogranin II (M-20), aldose reductase (Fl-316) and VGF (D-20).

Microscopy

Indirect immunofluorescence (IF) was performed as described (Gonzalez-Alegre and Paulson, 2004). In brief, cells were seeded in collagen coated slides and processed by fixation in 4% paraformaldehyde for 15 min, following by blocking in 5% serum, sequential incubation with the indicated primary and FITC or Texas Red conjugated secondary antibodies. DAPI was used for nuclear staining, slides were mounted using SlowFade Antifade (Invitrogen), visualized with a Zeiss Axioplan fluorescence microscope and images collected using an Axiocam HRm (Zeiss) digital camera. Digital images were assembled using Adobe Photoshop 6.0.

Transmission electron microscopy (TEM) was performed as previously described (Gonzalez-Alegre and Paulson, 2004). In summary, cells were submerged in 2.5% glutaraldehyde, postfixated in osmium tetroxide followed by 2.5% aqueous uranyl acetate, dehydrated in ethanol and embedded in Eponate-12 resin (Ted Pella Inc., Redding, CA). Ultrathin sections of 100 nm were counterstained with uranyl acetate and lead citrate before collecting digital pictures on a EOL JEM-1230 electron microscope.

RESULTS

Transcriptional profiling

We first asked whether the abnormal accumulation of torA(Δ E) in the NE causes transcriptional dysregulation. To answer that question, we queried expression of 31,099 genes in PC6-3 cells that inducibly express torA(Δ E), as these cells differentiate into neural phenotype and exhibit a significant redistribution of the mutated protein to the NE. To control for the potential transcriptional effects of torA expression in the secretory pathway and of the DOX treatment, we used a clone inducibly expressing torA(wt) and the parent cell line that expresses the tet-repressor but not a tet-operator (Gonzalez-Alegre and Paulson, 2004). By employing these clones with and without DOX each line acted as its own control, minimizing potential confounding effect derived from transcriptional heterogeneity among clones. TorA expression

was verified by western blot analysis of the protein fraction obtained from each sample, demonstrating similar transprotein levels in every replicate (representative blot shown in figure 1A). IF analysis performed in parallel confirmed the previously reported striking different subcellular localization for each form of torA (figure 1B). Surprisingly, neither treatment with DOX nor overexpression of torA(wt) or torA(Δ E) lead to significant expression changes in any single gene at the preset threshold (1.5 fold change). Even in the absence of significant expression changes in single genes, we considered plausible that torA could influence expression of multiple genes from a given pathway below the preset threshold, modifying the transcriptional phenotype of the cell. However, hierarchical clustering of the expression profiles obtained under each condition demonstrated that individual clones were highly homogeneous, irrespective of the treatment with DOX (figure 1C), thus arguing against any transcriptional effect of torA expression. Due to the presumed loss of torA function caused by the dominant negative effect of torA(Δ E), we hypothesized that its overexpression could trigger compensatory transcriptional upregulation in the other members of the mammalian torsin gene family (torsinB, torp2A and torp3A). Probes for every torsin gene were included in the arrays, including endogenous torA, showing no changes in their levels (figure 1D). Western blot analysis of the more closely related protein, torsinB, did not show alteration in steady-state levels in the setting of either torA(wt) or torA(Δ E) overexpression (not shown), arguing against a post-transcriptional stabilization of torsinB. To our knowledge, there are no antibodies that reliably detect endogenously expressed torp2A and torp3A.

Proteomic analysis

The experiments just described demonstrated that the accumulation of torA(Δ E) in the NE is not sufficient to induce significant transcriptional dysregulation. However, lack of transcriptional changes does not exclude differences at the protein level through several potential mechanisms, such as a transcription-translation mismatch, post-translational stabilization or modification, among others, that could help us identify biological pathways influenced by torsinA expression. To test this possibility, we profiled the proteome of the same cell lines. Whole cell lysates obtained from clones inducibly expressing either form of torA and non-induced controls were differentially labeled with Cy3 and Cy5, and subjected to analytical 2-DIGE, identifying a total of 2191 and 2146 spots in the torA(wt) and torA(Δ E) gels, respectively. Upon induction of torA(wt) expression, 390 spots (17.8%) were downregulated and 190 (8.7%) upregulated, whereas torA(Δ E) expression caused downregulation of 287 spots (13.4%) and upregulation of 135 (6.3%). Those with a volume change above the preset threshold of ≥ 1.5 , which included 32 spots in cells expressing torA (wt) and 35 for torA(Δ E) (figure 2), were selected and picked for protein identification by MALDI-TOF/TOF. All but 2 spots in the torA(wt) gel and 3 in the torA(Δ E) gel were identified with high confidence (table 1).

To validate these results, we selected 6 proteins differentially expressed upon torA(wt) or torA(Δ E) expression to complete western blot analysis in 3 independent experiments in lysates obtained from PC6-3 cells grown in the same conditions. These experiments confirmed significant differences or non-significant trends for selective changes in expression of annexin V, α SNAAP and VGF by torA(wt) and secretogranin II, tropomyosin 4 and aldose reductase by torA(Δ E) (figure 3A-B). Probes for all of these specific transcripts were included in the mRNA profiling studies showing no expression changes (figure 3C), suggesting, although not proving, that the differential expression detected arises at the post-translational level.

To determine that these changes in protein expression are a direct result of torA expression and not an artifact of the system employed, we generated a non-neuronal cell system where stable transgenic torA expression is not controlled by DOX. HEK293 cell lines stably expressing a single copy of human torA cDNA (wt or Δ E) were generated, transgene expression

shown by western blot analysis (figure 4A), ER and NE localization of torA(wt) and torA(Δ E), respectively, demonstrated by IF (not shown) and torA(Δ E)-induced disruption of NE structure confirmed by TEM (supplementary figure 1). In these cell lines, western blot analysis successfully detected annexin V, α SNAP and aldose reductase expression levels, demonstrating changes in expression levels following those observed in PC6-3 cells (figure 4 C-D). VGF, secretogranin II and tropomyosin 4 were not detected, likely due to lack of expression in this non-neural cell line. These experiments confirmed that the changes detected in PC6-3 cells result from torA expression independently of the system used.

Next, we inspected the list of proteins to ask whether they cluster into specific functional pathways. Interestingly, the main functional group identified included proteins involved in energy-generating catabolic pathways (glycolysis, acetyl CoA metabolism, Krebs cycle and electron transport chain) and proteins with oxidoreductase activity, whereas the remainder were implicated in cytoskeletal organization, secretory/synaptic functions and regulation of gene expression (table 2). We hypothesized that, if one these biological pathways is specifically implicated in DYT1 pathogenesis, it could also play a role in other forms of dystonia. For that reason, we investigated whether any of the identified proteins have been linked to human diseases that clinically manifest dystonia either directly (i.e., genetics defects in their coding gene) or indirectly (i.e., genetics defects in other components of functional complexes that include this protein). A total of 5 proteins identified by our proteomic analysis have been implicated in the pathogenesis of inherited disorders that manifest dystonia (table 3).

DISCUSSION

In this work, we have taken an unbiased approach combining transcriptional and proteomic profiling in cell-based neural systems that recapitulate aspects of torA biology linked to DYT1 dystonia. Several conclusions can be drawn from these experiments. First, the accumulation of torA(Δ E) in the NE is not sufficient to cause transcriptional dysregulation. Second, torA dysfunction is not coupled to compensatory upregulation of the torsin gene family. Third, torA could influence several functional pathways important for neuronal function, such as the regulation of energy metabolism and redox state, cytoskeletal organization or synaptic function. Finally, together with previous reports, our findings suggest that defects in energy metabolism and redox state might represent a common biological pathway in the pathogenesis of different forms of dystonia.

TorsinA, the nuclear envelope and gene expression

The remarkable lack of expression changes in neural cells overexpressing torA(Δ E) demonstrates that its abnormal interaction with NE proteins is not sufficient to cause transcriptional dysregulation. Genetic defects in NE proteins have been identified as the cause of several human diseases, and their dysfunction has been proposed to influence transcriptional programs. Our findings suggest that this is not the mechanism underlying DYT1 pathogenesis. Nevertheless, it remains possible that transcriptional dysregulation occurs in DYT1, but is context-dependent. Although the precise anatomical origin for DYT1 dystonia remains to be elucidated, its phenotype seems to derive from neuronal dysfunction restricted to specific brain regions (Breakefield et al., 2008). However, torA(Δ E) accumulates in the NE of all cellular types, including DYT1 patient fibroblasts (Goodchild and Dauer, 2004). PC6-3 cells do not represent true neurons and perhaps model unaffected somatic cells rather than dysfunctional neurons. Nevertheless, our findings convincingly demonstrate that the accumulation of torA in the NE by itself is not sufficient to cause transcriptional dysregulation. Expression profiling in central neurons derived from animal models of DYT1 should be completed to exclude the transcriptional hypothesis of DYT1 pathogenesis.

Only about a third of DYT1 mutation carriers develop the disease. We speculated that compensatory upregulation of other torsin genes would occur in the setting of the DYT1 mutation acting as modifiers of penetrance. However, the lack of transcriptional activation in those genes in cells expressing torA(Δ E) suggests that either they are not redundant or that functional overlap is not coupled to transcriptional regulation. The latter possibility is supported by the lack of feed-back regulation of endogenous rat torA expression by human torA(wt) or torA(Δ E) expression in PC6-3 cells. These findings, however, do not exclude a post-translational compensatory function of torsin proteins in DYT1.

Our findings also illustrate the importance of including appropriate controls in profiling studies. A previously reported mRNA expression analysis in stably transfected cells expressing torA(wt) or torA(Δ E) found differentially expressed genes between both conditions (Baptista et al., 2003). In light of our results, we speculate that those differences might not have arisen from torA expression, but from baseline transcriptional differences between the transfected cells. In our inducible clonal cells, even though they were derived from the same parent cell line, we found significant heterogeneity. If we had only considered the DOX conditions for the cell lines employed (equivalent to comparing stably transfected cells), we would have found significant differences that would have been erroneously attributed to the expression of either form of torA (figure 1). By using non-induced lines as controls, we determined that those differences are unrelated to torA expression. Even when experimental samples share the same origin, it cannot be assumed they are transcriptionally equivalent and proper controls should always be employed.

TorsinA, energy metabolism and redox state

Attempting to identify pathways that are influenced by torA function, we clustered all differentially expressed proteins identified through our proteomic analysis in torA(wt) or torA(Δ E) expressing cells. These experiments identified several functional groups of proteins, including different components of energy-generating catabolic pathways and oxidoreductases. We find this interesting, adding to multiple pieces of evidence that potentially implicate energy-metabolism/redox regulation on DYT1 pathogenesis. First, a torA homolog from *Caenorhabditis elegans* was recently found to couple redox sensing to nucleotide binding through the action of an essential cysteine, thus integrating two key elements that reflect the metabolic status of the cell (Zhu et al., 2008). Second, we and others have described abnormal intermolecular disulfide-link dependent oligomerization of torA(Δ E) (Gonzalez-Alegre and Paulson, 2004, Torres et al., 2004, Gordon and Gonzalez-Alegre, 2008). It is tempting to speculate that this abnormal oligomerization of torA(Δ E) would involve the cysteines that are essential for torA function, thus losing their “sensing” ability. Third, torA function has been linked by different studies to cellular responses to oxidative stress (Hewett et al., 2003, Kuner et al., 2003, Shashidharan et al., 2004). Fourth, dysfunction of the mitochondrial respiratory chain can lead to generalized dystonia (Gonzalez-Alegre, 2007). Finally, the proteins identified in this study and that have been previously implicated in other forms of dystonia mostly involve metabolic/redox control pathways. Because several animal models of DYT1 have been generated, some exhibiting relatively minor motor dysfunction and others without an obvious phenotype (Jinnah et al., 2008), the significance of these findings could be explored, for instance, through pharmacological challenge of metabolic pathways in central neurons of these animal models, both in culture and *in vivo*. If confirmed by these functional assays, the significance of our findings could go beyond adding to our understanding of DYT1 pathogenesis, as they could also provide us with a biological mechanism underlying other dystonias and lead to the development of common therapeutic strategies that target many different forms of inherited and perhaps sporadic dystonia.

Other biological pathways potentially implicated in dystonia pathogenesis

We also identified proteins that are very important for the proper function of central neurons, as they regulate cytoskeletal and synaptic function, and membrane biogenesis. For instance, secretogranin II was found to be downregulated in torA(Δ E) expressing cells. This protein is a pro-peptide that generates secretoneurin, a protein implicated in dopamine release (Saria et al., 1993). TorA(Δ E) causes defects in dopamine release in DYT1 transgenic mice through unknown mechanisms (Balcioglu et al., 2007). Reduced levels of secretogranin II could play a role in this phenotype. Other gene products identified that are very important for striatal synaptic function include protein phosphatase 1 (catalytic subunit, gamma isoform), G-protein alpha-activating activity polypeptide O, NSF and NSF-attachment protein alpha.

In conclusion, our experiments demonstrate that the accumulation of torA(Δ E) in the NE is not sufficient to cause transcriptional dysregulation and, together with previous publications, suggests a potential role of torA on different neurobiological pathways, including energy-metabolism and regulation of the redox state.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

2-DIGE, 2 dimensional fluorescence difference in gel electrophoresis
 α SNAP, N-ethylmaleimide sensitive fusion protein attachment protein alpha
 AAA, family of proteins (ATPases Associated with diverse cellular Activities)
 Ald. Red., aldose reductase
 Ann. 5, annexin 5
 DOX, doxycycline
 ER, endoplasmic reticulum
 NE, nuclear envelope
 NGF, nerve growth factor
 Sg-II, secretogranin-II
 TM-4, tropomyosin-4
 torA, torsinA
 VGF, VGF nerve growth factor

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Figure 1. TorsinA overexpression does not influence gene expression in PC6-3 cells

(A) Representative western blot analysis for human torA expression in PC6-3 cells overexpressing human torA(wt), torA(ΔE) and controls. α-tubulin is shown as a loading control. Protein lysates were obtained from one of the replicates used for mRNA expression profiling. The TA913 antibody, which identifies human but not rat torA, was used to detect torA expression. (B) Indirect immunofluorescence analysis of torA in PC6-3 demonstrating dramatic differences in subcellular distribution between torA(wt) and torA(ΔE) with the TA913 antibody. Note that the accumulation of torA(ΔE) in the NE triggers the generation of NE-derived cytoplasmic inclusions known as spheroid bodies. (C) Hierarchical clustering of gene expression profiles obtained from PC6-3 clonal cell lines in three independent experiments demonstrates differential gene expression between different clones, but not due to the presence or absence of DOX treatment. Each clone is represented in a different color (purple, green and orange). Presence or absence of DOX is represented in red or blue, respectively. (D) Normalized expression of probes for each one of the torsin rat genes expressed in PC6-3 cells.

Figure 2. Proteomic analysis of PC6-3 cells inducibly expressing torA

PC6-3 cells were cultured in parallel with or without DOX and processed as described in experimental procedures. Lysates obtained from cells under no DOX and DOX conditions were labeled with Cy3 and Cy5, respectively, mixed and subjected to 2-DIGE (separated images in top panel, overlap in lower panel). Spots differentially expressed upon the addition of DOX were identified by DeCyder and picked for MS identification (lower panel).

Figure 3. Confirmatory western blot for candidate proteins in PC 6-3 cells

Western blot analysis of lysates obtained from PC6-3 cells treated with or without DOX for 72 hrs, detecting expression levels of (A) annexin-V and secretogranin-II specifically after induction of torA(wt) and torA(ΔE), respectively, and of (B) SNAP and VGF upon torA(wt) expression, and tropomyosin-4 and aldose reductase after torA(ΔE) expression. Top panels show a representative western blot and lower panels the quantification of three independent experiments. α-tubulin is used as loading control. Statistical differences were determined by paired *t* test. * = *p* < 0.05. (C) Normalized expression of probes included in the microarray chips for each one of the indicated genes in PC6-3 cells.

Figure 4. Confirmatory western blot for candidate proteins in HEK293 cells

HEK293 cells expressing a single copy of the *torA*(wt) or *torA*(ΔE) transgenes were generated as described in the manuscript. (A) Western blot analysis of lysates obtained from cells expressing a single copy of *torA*(wt), *torA*(ΔE) or the parent (control) cell line demonstrating high transgene expression levels. Western blot analysis was completed to detect expression of aldose reductase in *torA*(ΔE) and control cells (B), and for annexin-5 (C) and αSNAP (D) in *torA*(wt) and control cells. Top panels show a representative western blot and lower panels the quantification of three independent experiments. α-tubulin is used as loading control. Statistical differences were determined by paired *t* test. * = *p* < 0.05.

Differentially expressed proteins identified by MALDI-TOF/TOF

Table 1

Spot	Top Ranked Protein (Gene symbol)	Accession No.	Protein		Peptide Count	Protein Score	Protein Score C.I. %	Total Ion Score	Total Ion C.I. %	Volume Change
			MW	PI						
Torsin A(wt)										
1	Leucine rich protein 157 precursor (Lrpprc)	gi 34539486	156553.5	6.2	18	127	100	18	0	1.81
2	Chain B, Rat Brain Hexokinase Type I Complex With Glucose And Inhibitor Glucose-6-Phosphate (Hk1)	gi 5542104	102455.2	6.29	10	74	99.875	5	0	1.81
3	Eukaryotic translation elongation factor 2 (Eef2)	gi 56082	95222.9	6.41	17	186	100	31	71.878	-1.71
4	Staphylococcal nuclease domain containing 1 (Snd1)	gi 77404395	101888.5	6.76	10	72	99.606	0	0	1.51
5	ATP-citrate lyase (Acy)	gi 17028103	92420	8.1	13	107	100	4	0	1.56
6	VGF nerve growth factor inducible (Vgf)	gi 13591864	68137.8	4.68	28	270	100	38	97.352	-2.18
7	Ezrin (Ezr)	gi 52138521	69347.6	5.83	8	77	99.863	34	57.193	1.52
8	N-ethylmaleimide sensitive fusion protein (Nsf)	gi 13489067	82600	6.55	21	252	100	67	99.993	1.76
10	M2 pyruvate kinase (Pkm2)	gi 206205	57744	7.15	15	310	100	156	100	-2.08
11	Bat1 (Bat1)	gi 19773876	48943.9	5.43	6	263	100	191	100	1.52
12	Solute carrier family 25 (mitochondrial carrier, phosphate carrier), member 24 (Slc25a24)	gi 189011598	52857.3	8.11	10	180	100	66	99.986	1.55
13	Phosphoglycerate kinase 1 (Pgk1)	gi 40254752	44510	8.02	20	264	100	77	100	1.54
14	Phosphoglycerate kinase 1 (Pgk1)	gi 40254752	44510	8.02	3	68	98.964	51	99.239	1.57
15	Laminin receptor 1 (Lamr1)	gi 8393693	32803.4	4.8	12	281	100	150	100	-1.96
16	G-protein alphaactivating activity polypeptide O (Gnao1)	gi 149032469	34604.9	5.18	6	192	100	138	100	1.52
17	Acetyl-Coenzyme A acetyltransferase 2 (Acat2)	gi 33086552	103243.9	6.09	7	66	99.092	11	0	-1.79
18	Annexin V (Anxa5)	gi 2981437	33944.4	4.99	15	453	100	227	100	2.33
19	N-ethylmaleimide sensitive fusion protein attachment protein alpha (Napa)	gi 18034791	33171.2	5.3	18	298	100	91	100	1.69
20	Pyruvate dehydrogenase (lipoamide) beta (Pdhb)	gi 56090293	38957	6.2	8	250	100	182	100	1.52
21	Protein phosphatase 1, catalytic subunit, gamma isoform (Ppp1cc)	gi 31980772	38479.4	5.8	10	84	99.987	7	0	1.6
22	TorsinA (Dyt1)	gi 22651450	37954.5	6.76	7	101	100	57	99.958	2.13
23	TorsinA (Dyt1)	gi 22651450	37954.5	6.76	6	61	97.556	9	0	3.55
24	Annexin A2 (Anxa2)	gi 37590785	38653.8	7.55	19	405	100	199	100	1.63
25	Electron-transfer-flavoprotein, alpha polypeptide(EtfA)	gi 57527204	34929.5	8.62	13	281	100	155	100	1.64
26	Heat shock protein 27(Hspb1)	gi 204665	22878.6	6.12	13	530	100	373	100	-1.63
27	Biliverdin reductase B (Blvrb)	gi 34855391	22080.3	6.29	8	154	100	64	99.987	-1.72
28	Glutathione S-transferase Yb-1 subunit (Gstm1)	gi 1943434	25752.1	8.42	18	277	100	38	95.52	-2.29
29	Splicing factor, arginine/serine-rich 3 (Sfrs3)	gi 34852270	14947.5	9.63	9	136	100	21	0	-1.78
30	Splicing factor, arginine/serine-rich 3 (Sfrs3)	gi 34852270	14947.5	9.63	8	157	100	59	99.957	1.69
TorsinA(ΔE)										
1	Leucine rich protein 157 precursor (Lrpprc)	gi 34539486	156553.5	6.2	23	226	100	44	98.921	1.74
2	Chain B, Rat Brain Hexokinase Type I Complex With Glucose And Inhibitor Glucose-6-Phosphate (Hk1)	gi 5542104	102455.2	6.29	24	203	100	3	0	2.02
3	Eukaryotic translation elongation factor 2 (Eef2)	gi 56082	95222.9	6.41	9	68	99.414	?	?	?
4	Staphylococcal nuclease domain containing 1 (Snd1)	gi 77404395	101888.5	6.76	15	288	100	155	100	1.54
5	ATP citrate lyase (Acy)	gi 8392839	120558.7	6.96	37	408	100	58	99.974	1.71
6	Eukaryotic translation elongation factor 2 (Eef2)	gi 56082	95222.9	6.41	29	296	100	37	95.637	-1.83
7	Secretogranin II (Seg2)	gi 12083601	70987.4	4.71	21	187	100	47	99.719	-1.88
8	Stress-70 protein, mitochondrial (Hspa9)	gi 55584140	73698.8	5.87	26	466	100	269	100	1.74
9	N-ethylmaleimide sensitive fusion protein (Nsf)	gi 13489067	82600	6.55	23	256	100	5	0	1.78
10	Acyl-CoA synthetase long-chain family member 1, isoform CRA_a (Acs11)	gi 149021423	67421.8	6.77	11	229	100	131	100	1.53
11	Retinoblastoma binding protein 4 (Rbbp4)	gi 157817007	51623.9	4.79	8	58	94.138	?	?	-2.9
12	Protein disulfide isomerase family A, member 6 (Pdia6)	gi 52345385	47190.7	4.95	14	495	100	356	100	1.62
13	Glutamate dehydrogenase 1 (Glud1)	gi 56200	61389.3	8.05	15	146	100	32	88.241	-1.76
14	M2 pyruvate kinase (Pkm2)	gi 206205	57744	7.15	20	343	100	115	100	-1.75
15	Sucl2a2 protein (Sucl2a2)	gi 187469277	47357.6	6.06	10	150	100	58	99.892	1.51

Spot	Top Ranked Protein (Gene symbol)	Accession No.	Protein		Peptide Count	Protein Score	Protein Score C.I. %	Total Ion Score	Total Ion C.I. %	Volume Change
			MW	PI						
16	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 6 (Serpinb6a)	gi 40018548	42990.9	5.79	9	84	99.987	14	0	1.76
17	Solute carrier family 25 (mitochondrial carrier, phosphate carrier), member 24 (Slc25a24)	gi 189011598	65127.5	8.95	14	178	100	90	100	1.62
19	Phosphoglycerate kinase 1 (Pgk1)	gi 40254752	44510	8.02	2	94	99.997	79	99.999	1.59
20	Tropomyosin 4 (Tpm4)	gi 6981672	28492.5	4.66	14	131	100	3	0	-1.81
21	Microtubule-associated protein, RP/EB family, member 1 (Mapre1)	gi 78097100	29985.2	5.12	9	259	100	149	100	1.56
22	Acidic ribosomal phosphoprotein P0 (Arbp)	gi 11693176	34193.8	5.91	13	593	100	413	100	1.58
23	Aldo-keto reductase family 1, member B1 (Akr1b1)	gi 6978491	35774.3	6.26	6	62	97.719	?	?	3.79
24	Aldo-keto reductase family 1, member B1 (Akr1b1)	gi 6978491	35774.3	6.26	12	208	100	95	100	?
25	TorsinA (Dyt1)	gi 22651450	37954.5	6.76	5	76	99.924	42	98.142	17.69
26	Annexin A2 (Anxa2)	gi 37590785	38653.8	7.55	21	310	100	80	100	1.83
27	Glyceraldehyde-3-phosphate dehydrogenase (Gapdh)	gi 8393418	35805.2	8.14	11	212	100	118	100	1.53
28	Actin related protein 2/3 complex, subunit 2 (Arpc2)	gi 34876797	34325.5	6.84	14	164	100	8	0	1.83
29	Electron-transfer-flavoprotein, alpha polypeptide (EtfA)	gi 57527204	34929.5	8.62	13	607	100	472	100	1.57
30	Heat shock protein 27 (Hspb1)	gi 204665	22878.6	6.12	12	562	100	412	100	-1.64
33	Cofilin 1 (Cfl1)	gi 55778270	18520.7	8.22	5	71	99.732	16	0	-2.64

Table 2

Functional clustering of differentially expressed proteins (official gene symbols)

Energy/Redox	Other
Glycolysis Hk1 (B) Pgk1 (B) Pkm2 (B) Pdhb (WT) Gapdh (ΔE) Acetyl CoA/ Mitochondria Acly (B) Etfb (B) Slc25a24 (B) Lrppe (B) Acat2 (WT) Glud1 (ΔE) Acs11 (ΔE) Suc1a2 (ΔE) Hspa9 (ΔE) Redox Hspb1 (B) Gstm1 (WT) Blvrb (WT) Akr1b1 (ΔE) Pdia6 (ΔE)	Cytoskeleton Ezr (WT) Arpc2 (ΔE) Tpm4 (ΔE) Cfl1 (ΔE) Mapre1 (ΔE) Synapsis Nsf (B) Vgf (WT) Ppp1cc (WT) Napa (WT) Gnao1 (WT) Scg2 (ΔE) Gene expression Snd1 (B) Eef2 (B) Sfrs3 (WT) Bat1 (WT) Rbbp4 (ΔE) Other Anxa2 (B) Anxa5 (WT) Lamr1 (WT) Serpinb6a(ΔE) Arbp (ΔE)

Table 3

Identified proteins implicated in other forms of dystonia

	Human HomologueHuman Disease	Reference
<i>Direct</i>	SUCLA2mtDNA depletion/Leigh-like sdr. GLUD1Hyperinsulinism-hyperammonaemia sdr. ETFAglutaric aciduria type 2	Spinazzola et al. (2009) Bahi-Buisson et al. (2008) Schiff et al. (2006)
<i>Indirect</i>	LRPPRCLeigh syndrome, French Canadian variant PDHBPyrivate dehydrogenase complex deficiency	Mootha et al. (2003) Head et al. (2005)