Published in final edited form as:

Neuroscience. 2008 August 13; 155(2): 439–453. doi:10.1016/j.neuroscience.2008.04.053.

Glial Elements Contribute to Stress-induced TorsinA Expression in the Central and Peripheral Nervous Systems

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Abstract

DYT1 dystonia is caused by a single GAG deletion in Exon 5 of *TOR1A*, the gene encoding torsinA, a putative chaperone protein. In this study, central and peripheral nervous system perturbations (transient forebrain ischemia and sciatic nerve transection, respectively) were used to examine the systems biology of torsinA. After forebrain ischemia, quantitative real-time RT-PCR identified increased torsinA transcript levels in hippocampus, cerebral cortex, thalamus, striatum, and cerebellum at 24 h and 7 d. Expression declined toward sham values by 14 d in striatum, thalamus and cortex, and by 21 d in cerebellum and hippocampus. TorsinA transcripts were localized to dentate granule cells and pyramidal neurons in control hippocampus and were moderately elevated in these cell populations at 24 h after ischemia, after which CA1 expression was reduced, consistent with the loss of this vulnerable neuronal population. Increased *in situ* hybridization signal in CA1 stratum radiatum, stratum lacunosum-moleculare, and stratum oriens at 7 d after ischemia was correlated with the detection of torsinA immunoreactivity in interneurons and reactive astrocytes at 7 and 14 days. Sciatic nerve transection increased torsinA transcript levels between 24 h and 7 days in both ipsilateral and contralateral dorsal root ganglia (DRG). However, increased torsinA immunoreactivity was localized to both ganglion cells and satellite cells in ipsilateral DRG but was restricted to satellite cells contralaterally. These results suggest that torsinA participates in the response of neural tissue to central and peripheral insults and its sustained up-regulation indicates that torsinA may contribute to remodeling of neuronal circuitry. The striking induction of torsinA in astrocytes and satellite cells points to the potential involvement of glial elements in the pathobiology of DYT1 dystonia.

Keywords

DYT1; dystonia; reactive astrocytes; hippocampus; satellite cells; dorsal root ganglia

DYT1 dystonia obeys an autosomal dominant inheritance pattern with reduced penetrance, usually begins in childhood and frequently generalizes. The causal mutation is a GAG deletion in the *TOR1A* gene that removes a single glutamic acid residue near the carboxy terminus of the encoded protein torsinA (Ozelius et al., 1997; Ozelius et al., 1999). TorsinA belongs to the

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ATPases associated with a variety of cellular activities (AAA+) superfamily of proteins. TorsinA possesses an N-terminal signal sequence, a single AAA+ module that includes Walker A and Walker B nucleotide binding motifs, sensor 1 and sensor 2 regions, and two biochemically-confirmed glycosylation sites (Ozelius et al., 1997; 1999; Neuwald et al., 1999; Kamm et al., 2004; Callan et al., 2007). Members of the AAA+ family function as molecular chaperones for protein quality control (protein complex assembly, operation, disassembly, protein folding, unfolding, and degradation), membrane fusion and vesicular transport, and cytoskeletal regulation (Neuwald et al., 1999; Vale, 2000; Ogura and Wilkinson, 2001).

In cultured cells, the majority of torsinA is located in lumen of the endoplasmic reticulum (ER) and associates peripherally with the ER membrane, possibly via binding to an integral ER membrane protein (Hewett et al., 2003; Kuner et al., 2003; Liu et al., 2003; Callan et al., 2007). When overexpressed, torsinA is enriched at the nuclear envelope (Goodchild and Dauer, 2004; Naismith et al., 2004). In normal brain, torsinA is present in neuron perikarya and extends distally to the tips of dendrites and axons (Konakova et al., 2001; Konakova and Pulst, 2001; Augood et al., 2003; Kamm et al., 2004).

An assortment of studies has indicated that torsinA may function as a chaperone for unfolded or degraded proteins and may facilitate movement of polytopic proteins to the cell surface (Torres et al., 2004). TorsinA has been found localized to Lewy bodies in Parkinson's disease brain and inclusion bodies in trinucleotide repeat diseases (Shashidharan et al., 2000; Sharma et al., 2001; Walker et al., 2003). Overexpression of torsinA suppresses aggregation of α -synuclein in human H4 neuroglioma cells (McLean et al., 2002), and polyglutamine-induced protein aggregation in *C. elegans* (Caldwell et al., 2003). TorsinA facilitates clearance of another dystonia-related protein, ϵ -sarcoglycan, by the ubiquitin proteasome system and protects PC12 cells against a variety of cellular insults including serum deprivation and oxidative stress (Kuner et al., 2003; Shashidharan et al., 2004; Esapa et al., 2007). Similarly, torsinA protects dopaminergic neurons from oxidative stress in mice (Kuner et al., 2004) and *C. elegans* (Cao et al., 2005).

The chaperone functions of torsinA are critical during developmental processes which seemingly involve interaction with cytoskeletal elements (Ferrari-Toninelli et al., 2004; Kamm et al., 2004; Hewett et al., 2006). The expression of torsinA is developmentally regulated with the highest levels of transcript and protein seen during the prenatal and early postnatal periods (Xiao et al., 2004). The expression of torsinA is particularly intense in cerebellar cortex and striatal cholinergic interneurons at Postnatal Day 14, a period of intense dendritogenesis and synaptogenesis in these regions (Xiao et al., 2004; Vasudevan et al., 2006). A torsinA homologue, OOC-5, present in *C. elegans*, plays an essential role in Par protein localization. Mutations of *ooc-5* result in polarity defects in *C. elegans* embryos (Basham and Rose, 2001). Attenuated torsinA expression promotes neurite outgrowth in SH-SY5Y human neuroblastoma cells (Ferrari-Toninelli et al., 2004). Conversely, overexpression of mutant torsinA interferes with neurite extension (Hewett et al., 2006). In aggregate, these studies suggest that torsinA may be part of the molecular machinery required for topologically-precise neuritogenesis and/or associated nuclear rotation.

Since penetration of the DYT1 *TOR1A* Δ GAG deletion is only 30-40% (Ozelius et al., 1997), environmental factors may contribute to the development of dystonia in individuals who harbor this mutation. Trauma to the central (CNS) and peripheral (PNS) nervous systems, hyperthermia, cerebral ischemia and structural disease of the brain and spinal cord are known causes of secondary dystonia (Jankovic and Van der Linden, 1988; LeDoux and Brady, 2003). Similarly, these and other stressors (e.g., intense sensorimotor training) may trigger the onset of dystonia in genetically predisposed individuals (Treves and Korczyn, 1986). Despite

these important clinical associations, understanding of torsinA responses to neural perturbations *in vivo* is limited. Our study evaluated the temporal and spatial expression of torsinA in response to central and peripheral nervous system injury in models of transient forebrain ischemia and sciatic nerve transection, respectively.

Experimental Procedures

Animals

All experiments were performed in accordance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* and with approval of the Institutional Animal Care and Use Committee. Wistar rats (Hilltop Lab Animals, Inc., Scottsdale, PA, USA) were used for transient forebrain ischemia since the effects of 4-vessel occlusion (4-VO) have been well characterized in animals of this source and strain (Pulsinelli and Brierley, 1979; Ueda and Nowak, 2005). The sciatic nerve transections were performed in Sprague-Dawley rats (Harlan, Indianapolis, IN, USA).

Transient forebrain ischemia

Adult male Wistar rats weighing 250 – 300 g were subjected to transient 4-VO ischemia (Pulsinelli and Brierley, 1979) with direct current (DC) potential monitoring as previously described (Ueda and Nowak, 2005). In brief, surgical procedures were carried out under general inhalational anesthesia with 1-2% halothane in 70% N₂ and 30% O₂. The vertebral arteries were electrocauterized at the first cervical segment and silastic occluding devices were placed around the common carotid arteries. On the following day, rats were re-anesthetized and placed in a stereotaxic frame. Epidural and rectal temperatures were monitored and maintained at 37°C. Hippocampal DC potentials were recorded using glass microelectrodes. The carotid occluding devices were tightened to produce cerebral ischemia, as verified by 10 - 20 mV shifts in DC potential. All hemispheres included in these studies exhibited ischemic depolarizations of 7 - 9 min duration in hippocampus, previously shown to produce consistent loss of CA1 neurons (Ueda and Nowak, 2005). After release of occlusions, scalp incisions were closed, and rats were allowed to recover from anesthesia. Control rats underwent sham surgical procedures that included vertebral artery cauterization and electrode placement without carotid occlusion. Brain tissues for RNA extraction and *in situ* hybridization were obtained at five post-ischemic survival intervals (6 hr, 24 hr, 7 d, 14 d and 21 d), whereas tissues for immunocytochemistry were collected at three post-ischemic survival intervals (24 hr, 7 d, and 14 d). Preparations from three ischemic and three sham control rats were obtained at each survival interval.

For RNA extraction and *in situ* hybridization, rats were anesthetized with 5% halothane prior to decapitation. Brains were rapidly removed from the cranial vault and sectioned in the mid-sagittal plane. For *in situ* hybridization hemispheres were frozen in isopentane (-40 °C), sectioned parasagittally at 16 µm and stored at -80 °C in sealed slide boxes with desiccant capsules. For RNA extraction, cerebellum, striatum, thalamus, hippocampus and cerebral cortex were dissected from the hemisphere and homogenized in RNeasywiz™ (Ambion, Austin, TX, USA) on ice within 2-3 min after decapitation.

For immunocytochemistry, rats were overdosed with pentobarbital (100 mg/kg, IP) prior to transcardiac perfusion with heparinized saline and then 4% paraformaldehyde/0.1 M phosphate buffer (PB). Brains were post-fixed for 2 hrs, blocked, and incubated in a cryoprotectant solution (30% sucrose/0.1 M PB, pH 7.4) for at least 48 hrs. Blocks were sectioned at 20 µm and collected on SuperFrost®-Plus glass slides (Fisher Scientific, Pittsburgh, PA, USA).

Sciatic nerve transection

Three-month old male Sprague-Dawley rats (250-300 g) were subjected to left sciatic nerve transection under ketamine/xylazine (87/13 mg/kg, IP) anesthesia. The rat sciatic nerve was readily identified in the posterior thigh between the vastus lateralis and caput vertebralis muscles. In each rat, the left sciatic nerve was completely transected except for the medial epineurium which was left intact to allow for apposition of the cut ends to facilitate axonal regeneration. Wounds were closed and animals were allowed to recover from anesthesia in individual cages. Four age-, weight- and gender-matched non-surgical control rats were only subjected to anesthesia. Four surgical rats were employed at each of five post-transection intervals (24 hr, 3 d, 7 d, 14 d and 28 d). Three rats in each group were utilized for RNA extraction and one was used for immunocytochemical examination of the dorsal root ganglia (DRG).

For RNA extraction, rats were overdosed with pentobarbital (100 mg/kg, IP) prior to transcardiac perfusion with saline and then RNAlater (Ambion) as described in LeDoux et al. (2006). The vertebral column was sharply dissected from the remainder of the carcass and placed in a 15 cc conical tube containing RNAlater. The vertebral column was kept moist with RNAlater while the right and left lumbar (L3-L6) DRG were microsurgically isolated and collected into separate pools for subsequent RNA extraction.

For immunocytochemistry, rats were overdosed with pentobarbital (100 mg/kg, IP) prior to transcardiac perfusion with heparinized saline and then 4% paraformaldehyde/0.1 M PB. The vertebral column was dissected and kept moist with fixative while the right and left lumbar (L4-L5) DRG were isolated and collected into separate vials. DRG were post-fixed for an additional 2 hr and then transferred to a cryoprotectant solution for at least 48 hr. DRGs were sectioned at 15 μ m with a cryostat and collected on SuperFrost®-Plus glass slides.

Relative quantitative multiplex real-time RT-PCR (QRT-PCR)

Relative levels of torsinA mRNA were established in all brain and DRG tissues collected for QRT-PCR. In brief, total RNA was extracted with RNAwiz™. RNA purity and concentration were analyzed with a NanoDrop® spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). DNA was removed with DNA-free™ (Ambion). Reverse transcription was performed with Ambion's RETROscript™ kit using 200 ng total RNA as template. The reaction mix was incubated at 44 °C for 1 hr and then at 92 °C for 10 min. QRT-PCR was performed using Taqman® probes (Applied Biosystems, Foster City, CA, USA) for both the target gene (torsinA) and endogenous controls (18S rRNA for brain tissues and GAPDH for DRG). Primer and probe sequences and concentrations along with additional QRT-PCR technical details are provided in Xiao et al. (2004). Of note, technical triplicates were performed on each sample and median values were used for subsequent analyses.

Differential expression of torsinA was determined using the comparative threshold cycle (C_T) method. In particular, the expression levels of torsinA transcript in both ischemic and sham brain at each post-ischemic interval were calculated relative to the mean C_T value for the sham controls at the corresponding time points. A two-factor (treatment and post-ischemic interval) analysis of variance (ANOVA) was used for statistical analysis with SAS (SAS Institute, Cary, NC, USA).

For relative quantitative analysis of torsinA mRNA in the DRG, expression levels were calculated in reference to the mean C_T value for all right and left (L3-L6) DRG from the three non-surgical control rats. One-factor ANOVAs were used for independent analysis of data from the right and left DRG. Single degree-of-freedom post-hoc contrasts were limited to comparisons between individual post-surgical time points and ipsilateral control DRG.

Analysis of the ipsilateral (left) DRG at 28 d was limited to two rats since poor quality RNA was obtained from the third animal in this group.

***In situ* hybridization**

Radiolabeled (^{35}S -UTP) complementary RNA (cRNA) probes were used to localize torsinA transcript in cryostat sections of rat brain. Probe and primer pair sequences along with a detailed protocol are provided in Xiao et al. (2004). In brief, probes were made by *in vitro* transcription with T7 RNA polymerase (Ambion). After fixation, acetylation and dehydration through a graded series of ethanol, slides were incubated overnight in the hybridization buffer with radioactive probes (final concentration - 3×10^4 cpm/ μl). After treatment with RNase A (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 30 min followed by a high stringency wash at 60 °C for 1 h, slides were dehydrated and then exposed to Kodak Biomax MR Film along with ^{14}C Microscale™ autoradiography standards (RPA504, Amersham Biosciences, Piscataway, NJ, USA) for 5 days. Each sheet of film was exposed to paired ischemia and sham microscopic slides. Autoradiographic images were acquired in transmission mode (ScanMaker 9800 XL, Microtek, Carson, CA, USA) and imported into ImageJ (Java version of NIH Image, <http://rsb.info.nih.gov/ij/>) for region of interest (ROI) quantification of radioactivity. Optical density was calibrated to the set of autoradiography standards to generate measures of radioactivity (nCi/g tissue) for each ROI. Hippocampal ROIs included internal and external blades of the dentate granule cell layer (iDG, eDG), four regions of stratum pyramidale encompassing CA1 (Z1C, Z2C) and CA3 (Z3C, Z4C), and stratum oriens (SO), stratum radiatum (SR) and stratum lacunosum-moleculare (SLM) of the CA1 region. Signal was acquired from a total of 15 pixels (3×5 rectangle) in the center of each ROI, from seven sections per animal and the average was expressed relative to the mean obtained from sham sections hybridized in each of the experimental runs. Two-factor (treatment and post-ischemic interval) ANOVA was used for statistical analysis of relative radioactivity within each ROI with SAS.

Immunocytochemistry

The primary and secondary antibodies used for immunocytochemical studies are listed in Table 1. Cryostat brain and DRG sections were collected in eight and six series, respectively. One series of slides was air dried overnight and stained with cresyl violet (brain) or hematoxylin and eosin (DRG). The other series were processed for immunohistochemical detection of torsinA and biomarkers for hippocampal interneurons (somatostatin and parvalbumin), astrocytes (glial fibrillary acidic protein [GFAP]), microglia (OX-42), intermediate filaments (vimentin) and synaptic vesicles (synaptophysin). Anatomically-equivalent sections from animals representing each of the three post-ischemic survival intervals and corresponding sham controls were processed simultaneously. Ipsilateral and contralateral DRG sections from all post-surgical intervals were processed as a group.

For peroxidase-based detection of torsinA, sections were first incubated in the quenching buffer (10% methanol and 3% H_2O_2 in 0.02 M phosphate buffered saline [PBS]) for 5 min. Sections were then blocked and permeabilized in PBS containing 2% nonfat dry milk and 0.3% Triton X-100 (Sigma). Next, sections were incubated overnight with primary antibody diluted in PBS with 3% normal serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and 0.1% Triton X-100. Sections were then incubated with biotinylated secondary antibody diluted in PBS with 2% normal serum and 0.1% Triton X-100 for 4 hrs, peroxidase-labeled streptavidin (Vector Laboratories, Burlingame, CA, USA) for 1 hr and then nickel-intensified diaminobenzidine (Vector Laboratories) for visualization. Between each step, slides were thoroughly rinsed with PBS. After the final rinse, slides were air dried overnight, dehydrated, cleared and coverslipped with Permount (Fisher Scientific).

Double-label fluorescent immunocytochemistry was used to examine the relationships between torsinA and cell-type specific biomarkers (Table 1). Two primary antibodies recognizing torsinA were used in these studies, a rabbit polyclonal, TA1, and a mouse monoclonal, D-M2AB. When TA1 was employed, Cy2- or rhodamine red-X-tagged donkey anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories) were used to visualize torsinA-immunoreactivity (IR). With D-M2A8, biotinylated horse anti-mouse was used as a secondary antibody followed by Cy2-tagged streptavidin (Jackson ImmunoResearch Laboratories). Slides were thoroughly rinsed, dehydrated, cleared and coverslipped with 1,3-diethyl-8-phenylxanthine mounting medium (Sigma-Aldrich). Sections were visualized with both epifluorescence (Leica DM6000, Leica Microsystems Inc, Bannockburn, IL, USA) and confocal laser-scanning (Bio-Rad Laboratories, Hercules, CA, USA) microscopes. For consistency of illustration, all torsinA-IR was transferred to the RGB green channel and other biomarker-IR to the RGB red channel within Adobe Photoshop (San Jose, CA, USA).

Results

TorsinA transcript is up-regulated after transient forebrain ischemia

QRT-PCR was performed to evaluate the spatial and temporal expression of torsinA mRNA after transient forebrain ischemia, using 18S rRNA as an endogenous control. The efficiencies of torsinA (1.74) and 18S rRNA (1.75) amplification were practically identical; the slope of ΔC_T versus $\log_{10}$ ng total RNA was 0.054. The C_T values associated with both torsinA and 18S rRNA amplification showed strong linear relationships with $\log_{10}$ ng total RNA (torsinA: $R^2=0.978$; 18S rRNA: $R^2=0.990$).

Up-regulation of torsinA mRNA was not detected at the 6 hr post-ischemic interval. However, torsinA mRNA levels were significantly elevated within 24 hr after ischemia in all regions tested (Fig. 1). Transcript levels remained elevated at 7 days in all regions, and then declined toward sham values by 14 d in striatum, thalamus and cortex, and by 21 d in cerebellum and hippocampus.

In hippocampus and cerebellum, there were strong overall effects of ischemia (hippocampus [$F_{1, 20} = 21.63$, $P = 0.0002$], cerebellum [$F_{1, 20} = 13.12$, $P = 0.0017$]), post-ischemic interval (hippocampus [$F_{4, 20} = 5.24$, $P = 0.0047$], cerebellum [$F_{4, 20} = 4.17$, $P = 0.0129$]) and their interaction (hippocampus [$F_{4, 20} = 5.34$, $P = 0.0043$], cerebellum [$F_{4, 20} = 4.48$, $P = 0.0095$]) on torsinA mRNA levels (Fig. 1); in these two regions, increased expression of torsinA transcript were present at 24 hr, 7 d, and 14 d but not at 6 hr or 21 d. In the other three regions examined, the effects of ischemia were also considerable (striatum [$F_{1, 20} = 13.96$, $P = 0.0013$], thalamus [$F_{1, 20} = 8.43$, $P = 0.0088$], cerebral cortex [$F_{1, 20} = 17.72$, $P = 0.0004$]), although there were no significant effects of post-ischemic interval and the ischemia $\times$ post-ischemic interval interaction on the relative levels of torsinA transcript. As seen in Fig. 1, individual contrasts between the ischemia and sham groups were significant ($P < 0.05$) in all regions tested at the 24 hr and 7 d post-ischemic intervals and, additionally, in cerebellum and hippocampus at the 14 d post-ischemic interval. The degree of torsinA mRNA up-regulation was greatest in ischemic cerebral cortex and hippocampus (>3 fold). TorsinA mRNA up-regulation in thalamus and striatum was also substantial (>2 fold compared to control).

The distribution of torsinA mRNA expression determined by *in situ* hybridization was consistent with its generalized expression in major neuron populations, and this was not grossly impacted following ischemia (Fig. 2). A decline in signal was evident in the hippocampal CA1 pyramidal layer at longer post-ischemic intervals, consistent with the loss of these neurons. In addition, a more diffuse increase in torsinA hybridization was apparent in hippocampal neuropil at 7 and 14 days, as further supported by quantitative analyses. Among the 9 hippocampal ROIs analyzed, SLM ($F_{1, 16} = 18.13$, $P < 0.0006$) and iDG ($F_{1, 16} = 6.88$, $P <$

0.0185) showed the largest overall effects of ischemia on relative torsinA radioactivity (Fig. 3). The ischemia $\times$ post-ischemic interval interaction was also significant in iDG ($F_{3, 16} = 4.89$, $P < 0.0134$) and SLM ($F_{3, 16} = 5.71$, $P < 0.0074$). In SLM, for example, relative torsinA radioactivity was only increased at the 7 d post-ischemic interval. In Z1C and Z2C, the effects of post-ischemic interval ($F_{1, 16} = 13.03$, $P < 0.0001$ and $F_{1, 16} = 16.86$, $P < 0.0001$, respectively) and the ischemia $\times$ post-ischemic interval interaction ($F_{3, 16} = 10.32$, $P < 0.0005$; $F_{3, 16} = 17.33$, $P < 0.0001$) were highly significant due to increased signal in post-ischemic tissue at 24 hr and decreased signal at 7 d. Other noteworthy findings were the effects of ischemia in SR ($F_{1, 16} = 9.26$, $P < 0.0077$) and the ischemia $\times$ post-ischemic interval interaction in eDG ($F_{1, 16} = 8.37$, $P < 0.0014$). Individual contrasts between the ischemia and sham groups showed that relative torsinA radioactivity first became elevated at the 24 hr post-ischemic interval in iDG, eDG, Z2C, and Z1C ($P < 0.05$, for all). Then, at the 7 d post-ischemic interval, relative torsinA radioactivity was significantly up-regulated in SO, SR and SLM ($P < 0.05$, for all) and down-regulated in Z2C and Z1C ($P < 0.05$, for both). By 21 d post-ischemia, torsinA hybridization returned toward sham values in all regions except Z2C.

TorsinA is up-regulated in hippocampal astrocytes and interneurons

Changes in torsinA immunocytochemistry were consistent with the hybridization results and permitted signal localization to specific cell types. The initial increase in torsinA-IR at 24 hr occurred in the major hippocampal neuron populations that exhibited endogenous expression, whereas later diffuse increases overlapped the distributions of glial activation and synaptophysin up-regulation that took place in regions of CA1 neuron loss (Fig. 4).

Double labeling (Fig. 5) identified torsinA co-localization in GFAP-positive astrocytes but not OX-42-labeled microglia. Additional cells strongly-IR for torsinA included parvalbumin and somatostatin-IR interneurons. As seen in Fig. 6, hippocampal somatostatin and parvalbumin interneurons were torsinA-IR in both post-ischemic and sham tissue. However, after ischemia, the relative intensity of torsinA-IR was higher in these cell types than in adjacent neurons. Many of the parvalbumin-IR interneurons were scattered among pyramidal cells in the hippocampal pyramidal cell layer and granule cells in the dentate gyrus whereas others were localized to the dentate hilus and near the edge of the SO. Most of the somatostatin-IR interneurons were found in the dentate subgranular proliferative zone, dentate hilus, SO and the boundary region between the SR and SLM.

As seen in Fig 7, torsinA-IR was increased in neurons in the subgranular proliferative zone and hilus of the dentate gyrus and pyramidal cells of the CA2 and CA1 regions at 24 hr post-ischemia. At 7 d post-ischemia, torsinA-IR was mildly decreased in CA1 pyramidal cells and increased in neurons in the subgranular proliferative zone and hilus of the dentate gyrus, CA3/CA2 pyramidal cells and the cells in the SR. In the 14 d post-ischemic hippocampus, robust torsinA-IR was apparent in cells scattered within the SR and hilus of the dentate gyrus and moderately-IR cells were sparsely distributed within the SO.

TorsinA transcript and protein are up-regulated in bilateral DRG after unilateral sciatic nerve transection

TorsinA mRNA expression was increased after sciatic nerve transection (Fig. 8), as assessed by QRT-PCR. The amplification efficiencies for torsinA and GAPDH were 0.95 and 1.01 respectively and the slope of ΔC_T versus $\log_{10}$ ng total RNA was -0.043. The C_T values associated with torsinA and GAPDH amplification showed strong linear relationships with $\log_{10}$ ng total RNA (torsinA: $R^2 = 0.94$; GAPDH: $R^2 = 0.99$). The overall effect of sciatic nerve transection was robust in contralateral DRG ($F_{5, 12} = 8.31$, $P < 0.0015$) but did not reach statistical significance in ipsilateral DRG ($F_{5, 11} = 2.47$, $P < 0.098$). Post-hoc contrasts showed that torsinA expression differed between the control and surgical groups in both ipsilateral and

contralateral DRG at 24 hr and 3 d ($P < 0.05$, for all), with a significant increase persisting in the contralateral DRG at 7 d ($P < 0.05$).

In control DRG, torsinA-IR was detected in satellite cells, the somas of ganglion cells, and axons (Fig. 9). After sciatic nerve transection, torsinA-IR increased in both ganglion and satellite cells ipsilateral to the lesion, but only in satellite cells contralaterally. In ipsilateral ganglion cells, torsinA-IR was most prominent at 3 and 7 d after sciatic nerve transection. GFAP-IR co-labeled torsinA-positive satellite cells (Fig. 10).

TorsinA and vimentin co-localize in neuronal and glial elements in the CNS and PNS

Given the apparent up-regulation of GFAP after both CNS and PNS insults and prominent expression of torsinA in GFAP-IR reactive astrocytes and satellite cells, we elected to localize a closely related intermediate filament protein, vimentin, in hippocampus and DRG (Fig. 11). Vimentin-IR interneurons and reactive astrocytes were readily apparent in post-ischemic hippocampus. In interneurons, vimentin-IR was diffuse and co-localized with torsinA-IR. Although many reactive astrocytes expressed both vimentin and torsinA, co-localization of vimentin- and torsinA-IR was weak in these cells. After sciatic nerve transection, vimentin-IR was readily visualized in satellite cells of the ipsilateral and contralateral DRG.

Discussion

In normal brain, expression of torsinA is high in neurons and low in glia, and its developmental regulation indicates that torsinA may play a role in postnatal maturational events in the CNS (Xiao et al., 2004). The present results indicate an altered pattern of torsinA expression, notably involving astroglial cell types, after challenges to both the central and peripheral nervous systems. The similar quantitative and temporal features of torsinA up-regulation after sciatic nerve transection and 4-VO suggest the presence of common transcriptional control mechanisms, as well as a common role for this protein in the reparative and/or adaptive responses to such perturbations.

Post-ischemic torsinA expression in the CNS

Brief durations of global ischemia such as used in these studies result in selective loss of hippocampal CA1 pyramidal cells and interneurons of dentate hilus (Pulsinelli et al., 1982a; Nishino and Nowak, 2004; Ueda and Nowak, 2005). However, 4-vessel occlusion produces severe reductions in blood flow throughout forebrain structures, as well as appreciable perfusion deficits in cerebellum and brainstem (Pulsinelli et al., 1982b). The rapid, generalized increase in torsinA transcript levels throughout the brain (Fig. 1), maintaining the distribution in major neuron populations seen in control brain (Fig. 2), appears to reflect a relatively homogeneous response to this initial ischemic insult. This parallels in many respects the acute post-ischemic induction of many ischemia-responsive genes including many heat shock proteins (Nowak, 1991; Kawagoe et al., 1992; Xue et al., 1998; Nowak and Kiessling, 1999; Yagita et al., 2001; Tanaka et al., 2002). Studies in other *in vivo* and *in vitro* models also suggest a role for torsinA in response to oxidative stress (Hewett et al., 2003; Kuner et al., 2004; Cao et al., 2005). In contrast, the delayed component of torsinA increase includes prominent expression in reactive astrocytes in regions of hippocampus (e.g., CA1) known to undergo neuron loss and synaptic reorganization after ischemia (Arabadzisz and Freund, 1999; Briones et al., 2004). In agreement with previous work, astrocytic and microglial markers showed the most prominent post-ischemic response in the CA1 region (Schmidt-Kastner et al., 1990; Morioka et al., 1991; Gottlieb and Matute, 1999). Although not specifically evaluated in this study, the persistent modest increase in torsinA expression noted in cerebellum (Fig. 1) may be a correlate of glial activation in response to Purkinje cell vulnerability that also has been described after global ischemia (Diemer and Siemkovicz, 1981).

Astrocytes are believed to carry out protective functions such as maintenance of ionic homeostasis, prevention of excitotoxicity (via glutamate uptake), scavenging free radicals, provision of nutrients and growth factors, and support of synaptogenesis and neurogenesis (Panickar and Norenberg, 2005). Astrocytes are associated with the synapses, enwrapping many pre- and post-synaptic terminals, facilitating synaptic formation and synaptic neurotransmitter release (Araque et al., 1999; Grosche et al., 1999; Ventura and Harris, 1999; Riquelme et al., 2002; Liu et al., 2004; Sobkowicz et al., 2006). The up-regulation of torsinA in reactive astrocytes may facilitate one or more protective functions of astrocytes such as glutamate uptake via movement of the polytopic glutamate transporter to the cell surface. Alternatively, torsinA may contribute to the morphological and topological changes that reactive astrocytes must undergo in order to facilitate synaptogenesis and network reorganization (Lepekhn et al., 2001; Witcher et al., 2007).

These results also demonstrate up-regulation of torsinA expression in surviving somatostatin- and parvalbumin-IR interneurons (Fig. 6), which likely contribute to the partial maintenance of torsinA transcript levels in the CA1 stratum pyramidale at late post-ischemic intervals despite the death of most pyramidal neurons (Fig. 3). It is well established that parvalbumin-IR interneurons in the hippocampus are resistant to ischemic stress (Nitsch et al., 1989; Ferrer et al., 1995). Similarly, somatostatin-IR interneurons in the CA1 region are also relatively resistant to ischemia (Bering and Johansen, 1993; Bering et al., 1997). Surviving interneurons in the CA1 region undergo significant morphological changes and may serve as substitute targets for Schaffer collateral and other excitatory synaptic input after hippocampal ischemia (Arabadzisz and Freund, 1999). Accordingly, it is rational to postulate that torsinA may participate in structural changes and/or transport of cell surface receptors in these hippocampal interneurons.

Transient global ischemia is followed by neurogenesis in the subgranular proliferative zone (Sharp et al., 2002; Kokaia and Lindvall, 2003) and there is some evidence to suggest a parallel response in the posterior periventricular zone adjacent to the hippocampus (Nakatomi et al., 2002). Neuroblasts originating from the subgranular proliferative zone give rise to neurons within the granule cell layer and GFAP-IR astrocytes in the dentate hilus. Although BrdU labeling was not examined in this study, the numerous cells robustly-IR for torsinA in the subgranular proliferative zone (Fig. 7) may be such neuroblasts. Developmental profiles of torsinA transcript and protein expression are also consistent with a role for torsinA during neurogenesis in brain (Xiao et al., 2004).

TorsinA in the injured PNS

TorsinA is constitutively expressed in the PNS, where it is present in both neuronal and glia elements (ganglion and satellite cells, respectively) in the DRG. After unilateral sciatic nerve transection torsinA transcript increased bilaterally (Fig. 8). TorsinA immunoreactivity was detected in both cell types in the DRG ipsilateral to the lesion, but was more prominently increased in satellite cells contralaterally (Figs. 8-10). Bilateral effects of sciatic nerve transection are consistent with work from other laboratories. Ryoike and colleagues (2000) reported that a conditioning lesion (i.e., left sciatic nerve transection) enhanced expression of IL-1 β and TGF- β in the contralateral DRG and promoted nerve regeneration. Similarly, increased expression of four cytokines (IL-1 β , TGF- β 1, TNF- α , and IL-10) was apparent in contralateral DRG for up to 35 days after sciatic nerve transection (Ruohonen et al., 2002). Up-regulation of torsinA in ipsilateral ganglion cells is consistent with its potential role in the response to cellular stress as noted above in the context of cerebral ischemia. However, the mechanism for up-regulation of torsinA in contralateral satellite cells is less obvious. Satellite cells appear to play important roles in the physiology of their contiguous ganglion cells (Dublin and Hanani, 2007; Kuo et al., 2007). Satellite cells exhibit increased gap-junction coupling in

response to the ipsilateral application of noxious stimuli (Dublin and Hanani, 2007). Up-regulation of p75 in satellite cells appears to be responsible for sympathetic sprouting in the DRG after peripheral nerve lesions (Hu and McLachlan, 2000). Since unilateral sciatic nerve transection is associated with compensatory changes in gait and appendicular loads, up-regulation of torsinA in contralateral satellite cells could be related to increased signaling demands on the adjacent ganglion cells. Alternatively, up-regulation of torsinA in satellite cells could be part of a generalized response to the surgical intervention, possible due to plasma TGF- β . Interestingly, work in *Drosophila* suggests that TGF- β signaling may be defective in DYT1 dystonia (Koh et al., 2004). Over-expression of Smad2, a downstream effector of TGF- β , suppressed ultrastructural defects at the neuromuscular junction in Δ GAG human torsinA flies.

TorsinA structure and function

TorsinA protein is structurally related to the ClpB/Hsp100 family of proteins which are required for thermotolerance in yeast and plants (Hong and Vierling, 2000; Ung et al., 2007). Yeast and plant Hsp104, which are members of the same ClpB/Hsp100 subfamily of AAA+ proteins, have been shown to interact with Hsp70 and Hsp40 co-chaperones in the process of returning aggregated proteins to their active states (Weibezahn et al., 2004). There are no legitimate Hsp104 orthologs in mammals. Accordingly, torsinA may play a similar role in mammalian systems (McLean et al., 2002; Kuner et al., 2003).

In addition to its role in thermotolerance, plant Hsp104 also plays a critical role in the development of chloroplasts (Ung et al., 2007). In similar fashion, torsinA may be essential for postnatal maturation of the CNS (Xiao et al., 2004; Goodchild et al., 2005). Hence, the appearance of dystonia in carriers of the DYT1 Δ GAG *TOR1A* mutation could be due to defects in neurodevelopment, the stress response, or a combination thereof. Ultimately, these defects may be the consequence of anomalies in CNS intercellular signaling since torsinA appears to play an important role in synaptic vesicle recycling (Granata et al., 2007). In transgenic mice, for example, overexpression of mutant torsinA is associated with increased striatal dopamine turnover (Zhao et al., 2008). Theoretically, torsinA may contribute to vesicle turnover in both neurons and glia.

Several, largely-independent lines of evidence indicate that cytoskeletal interactions at the nuclear envelope and endoplasmic reticulum are an important aspect of torsinA biology. TorsinA knock-out and homozygous Δ GAG knock-in mice develop morphological abnormalities of the nuclear envelope (Goodchild et al., 2005). TorsinA interacts with LAP1 in the nuclear envelope and Lull1 in the endoplasmic reticulum (Goodchild and Dauer, 2005). In a yeast two-hybrid study, torsinA was shown to interact with kinesin light chain (Kamm et al., 2004). In more recent work, torsinA co-immunoprecipitated with a multi-molecular complex that included vimentin, tubulin, actin, kinesin light chain, LAP1, LULL1 and nesprin (Hewett et al., 2006; Nery et al., 2007). *In vitro* studies have shown that mutant torsinA interferes with cytoskeletal events which involve vimentin (Hewett et al., 2006). Vimentin, a member of the intermediate filament family of proteins, is expressed in developing brain (Sancho-Tello et al., 1995; Hutchins and Casagrande, 1989) and is also induced in reactive glia (Kindy et al., 1992; Braun et al., 1998). Vimentin plays an important role in cellular morphology and organelle positioning (Goldman et al., 1996). As shown here (Fig. 11), the increased detection of vimentin in reactive astrocytes and surviving interneurons after 4-VO is compatible with previous studies.

Conceptually, cellular polarity is of obvious importance to neural and neuronal architecture (Arimura and Kaibuchi, 2005). Theoretically, mutations in torsinA could interfere with the asymmetric localization of Par proteins (Basham and Rose, 1999). The polarity protein Par-3 interacts with the p75 neurotrophin receptor at the axon-glial junction during myelination in

the PNS (Chan et al., 2006). It may be speculated that defective or deficient torsinA could disrupt normal structural relationships between neurons and glia. Microstructural changes have been identified in white matter in DYT1 mutation carriers with diffusion tensor magnetic resonance imaging (DT-MRI) (Carbon et al., 2004; Blood et al., 2006). Fractional anisotropy (FA), a measure of axonal integrity and coherence, was significantly reduced in the white matter underlying sensorimotor cortex in DYT1 mutant carriers (Carbon et al., 2004).

Conclusions

Neurodevelopmental abnormalities may be central to the pathobiology of DYT1 dystonia given that generalized dystonia rarely develops during adulthood in Δ GAG mutation carriers (Bressman et al., 2000; Carbon et al., 2008). On the other hand, the reduced penetrance of the Δ GAG *TOR1A* mutation suggests that a perturbation (e.g., trauma, hypoxia, severe febrile illness, intense sensorimotor training) to the CNS or PNS may be required for the appearance of a dystonic phenotype. Hypoxic-ischemic insults are believed to be pathogenic in cases of dystonic cerebral palsy (Kyllerman, 1982; Treves and Korczyn, 1986; Cerovac et al., 2007), an example of secondary dystonia, and it is conceivable that perinatal factors such as hypoxia-ischemia may increase expressivity of dystonia in carriers of the DYT1 mutation. Its well-demarcated gross anatomy and precise intrinsic connections make the post-ischemic rat hippocampus an ideal model system in which to study the reactive synaptogenesis, astrogliosis, and neurogenesis that follow loss of CNS neurons, and sciatic nerve transection offers comparable advantages in the PNS. The induction of torsinA in glia suggests a novel avenue through which to explore the cellular and systems pathophysiology of DYT1 dystonia.

Acknowledgements

This work was supported by the Dystonia Medical Research Foundation and the National Institute of Neurological Disorders and Stroke (R01-NS048458 and R03-NS050185 to MSL, and R01-NS032344 to TSN). TorsinA antibodies were generous gifts from Dr. Vijaya Ramesh, Department of Neurology, Harvard Medical School

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Abbreviations

4-VO	4-vessel occlusion
AAA+	ATPases associated with a variety of cellular activities
ANOVA	analysis of variance
CNS	central nervous system
cRNA	complementary RNA
C_T	threshold cycle
DC	direct current
DG	dentate gyrus
DRG	dorsal root ganglia
ER	endoplasmic reticulum
GFAP	glial fibrillary acidic protein
IR	immunoreactivity
PB	phosphate buffer
PBS	phosphate buffered saline
PNS	peripheral nervous systems
QRT-PCR	

relative quantitative multiplex real-time reverse transcriptase polymerase chain reaction

ROI

region of interest

SLM

stratum lacunosum-moleculare

SO

stratum oriens

SR

stratum radiatum

Fig. 1.

Quantitative RT-PCR analysis of regional torsinA expression after transient forebrain ischemia. TorsinA transcript levels in each brain region are expressed relative to the mean C_T value for the sham controls at each time point. A significant increase in torsinA transcript was observed in all regions at 24 hr and 7 d post-ischemic intervals, persisting in cerebellum and hippocampus at 14 d. Results are expressed as the mean $\pm$ SEM (*, $P < 0.05$ for the difference between ischemic and sham groups at each interval).

Fig. 2.

In situ hybridization for torsinA. Representative parasagittal sections are illustrated for a sham control and at indicated post-ischemic intervals, together with higher magnification images of the corresponding hippocampus. Evident changes included a decrease in torsinA expression in the CA1 pyramidal cell layer at late post-ischemic intervals, accompanied by elevated signal in neighboring neuropil. Scale bars indicate 5 mm for whole-brain images and 500 μ m for hippocampal images. Upper panel identifies ROIs subjected to further quantitative analysis (see Fig. 3).

Fig. 3.

Quantitative *in situ* hybridization analysis of hippocampal torsinA transcript levels. TorsinA hybridization was determined in the ROIs indicated in Fig. 2 and expressed relative to the mean value for sham sections hybridized in each experimental run. A slight but significant increase in torsinA transcript was observed in dentate granule cells (iDG and eDG) and CA1 neurons (Z1C and Z2C) at 24 hr after ischemia. Expression in the CA1 pyramidal layer decreased at later intervals, accompanied by increased expression in SR, SLM, and SO at 7 d. Results are expressed as the mean $\pm$ SEM (*, $P < 0.05$ for the difference between ischemic and sham groups at each post-ischemic interval).

Fig. 4.

Distribution of torsinA immunoreactivity and comparison with other markers. Torsin A-IR was moderately increased in the major hippocampal neuron populations at the 24 hr post-ischemic interval, and overlapped the distributions of astrocyte (GFAP) and microglial (OX-42) activation and synaptophysin upregulation at later intervals. Confocal gray scale images of fluorescent immunocytochemistry are shown for torsinA (antibody TA1), GFAP, and OX-42, and inverted gray-scale images of peroxidase detection are shown for synaptophysin. Scale bar=500 μ m.

Fig. 5.

Cellular localization of torsinA immunoreactivity. High-magnification confocal images of double-label fluorescent immunocytochemistry for simultaneous detection of torsinA (green, right column gray scale) and cell markers GFAP, OX-42 (red, middle column gray scale) in post-ischemic (7 d) hippocampus. TorsinA co-localizes with GFAP-positive astrocytes but not OX-42-labeled microglia. Double-labeled cells appear yellow in the merged images. Scale bar=50 μ m.

Fig. 6. Cellular localization of torsinA immunoreactivity. High-magnification confocal images of double-label fluorescent immunocytochemistry for simultaneous detection of torsinA (green, right column gray scale) and cell markers parvalbumin and somatostatin (red, middle column gray scale) in sham and post-ischemic (7 d) hippocampus. TorsinA-IR is prominent in neurons expressing parvalbumin and somatostatin (arrows). Double-labeled cells appear yellow in the merged images. Scale bar=50 μ m.

Fig. 7.

Immunoperoxidase detection of torsinA in post-ischemic hippocampus. Using the TA1 polyclonal antibody, increased torsinA-IR was detected in the cells in the subgranular proliferative zone (arrow) and hilus of the dentate gyrus, and pyramidal cells in CA3 and CA2 region at all time points after ischemia, and in cells scattered within the SR and SO of CA1 at 14 d after ischemia. M, molecular layer; G, granule cell layer; and H, hilus of the dentate gyrus. Scale bar = 200 μ m.

Fig. 8.

Temporal profiles of torsinA mRNA expression in DRG after unilateral sciatic nerve transection. TorsinA mRNA expression levels in ipsilateral and contralateral DRGs were calculated in reference to the mean C_T value for all DRG from control rats (C). Prominent bilateral increases in torsinA transcript were observed bilaterally at 1 and 3 days. Symbols indicate the mean $\pm$ SEM (*, $P < 0.05$ for the difference between lesioned and control animals).

Fig. 9.

Immunocytochemical localization of torsinA expression in DRG after unilateral sciatic nerve transection. TorsinA-IR increases were detected ipsilaterally in both ganglion cells (G) and satellite cells (arrow), but only in satellite cells contralaterally. C, non-surgical control. Scale bar = 100 μ m.

Fig. 10.

Co-localization of torsinA and GFAP in satellite cells. Confocal images of fluorescent immunocytochemistry illustrate co-localization in satellite cells (arrow) of ipsilateral DRG 14 d after sciatic nerve transaction. Co-localization appears yellow in the merged images. G, ganglion cell. Scale bar=50 μ m.

Fig. 11.

Co-localization of torsinA and vimentin expression after ischemia and sciatic nerve transection. Confocal images of fluorescent immunocytochemistry illustrate co-localization in a subset of CA3 interneurons and CA1 astrocytes in 7 d post-ischemic hippocampus. Vimentin also co-localizes with the component of torsinA expression in satellite cells (arrow) of ipsilateral DRG 14 d after sciatic nerve transection. Co-localization appears yellow in the merged images. G, ganglion cell. Scale bars=50 μ m (top three panels) and 25 μ m (other panels).

Table 1

Primary and secondary antibodies

Antibody name	Type	Target	Concentration	Source
TA1	rabbit polyclonal	torsinA	1:500	Dr. Vijaya Ramesh, Harvard Medical School
D-M2A8	mouse monoclonal	torsinA	1:300	Dr. Vijaya Ramesh, Harvard Medical School
Anti-somatostatin	rabbit polyclonal	somatostatin	1:250	Chemicon International, Temecula, CA, USA
Parv-19	mouse monoclonal	parvalbumin	1:1000	Sigma, St. Louis, MO, USA
GA5	mouse monoclonal	GFAP	1:500	Chemicon
OX-42	mouse monoclonal	microglia	1:500	Chemicon
V9	mouse monoclonal	vimentin	1:10	Abcam, Cambridge, MA, USA
SY38	mouse monoclonal	synaptophysin	1:100	Abcam
biotinylated horse anti-mouse	horse polyclonal	mouse IgG (H+L)	1:500	Vector, Burlingame, CA, USA
biotinylated goat anti-rabbit	goat polyclonal	rabbit IgG (H+L)	1:500	Vector
Cy2-tagged donkey anti-mouse	donkey polyclonal	mouse IgG (H+L)	1:250	Jackson ImmunoResearch Laboratories
Cy2-tagged donkey anti-rabbit	donkey polyclonal	rabbit IgG (H+L)	1:250	Jackson ImmunoResearch Laboratories
rhodamine red-X-tagged donkey anti-mouse	donkey polyclonal	mouse IgG (H+L)	1:250	Jackson ImmunoResearch Laboratories
rhodamine red-X-tagged donkey anti-rabbit	donkey polyclonal	rabbit IgG (H+L)	1:250	Jackson ImmunoResearch Laboratories