Cellular Distribution of Torsin A and Torsin B in Normal Human Brain

Marina Konakova, PhD; Duong P. Huynh, PhD; William Yong, MD; Stefan M. Pulst, MD

Background: Early-onset torsion dystonia is an hyperkinetic movement disorder caused by a deletion of 1 glutamic acid residue in torsin A protein, a novel member of the AAA family of adenosine triphosphatases. No mutation has been found so far in the closely related torsin B protein. Little is known about the molecular basis of the disease, and the cellular functions of torsin proteins remain to be investigated.

Objective: To study the regional, cellular, and subcellular distribution of the torsin A and torsin B proteins.

Methods: Expression of torsin proteins in the central nervous system was analyzed by Western blot analysis and immunohistochemistry in human postmortem brain tissues.

Results: We generated polyclonal antipeptide antibodies directed against human torsin A and torsin B proteins. In Western blot analysis of normal human brain homogenates, the antibodies specifically recognized 38-kd endogenous torsin A and 62-kd endogenous torsin B. Absorption controls showed that labeling was blocked by cognate peptide used for immunization. Immunolocalization studies revealed that torsin A and torsin B were widely expressed throughout the human central nervous system. Both proteins displayed cytoplasmic distribution, although torsin B localization in some neurons was perinuclear. Strong labeling of neuronal processes was detected for both proteins.

Conclusions: Torsin A and torsin B have similar distribution in the central nervous system, although their subcellular localization is not identical. Strong expression in neuronal processes points to a potential role for torsin proteins in synaptic functioning.

Arch Neurol. 2001;58:921-927

Primary Early-Onset Torsion Dystonia

Primary early-onset torsion dystonia is an autosomal dominant inherited disorder that has been linked to the genetic locus DYT1 on human chromosome 9q34.1,2 Two genes from this locus (TOR1A and TOR1B) have been recently identified through positional cloning.3,4 The TOR1A gene encodes a 37-kd protein called torsin A. The adjacent TOR1B gene encodes a closely related protein called torsin B of unknown molecular weight. A GAG deletion at codon 302 in torsin A results in loss of 1 glutamic acid and causes disease, although penetrance of this mutant allele is only 30%.

Sequence analysis of the complementary DNA encoding torsin A and torsin B indicated that these proteins belong to the AAA family of adenosine triphosphatases (ATPases), associated with diverse cellular activities. This family of ATPases is defined by a conserved ATPase domain that contains Walker homology sequences. Since AAA-type ATPases are implicated in a wide variety of functions, it is difficult to predict what cellular processes involve the torsin proteins.

Despite significant progress in understanding the genetic basis of early-onset torsion dystonia, the molecular mechanism by which this mutation results in the disease phenotype is not well understood. The process is thought to involve a deficiency in dopamine release in the substantia nigra.5,6 To address the molecular mechanisms of early-onset dystonia, several studies have examined the anatomical distribution of torsin proteins in the central nervous system (CNS). One of these studies focused on the expression of torsin A and torsin B messenger RNA (mRNA) expression in the normal human brain7—torsin A mRNA was found in many brain regions, including the dopamine neurons of the substantia nigra; however, no expression of torsin B mRNA was detected in the same regions. One study examined the expression of torsin A protein in the frontal cortex, cerebellum, and substantia nigra of rat and human brains.8 Localization was nuclear and cytoplasmic in neurons; the distribution of torsin B protein was not analyzed. Nuclear localization of torsin A in this study did not correspond to cytoplasmic
METHODS

PREPARATION OF ANTIPETIDE ANTIBODIES

Antisera against human torsin A and B were generated by immunizing rabbits with synthetic peptides conjugated to keyhole limpet hemocyanin. These peptides were synthesized with a COOH-terminal cysteine. Synthetic peptides used for immunization were as follows: peptide 1 (GQKRSLSREALQK [residues 51-65]) and peptide 2 (SGQVREDIKLKDIE [residues 224-237]) of human torsin A and peptide 3 (HEQKIKLQDYDQLQK [residues 77-90]) of human torsin B.

AFFINITY PURIFICATION OF ANTIBODIES

Individual peptides were coupled to cyanogen bromide-activated Sepharose 4B (Amersham Pharmacia Biotech, Piscataway, NJ) in 0.1-mol/L carbonate-bicarbonate buffer (pH 11) overnight. Antiserum samples were passed through the columns in a slow, regulated flow (0.5 mL/min). The columns were then washed with phosphate buffer and peptide-specific antibodies were eluted with 0.5-mol/L glycine hydrochloride buffer (pH 2.5). The eluants were immediately neutralized with 10% (vol/vol) Tris base buffer (pH 8.0), desalted, and concentrated with Centriprep 30 columns (Amicon Pharmaceuticals Inc, Beverly, Mass).

PROTEIN EXTRACTION

Nondenaturing Triton X-100 lysates of human trabecular bone (HTB) cells were made by resuspending cell pellets in ice-cold lysis buffer (100-mmol/L Tris hydrochloride, pH 7.5; 150-mmol/L sodium chloride; 5-mmol/L EDTA; 1% Triton X-100; and a cocktail of protease inhibitors). Lysates were clarified by centrifuging in a microcentrifuge at full speed for 15 minutes. Brain homogenates were prepared as described previously. To address the discrepancies in previous studies and to compare torsin A and torsin B protein distribution, we generated and characterized torsin A– and torsin B–specific antibodies. Using these antibodies, we performed a comprehensive comparative analysis of the cellular expression of torsin A and torsin B in the human CNS. We also made the first estimate of the molecular weight of torsin B.

GEL ELECTROPHORESIS AND WESTERN BLOTTING

Cell lysates were mixed with an equal volume of 2X loading buffer (133-mmol/L Tris hydrochloride, pH 6.8; 20% glycerol; 4% sodium dodecyl sulfate; 5% 2-mercaptoethanol; and 0.0025% bromphenol blue) and subjected to one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (4%-15% acrylamide gels) according to the method of Laemmlli. After electrophoresis, proteins were transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech) in transfer buffer (25-mmol/L Tris, 192-mmol/L glycine, and 20% methanol) using a tank blot apparatus. Blots were stained with Ponceau S to verify equal protein loading per lane. After 1 hour of blocking using 5% nonfat milk powder in Tris-buffered saline with Tween (20-mmol/L Tris-hydrochloride, pH 7.6; 137-mmol/L sodium chloride; and 0.05% Tween 20), blots were probed with antibodies (1 or 2 µg/mL, diluted in Tris-buffered saline with Tween containing 5% milk powder) overnight. The primary antibodies were detected with horseradish peroxidase-conjugated antirabbit antibodies (1:5000 dilution in Tris-buffered saline with Tween containing 5% milk powder) and an ECL detection system (Amersham Pharmacia Biotech).

IMMUNOHISTOCHEMISTRY

All immunohistological staining was performed on 7-µm sections. Tissue sections were dewaxed in xylene and rehydrated through washes in decreasing concentrations of ethanol (100%, 95%, and 70%). Sections were subsequently treated with a protease cocktail (Biomeda Corp, Hayward, Calif) and avidin-biotin and blocked with 3% normal goat serum. Sections were then incubated with torsin A and B antibodies, 20 µg/mL, overnight at 4°C. Primary antibodies were detected using a Vector avidin-biotin complex elite peroxidase kit (Vector Laboratories, Burlingame, Calif), enhanced by diaminobenzidine (Vector Laboratories, Burlingame, Calif), and visualized with diaminobenzidine- and 2-mercaptoethanol; and 0.0025% bromphenol blue) and subjected to one-dimensionally to known proteins. Two torsin A–specific antibodies (A1 and A2) and 1 torsin B–specific (B1) antibody were prepared. The A1 and A2 antibodies were generated using an N-terminal (residues 51-65) and a C-terminal (residues 224-237) torsin A peptide, respectively. The B1 antibody was raised against an N-terminal torsin B peptide (putative residues 77-90).

Expression of torsin A and B proteins in the brain was examined by Western blot analysis of whole human brain homogenates (Figure 1A). Analysis using A1 and A2 antibodies revealed that torsin A migrates as a single band with an apparent molecular mass of approximately 38 kd (Figure 1A, lanes 1 and 4), which is in agree-
with the molecular weight of 37.8 kd predicted from the torsin A complementary DNA sequence. Both anti–torsin A antibodies recognized the same protein band, indicating the specificity of the generated antibodies. The specificity was further verified by preabsorption of antibodies with peptides used for immunization. Thus, immunorecognition of the 38-kd band was completely blocked by preabsorption of A1 antibody with the aminoterminal peptide (peptide 1), 100 µmol/L, and A2 antibody with the carboxy-terminal peptide (peptide 2), 100 µmol/L (Figure 1A, lanes 2 and 5). Preabsorption of antibodies with unrelated peptides did not alter the specific immunorecognition of the 38-kd torsin A protein (Figure 1A, lanes 3 and 6).

Immunoblotting analysis using B1 antibody revealed that torsin B underwent electrophoresis as a 62-kd protein (Figure 1A, lane 7). This is the first estimate of the molecular weight of endogenous torsin B. As with torsin A antibodies, the immunoreactivity of torsin B–specific B1 antibody could be completely blocked by preabsorption with homologous peptide (peptide 3), 100 µmol/L (Figure 1A, lane 8). Preabsorption of the B1 antibody with the same amount of an unrelated peptide (peptide 1) did not affect its ability to recognize torsin B (Figure 1A, lane 9).

To gain additional information about specificity, we performed a Western blot analysis using Triton X-100 (Sigma Chemical Co, St Louis, Mo) extracts of human neuroblastoma HTB10 cells. This cell line expressed a single immunoreactive band when probed with anti–torsin A antibodies (Figure 1B, lanes 1 and 2). Both antibodies (A1 and A2) showed similar reaction patterns. In HTB10 cells, torsin A migrated as a protein with an apparent molecular weight of 35 kd. In contrast to torsin A, torsin B was not detectable in HTB10 cells (Figure 1B, lane 3).

**IMMUNOHISTOCHEMICAL ANALYSIS**

**Torsin A Expression in the CNS**

To determine the regional and cellular distribution of torsin A and B, we performed immunohistochemical analyses of normal human brain using paraffin-embedded sections. These studies demonstrated a widespread distribution of torsin proteins in the central nervous system. Both torsin A–specific A1 and A2 antibodies and torsin B–specific B1 antibody demonstrated strong immunoreactivity in all brain regions studied. In all regions examined, labeling for torsin A and B staining was neuronal and cytoplasmic. Expression was not restricted to neuronal cell bodies, and strong to moderate staining was observed in the neuropil in all areas of the human brain. Preabsorption of antibodies with the homologous peptide, 100 µmol/L (Figure 2A and Figure 3A), significantly reduced specific staining, confirming the specificity of antibodies on Western blots.

In the basal ganglia, weak torsin A–immunoreactive labeling was observed in the caudate and putamen (Figure 2B and 2C). In the matrix regions, torsin A–specific staining was detected in numerous cell popula-

tions although it was almost absent in the fibers. In contrast, no patch neurons were labeled, and torsin A immunolabeling was restricted to fibers. Moderate torsin A immunoreactivity was also observed in the globus pallidus (data not shown). In the substantia nigra, the cell bodies of dopaminergic neurons were weakly labeled with staining extending to the cell processes (Figure 2D).

In the cerebellum, the most intense labeling was observed in Purkinje cells and in cells in the dentate nucleus. In the Purkinje cells, torsin A–specific immunoreactivity was localized in cell bodies and dendrites (Figure 2E and 2F). No staining was observed in the glial cells of the molecular layer. Torsin A was not expressed in the cell bodies of granule neurons.

In the frontal cortex, torsin A labeling was detected in all layers and was especially prevalent in the pyramidal neurons of layers III and V (Figure 2G and 2H). Staining was not restricted to the cell bodies and extended into axons and dendrites. Light staining was also observed in the neuropil of all cortical areas and in the white matter.

Torsin A–positive cells were detected in all hippocampal subfields, including the dentate gyrus and subiculum as well as the CA1 and CA3 regions (Figure 2I and 2J). In the Purkinje cells, torsin A–specific immunoreactivity was localized in dendrites (Figure 2K). Some Golgi cells of the granular layer were faintly labeled. Interestingly, strong torsin A immunoreactivity was found in cerebellar glomeruli (Figure 2L), which consist of mossy fiber terminals, Golgi cell axons, and granule cell dendrites. The neurons of the dentate nuclei were moderately immunopositive whereas the surrounding fibers were strongly torsin A–positive.

The highest intensity of staining was found in the pyramidal neurons of CA3 and in the granule cells of the dentate gyrus. Light staining was also observed in the neuropil of the hippocampal formation.

In the midbrain, the most abundant torsin A–immunoreactive neurons were detected in the oculomotor nucleus (Figure 2M and 2N). Regions that exhibited moderate expression of torsin A were the nucleus of...
the geniculate body, the reticular formation, and the superior colliculus. No obvious labeling of pigmented neurons of the substantia nigra was observed. It is notable that strong to moderate expression of torsin A was observed in the fibers throughout all the midbrain areas, with the most intense signals detected in the brachium of the superior colliculus and the mesencephalic trigeminal nucleus.

In the thalamus, clusters of torsin A–positive cells were found in the anterior nuclear group and in the dorsomedial nucleus (Figure 2O and 2P). Immunostaining was also detected in neuronal fibers of the ventromedial nucleus. In the spinal cord, high immunoreactivity was seen in almost all regions (Figure 2Q and 2R). Prominent labeling was observed in the motor neurons of the anterior horn, which extended throughout the axons. Dense punctate torsin A immunolabeling was seen in the neuropil.

Torsin B Expression in the CNS

Immunohistochemical analysis of torsin B distribution revealed a pattern of expression similar to that of torsin A. Torsin B–immunopositive cells were detected in all brain regions studied. Torsin B exhibited a slightly different subcellular localization compared with torsin A; it was found exclusively in the cytoplasm, and unlike torsin A, its expression was polarized toward the cell edge. Torsin B labeling was completely abolished by addition of the immunizing peptide, confirming the specificity already shown on Western blots (Figure 3A).

In the basal ganglia, neurons in the caudate and putamen were strongly labeled (Figure 3B and 3C). As with torsin A, differential staining of patch and matrix putamen was observed. The matrix region contained numerous torsin B–immunopositive cells but was nearly devoid of

Figure 2. Immunolocalization of torsin A in the normal human brain. The distribution of torsin A immunoreactivity was determined with A1 antibody, 20 µg/mL. A–C, Patches and matrix compartments of the caudate nucleus. D, Dopaminergic neurons of the substantia nigra. E, Molecular and Purkinje cell layers of the cerebellum. F and K, Purkinje neurons of the cerebellum. G and H, Large pyramidal neurons of layer III of the cerebral cortex. I and J, Pyramidal neurons of layer CA1 of the hippocampus. L, Glomeruli in the granular layer of the cerebellum. M and N, Neurons of the oculomotor nucleus of the midbrain. O and P, Neurons of the anterior nuclear group of the thalamus. Q and R, Motor neurons of the spinal cord. In part A, antibody preabsorbed with peptide 1, 100 µmol/L, was used for staining (all sections were counterstained with hematoxylin; original magnification ×100 [parts A, B, G, Q, and P], ×250 [parts C, F, H, J, K, L, N, and P], and ×50 [parts D, E, I, M, and Q]).
immunoreactive fiber bundles, whereas the patch region exhibited intense immunolabeling of fibers with no cellular staining. In the globus pallidus, strong expression of torsin B was detected in some neuronal cells (data not shown). Pigmented dopamine-containing cells of the substantia nigra were moderately stained in their cell bodies and proximal processes. Stronger staining was observed in the cellular fibers in the neuropil (Figure 3D).

In the spinal cord, torsin B immunoreactivity was present in the cell bodies of motor neurons as well as in the surrounding neuropil (Figure 3E and 3F). Torsin B was strongly expressed in all cortical layers, including the pyramidal neurons of layers III and V (Figure 3G, 3H, and 3I).

Moderate torsin B immunoreactivity was present throughout the thalamus. Significant staining was observed in the neurons of the anterior nuclear group (Figure 3J, 3K, and 3L), while neurons of the dorsomedial nucleus were only weakly stained. In the midbrain, strong labeling was detected in the oculomotor neurons and in the medial geniculate body (Figure 3M and 3N). Similar to torsin A, intense fiber staining was seen throughout all areas of the midbrain. Torsin B expression was also observed throughout the hippocampal formation (Figure 3O and 3P). In this structure, labeling was prevalent in the pyramidal neurons of CA3 and the granule cells of the dentate gyrus. There was also significant staining within the neuropil.

Little staining for torsin B was seen in the cerebellum (Figure 3Q and 3R). In contrast to torsin A labeling, Purkinje cells as well as neurons of the dentate nucleus did not show any significant immunoreactivity for torsin B. However, there was some staining in the granules of the granular layer (not shown).
Torsins A and B are physically close on chromosome 9 and share significant homology (72% identity at the nucleotide level and 69% identity in the predicted amino acid sequence). We generated 3 different antibodies against torsin proteins, and several lines of evidence suggest that the antibodies are specific to the individual proteins.

SPECIFICITY

Two anti–torsin A antibodies, designated A1 and A2, specifically detected a single protein band of approximately 38 kd. This finding was in concordance with the predicted molecular weight of torsin A calculated from its deduced amino acid sequence. The 38-kd protein was not recognized by the torsin B antibody. This antibody recognized a single band of 62 kd, which in turn was not recognized by the torsin A antibodies. Since a full-length torsin B complementary DNA sequence has not yet been isolated, we cannot compare the observed with the predicted molecular weight. The finding of a larger torsin B protein, however, is consistent with the observation that the torsin B mRNA seen by Northern blotting is approximately 1 kb larger than the torsin A mRNA. The torsin B antibody did not recognize any proteins in HTB10 cells, which apparently only express torsin A. In addition, staining of Western blots and immunocytochemical labeling were completely eliminated when the antibodies were preincubated with their respective peptides (Figures 1, 2, and 3). This absorption reaction was specific, because absorption with unrelated peptides did not affect labeling (Figure 1).

In HTB10 cells, torsin A migrated slightly faster, demonstrating an apparent molecular weight of 35 kd. This may be a result of different posttranslational modifications of torsin A in cultured cells. Another torsin A antibody raised against a different C-terminal epitope has recently been described. This antibody detected a protein in human tissue with an apparent molecular weight of 48 kd. The recombinant torsin A protein, however, showed the predicted electrophoretic migration. Interestingly, this discrepancy in the migration pattern of endogenous and recombinant torsin A was eliminated if tissue homogenates were incubated with ATP; yet in another study, 3 rabbit polyclonal antibodies and mouse monoclonal antibody against torsin A were generated and shown to recognize a protein with a predicted size ranging from 37 to 39 kd in different cell types.

EXPRESSION MORE WIDESPREAD THAN DISEASE

Torsins A and B were widely expressed in all human brain regions studied, including areas affected by disease (the extrapyramidal system) and areas not implicated in the disease process (the cortex, cerebellum, and spinal cord). Torsin A and B expression was neuronal, with no detectable expression in glial cells. This expression pattern is in accordance with expression analysis of torsin A mRNA. The main expression site of torsin A and B in neurons was in the cytoplasm, which contrasts with a recent study that also detected nuclear labeling.

No neuronal cytoplasmic labeling was observed in the patch regions of the caudate and putamen. The striking contrast between patch and matrix compartments probably relates to their distinct input-output organization. Thus, the matrix area, containing cholinergic neurons, receives input from sensory and motor cortical layers and projects to the substantia nigra pars reticulata. The patch area, which is rich in enkephalin and substance P, receives input from the prelimbic cortex and hippocampus while it projects to the substantia nigra pars compacta. The dopaminergic neurons of the substantia nigra were faintly labeled with both torsin A– and torsin B–specific antibodies. However, the neuropil of the substantia nigra consistently showed stronger staining as compared with the neuronal cell bodies.

The distribution of torsin A and B labeling was almost identical except for the cerebellum. While strong torsin A immunoreactivity was observed in the Purkinje cells and dentate nucleus, immunolabeling for torsin B was significantly weaker in these structures. We also observed a slightly different pattern in the intracellular distribution of these 2 proteins. Torsin A staining was diffusely cytoplasmic, whereas torsin B staining was considerably polarized in the cytoplasm of some neurons. No nuclear torsin A or torsin B immunoreactivity was observed. This finding was in contrast to a recent study in which torsin A immunoreactivity was localized to both the nucleus and cytoplasm. However, the cytoplasmic localization of torsin A and its extension to neurites described herein appear to be similar to the previous findings obtained through immunofluorescence analysis of transfected cells. In these studies, torsin A was found throughout the cytoplasm with a high degree of colocalization with the endoplasmic reticulum.

Findings by other research groups in rodents and by us in the human brain clearly demonstrate the presence of torsin A and B proteins in brain structures unaffected by disease. Why these regions (which are apparently spared in disease) have high levels of torsin A and B expression will remain unclear as long as the cellular functions of these proteins continue to elude us. The widespread and abundant distribution of the torsin proteins detected in the present study suggests their involvement in a number of important functions within the human brain. Our data are consistent with the positron emission tomography scan analyses of dystonia patients that described increased metabolic activity in the midbrain, cerebellum, and thalamus. Of particular interest will be the analysis of human brains of dystonia patients, especially those with DYT1 gene mutations and those with sporadic dystonia, to determine whether there are any differences in the expression of these proteins between normal and diseased brains.

Both torsin A and torsin B contain 1 ATP-binding site and have sequence homology with heat-shock proteins of the Hsp100/CliP family. Moreover, their sequences include a conserved module of approximately 220 amino acids, which allows their classification as new members of the AAA protein family (ATPase associated with diverse activities). Members of this family include spastin and...
the PEX proteins. Mutations in these proteins cause spastic paraplegia or peroxisome biogenesis disorders, respectively.

Staining for torsins A and B was granular, suggesting labeling of synaptic vesicles. Torsin A may control dopamine release by regulating the transport of dopamine-containing vesicles or vesicle fusion with the plasma membrane, or both. In eukaryotic cells, several AAA ATPases have been shown to be involved in different aspects of intracellular vesicle trafficking. The distribution of torsins A and B in the human brain has a striking resemblance to that of parkin. Although parkin is a neuronal cytoplasmic protein, most of the labeling was detected in fibers. Similar to torsin A and B, parkin labels glomeruli in the granule cell layer of the cerebellum. These are regions of intense synaptic connection, suggesting that all 3 proteins may play a part in synaptic transmitter release. The conserved AAA ATPase domains further suggest that this class of proteins may be involved in regulating transmitter release in response to cellular ATP.

Accepted for publication December 15, 2000.

This work was supported by the Carmen and Louis Warschaw Endowment Fund for Neurology, by FRIENDs of Neurology, and by grant R01-NS33123 (Dr Pulst) from the National Institutes of Health, Bethesda, Md.

Corresponding author and reprints: Stefan M. Pulst, MD, Division of Neurology, Cedars-Sinai Medical Center, UCLA School of Medicine, 8700 Beverly Blvd, Los Angeles, CA 90048 (e-mail: stefan.pulst@cshs.org).

REFERENCES


©2001 American Medical Association. All rights reserved.

Downloaded From:  by a Non-Human Traffic (NHT) User  on 10/30/2018