

Viewpoints

Current Gaps in the Understanding of the Subcellular Distribution of Exogenous and Endogenous Protein TorsinA

N. Charles Harata*

Department of Molecular Physiology & Biophysics, University of Iowa Carver College of Medicine, Iowa City, IA, USA

Abstract

Background: An in-frame deletion leading to the loss of a single glutamic acid residue in the protein torsinA (ΔE -torsinA) results in an inherited movement disorder, DYT1 dystonia. This autosomal dominant disease affects the function of the brain without causing neurodegeneration, by a mechanism that remains unknown.

Methods: We evaluated the literature regarding the subcellular localization of torsinA.

Results: Efforts to elucidate the pathophysiological basis of DYT1 dystonia have relied partly on examining the subcellular distribution of the wild-type and mutated proteins. A typical approach is to introduce the human torsinA gene (*TOR1A*) into host cells and overexpress the protein therein. In both neurons and non-neuronal cells, exogenous wild-type torsinA introduced in this manner has been found to localize mainly to the endoplasmic reticulum, whereas exogenous ΔE -torsinA is predominantly in the nuclear envelope or cytoplasmic inclusions. Although these outcomes are relatively consistent, findings for the localization of endogenous torsinA have been variable, leaving its physiological distribution a matter of debate.

Discussion: As patients' cells do not overexpress torsinA proteins, it is important to understand why the reported distributions of the endogenous proteins are inconsistent. We propose that careful optimization of experimental methods will be critical in addressing the causes of the differences among the distributions of endogenous (non-overexpressed) vs. exogenously introduced (overexpressed) proteins.

Keywords: DYT1 dystonia, endogenous protein, localization, overexpression, torsinA

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*To whom correspondence should be addressed. E-mail: charles-harata@uiowa.edu

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Introduction

DYT1 dystonia is defined as “early-onset generalized isolated dystonia”.¹ It is characterized by involuntary muscle contractions and abnormal postures, and caused by a mutation in the *TOR1A* gene (c.904_906delGAG/c.907_909delGAG; p.Glu302del/p.Glu303del) that results in deletion of a glutamic acid residue from the torsinA protein (ΔE -torsinA).^{2–6} Patients with this autosomal dominant disorder are heterozygous for the mutation (*TOR1A*^{+/ ΔE}). TorsinA belongs to the AAA+ family (ATPases associated with various cellular activities).⁷ Its ATPase activity is regulated by proteins in the endoplasmic reticulum (ER) and nuclear envelope,⁸ and is thought to contribute to diverse cellular processes, including the unfolding of

proteins during their degradation, the disassembly of protein aggregates, and the disassembly of protein complexes.⁷ Although the biochemical properties of torsinA have been identified, how it contributes to neuronal function in the brain remains elusive. Because the first dystonia gene locus to be identified was for DYT1 dystonia, it serves as a paradigm for the pathogenesis and pathophysiology of this debilitating neurological disorder.

Knowledge of the subcellular distribution of torsinA is expected to provide insight into its function and the pathogenesis that occurs when it is mutated. According to a prevailing model for the localization and function of torsinA proteins, the wild-type form is present throughout the ER whereas the ΔE form is present in the nuclear envelope and

causes abnormalities in its ultrastructure (reviewed, e.g., in Granata et al.⁹). This proposed spatial redistribution of torsinA is thought to play a critical role in dystonia pathogenesis, because neuronal dysfunction can be caused by either a lack of wild-type torsinA or an accumulation of ΔE -torsinA in certain subcellular compartments. However, this model is based largely on studies in which expression of exogenous torsinA in host cells was forced (overexpression), and levels of the protein were much higher than in the endogenous state. As the total content of torsinA (wild-type plus ΔE -torsinA) in patient fibroblasts is similar to, or even slightly reduced from, that in normal subjects,^{10–12} it is possible that the results from overexpression studies reflect a cellular state that is distinct from DYT1 dystonia.

The objective of this article is to compare imaging-based results obtained from exogenous overexpression in host cells with those for endogenous expression in a natural context, and to identify issues that will need to be resolved in order to settle the question of endogenous torsinA localization.

Search strategy and selection criteria

PubMed searches of articles were carried out using the terms “DYT1 dystonia,” “torsinA,” “TOR1A,” “distribution,” or “localization.” No time limits were placed. The reference lists of the primary articles were also searched. Only mammalian studies reported in full-text articles written in English were analyzed.

Consistent localization results with overexpression systems

In overexpression systems, the human torsinA genes are exogenously introduced into model animals or cells. The distribution of overexpressed torsinA is typically evaluated by one of three methods: 1) torsinA is tagged with a fluorescent protein (fluorescence tag, e.g., green fluorescent protein [GFP], cyan fluorescent protein [CFP]) and its fluorescence is detected; 2) torsinA is tagged with a non-fluorescent peptide (epitope-tag, e.g., myc-tag, V5-tag, polyhistidine-tag) and is detected by immunocytochemistry using an anti-tag antibody; or 3) untagged torsinA is overexpressed and detected by immunocytochemistry using an anti-torsinA antibody. Western blotting was used to measure the fold increase in the level of protein overexpression. The value has been reported as ~ 1.3 to 1.5 ,¹³ ~ 1.3 to 2.3 ,¹⁴ ~ 2 to 6 ,¹⁵ in transgenic mice, and as high as ~ 2 to ~ 20 ,¹⁶ or ~ 10 to 50 in some cultured cells.^{17,18} In studies where the Western blotting results were not quantified, the signals from endogenous protein were very weak or not detectable, in comparison to the overexpressed protein in cultured cells.^{19–21}

Overexpression studies have provided relatively consistent results (Table 1). In Table 1, the reported data are entered according to the host cell types, and these are broadly classified into cultured non-neuronal cells, cultured tumor cells of neuronal origin, cultured neurons, and neurons *in situ*. All of the host cells had a wild-type background. For the sake of highlighting the potential variations among individual reports, the entries for a particular cell type are grouped together only where exactly the same result regarding either the subcellular distribution of torsinA or its colocalization with

organellar markers was reported by the same group of researchers. In the case of wild-type torsinA, overexpressed protein was localized mainly in the ER, resulting in diffuse cytoplasmic—or where the resolution was high enough tubular or reticular—staining. At the level of light microscopy, the ER domain was almost equivalent to the cytoplasmic domain. A minor component of the signal emanated from the nuclear envelope (which is contiguous with the ER), in a perinuclear, ring-like pattern around the nucleoplasm. In the case of ΔE -torsinA, by contrast, the overexpressed protein was present mainly at the nuclear envelope and/or in cytoplasmic inclusion bodies. A minor component was present in the ER. Notably, the distributions of wild-type and ΔE forms of torsinA were not specific to the host cell types used, to whether the cells examined were cultured or in brain slices (*in situ*), or to the brain regions analyzed. Rather the distributions corresponded to whether it was wild-type or ΔE -torsinA that was overexpressed. Thus the general conclusion from these findings was that the signature feature of DYT1 dystonia is the mislocalization of ΔE -torsinA.

Advantages of overexpression systems

Overexpression systems are widely used, in part because they make it possible to introduce recombinant forms of torsinA with modifications. Other advantages of overexpression systems include the following: a fluorescence tag can be used to reveal torsinA localization, and the intensity of the tag is much stronger than background noise (autofluorescence); an epitope-tag can be used to reveal torsinA localization, and it can be detected with antibodies with high specificity for antigens not normally present in tissue; and, when an anti-torsinA antibody is used in this context, the signal of the overexpressed protein will be well above the background noise or endogenous signal, even though its affinity for torsinA may be lower than those of anti-tag antibodies for their respective tags. These overexpression systems have advantages in addition to their usefulness in analyzing the subcellular localization of torsinA. For example, they have been used to identify interacting partners of torsinA (reviewed, e.g., in Warner et al.²²), to purify the torsinA protein,^{8,19} to measure the rate of turnover of torsinA protein,²³ to evaluate the effects of torsinA in specific types of neurons,²⁴ and to establish what effects torsinA has on cell biological processes such as the ER- and oxidative-stress responses, and the chaperoning of proteins (reviewed, e.g., in Bragg et al.⁶).

Disadvantages of overexpression systems

Overexpression systems are associated with several general issues, including potentially violating balanced gene dosage, and affecting the folding of proteins, the assembly of protein complexes, and the regulation of downstream signaling.²⁵ In addition, overexpression systems have at least two disadvantages specific to the study of torsinA. One is that the distribution of torsinA is known to change depending on the expression level (Table 2). When ΔE -torsinA expression is low, it is present in the nuclear envelope (e.g. two- to 10-fold increase from the endogenous level), whereas when its expression is high, it is preferentially localized to cytoplasmic inclusions (e.g. ~ 20 -fold

Table 1. Genotype-dependent Subcellular Distributions of Overexpressed Human TorsinA Proteins in Mammalian Systems

Cell Type/Name	WT-torsinA	ΔE -torsinA	References
Cultured non-neuronal cells			
Human astrocytes (brain region, not specified)	Diffuse cytoplasmic (colocalized with GFAP)	(-)	Armata et al. ⁷⁶
Human fibroblasts	Diffuse cytoplasmic (colocalized with PDI); no TEM abnormality of nuclear envelope	Inclusions; abnormal TEM of nuclear envelope	Hettich et al. ⁷⁷
Human glioma Gli36 cells	Diffuse cytoplasmic (colocalized with PDI); perinuclear staining	Inclusions; perinuclear	Bragg et al. ⁷⁸
Human glioma Gli36 cells	Diffuse cytoplasmic; perinuclear (colocalized with laminB, nucleoporin)	Perinuclear (colocalized with laminB, nucleoporin); with/without inclusions (not colocalized with laminB or nucleoporin), depending on expression levels	Bragg et al. ¹⁶
Human osteosarcoma U2OS cells	Diffuse cytoplasmic; minimal inclusions (<5% of cells)	Inclusions (~90% of cells); not perinuclear	Kock et al. ⁷⁹
Human osteosarcoma U2OS cells	Diffuse cytoplasmic; minimal perinuclear	Diffuse cytoplasmic; perinuclear; no inclusions	Vander Heyden et al. ⁸⁰
Human osteosarcoma U2OS cells	Diffuse cytoplasmic	(-)	Naismith et al. ⁸¹
Human osteosarcoma U2OS cells	Diffuse cytoplasmic (excluded from ER exit sites)	(-)	Vander Heyden et al. ⁸²
Human adenocarcinoma HeLa cells	Diffuse cytoplasmic (colocalized with KDEL)	Same as WT-torsinA overexpression	Giles et al. ²⁶
Human adenocarcinoma HeLa cells	Diffuse cytoplasmic; minimal perinuclear	Inclusions; minimal perinuclear	Zhu et al. ⁸³
Human embryonic kidney epithelial HEK293 cells	Diffuse cytoplasmic (colocalized with GRP78/BiP); perinuclear	Inclusions (not colocalized with GRP78/BiP); not perinuclear	Kustedjo et al. ¹⁹
Human embryonic kidney epithelial HEK293 cells	Diffuse cytoplasmic (colocalized with PDI)	Inclusions (in>95% of cells)	O'Farrell et al. ^{21,84}
Human embryonic kidney epithelial HEK293 cells	Diffuse cytoplasmic	Inclusions; overexpressed ΔE -torsinA recruits overexpressed WT-torsinA to inclusions	Torres et al. ⁸⁵
Human embryonic kidney epithelial HEK293 cells	Diffuse cytoplasmic	Inclusions; these form whether or not torsinA has a carboxy-terminal V5-His-tag	Grundmann et al. ¹⁵
Human embryonic kidney epithelial HEK293 cells	Diffuse cytoplasmic	(-)	Armata et al. ⁷⁶

Table 1. Continued

Cell Type/Name	WT-torsinA	ΔE -torsinA	References
Human embryonic kidney epithelial HEK293 cells	(-) (no light-microscopic data; normal TEM of nuclear envelope)	(-) (no light-microscopic data; abnormal TEM of nuclear envelope)	Zirn et al. ⁸⁶
Human embryonic kidney epithelial HEK293 cells	Diffuse cytoplasmic	Inclusions; overexpressed ΔE -torsinA recruits overexpressed WT-torsinA to inclusions	O'Farrell et al. ⁶⁸
Human embryonic kidney epithelial 293T cells	Diffuse cytoplasmic (colocalized with PDI)	Inclusions	Hettich et al. ⁷⁷
African green monkey kidney fibroblast-like COS-7 cells	Diffuse cytoplasmic (colocalized with GRP78/BiP); perinuclear; (N-terminus was modified to increase expression level in Kustedjo et al. ⁸⁷)	Inclusions (not colocalized with GRP78/BiP); not perinuclear; (N-terminus was modified to increase expression level in Kustedjo et al. ⁸⁷)	Kustedjo et al. ^{19,87}
African green monkey kidney fibroblast-like COS-7 cells	Diffuse cytoplasmic (colocalized with PDI)	Perinuclear; inclusions (not colocalized with PDI)	Naismith et al. ¹⁸
African green monkey kidney fibroblast-like COS-7 cells	Diffuse cytoplasmic	Inclusions	Torres et al. ⁸⁵
African green monkey kidney fibroblast-like COS-7 cells	Diffuse cytoplasmic; minimal perinuclear	Inclusions; minimal perinuclear	Zhu et al. ⁸³
African green monkey kidney fibroblast-like COS-7 cells	Diffuse cytoplasmic (colocalized with PDI)	(-)	Nery et al. ⁸⁸
African green monkey kidney fibroblast-like COS-7 cells	Diffuse cytoplasmic (only partly colocalized with calreticulin, the soluble luminal protein present throughout the ER, indicating that torsinA prefers ER flat sheet to ER tubules)	(-)	Vander Heyden et al. ⁸²
African green monkey kidney fibroblast-like COS-1 cells	Diffuse cytoplasmic	(-)	Shashidharan et al. ⁴⁸
African green monkey kidney epithelial Vero cells	Diffuse cytoplasmic (colocalized with KDEL); weak perinuclear	(-)	Maric et al. ⁸⁹
Mouse fibroblast NIH-3T3 cells	Diffuse cytoplasmic	Perinuclear	Jungwirth et al. ⁹⁰

Table 1. Continued

Cell Type/Name	WT-torsinA	ΔE -torsinA	References
Baby hamster kidney fibroblast BHK21 cells	Diffuse cytoplasmic (colocalized with KDEL); weak perinuclear (~20% of cells); no inclusions (~0%)	Perinuclear (~70% of cells); with/without inclusions (inclusions in ~70% of cells), depending on expression levels; overexpressed ΔE -torsinA recruits overexpressed WT-torsinA to perinuclear distribution	Goodchild and Dauer ¹⁰
Baby hamster kidney fibroblast BHK21 cells	Diffuse cytoplasmic	Perinuclear; with/without diffuse cytoplasmic staining, depending on expression levels	Goodchild and Dauer ⁹¹
Baby hamster kidney fibroblast BHK21 cells	Diffuse cytoplasmic (colocalized with KDEL in 100% of cells); inclusions (10% of cells); minimal perinuclear (colocalized with laminB1 in 6% of cells)	Inclusions (79% of cells; inclusions were smaller than those in overexpressed WT-torsinA); colocalized with laminB1 in 89%; colocalized with KDEL in 12%; not colocalized with lysosomal or autophagosomal markers	Calakos et al. ⁹²
Baby hamster kidney fibroblast BHK21 cells	Diffuse cytoplasmic (colocalized with PDI); perinuclear (not colocalized with PDI)	(-)	Jungwirth et al. ⁶⁵
Baby hamster kidney fibroblast BHK21 cells	Diffuse cytoplasmic	Perinuclear	Jungwirth et al. ⁹⁰
Chinese hamster ovary epithelial-like CHO cells	Diffuse cytoplasmic	Perinuclear; with/without inclusions, depending on expression levels	Naismith et al. ¹⁸
Chinese hamster ovary epithelial-like CHO cells	Diffuse cytoplasmic (porcine torsinA)	Same as WT-torsinA overexpression (porcine torsinA)	Henriksen et al. ⁹³
Chinese hamster ovary epithelial-like CHO cells	Diffuse cytoplasmic (colocalized with GRP78/BiP)	(-)	Josse et al. ⁹⁴
Rat oligodendrocyte OLN cells	Punctate (porcine torsinA)	Same as WT-torsinA overexpression (porcine torsinA)	Henriksen et al. ⁹³
Cultured neuronal tumor cells			
Human neuroblastoma SH-SY5Y cells	Diffuse cytoplasmic (colocalized with PDI)	Inclusions (colocalized with VMAT2 but not with PDI); not perinuclear	Misbahuddin et al. ¹⁷
Human neuroblastoma SH-SY5Y cells (differentiated)	Diffuse cytoplasmic	Perinuclear; inclusions	Hewett et al. ⁴⁰
Human neuroblastoma SH-SY5Y cells	Diffuse cytoplasmic (colocalized with PDI)	Perinuclear; inclusions (colocalized with snapin)	Granata et al. ³⁷

Table 1. Continued

Cell Type/Name	WT-torsinA	ΔE -torsinA	References
Human neuroblastoma SH-SY5Y cells	Diffuse cytoplasmic (colocalized with KDEL); perinuclear	Diffuse cytoplasmic (colocalized with KDEL); more preferential perinuclear staining than with WT-torsinA	Giles et al. ^{26,95}
Human neuroblastoma SH-SY5Y cells	Diffuse cytoplasmic (porcine torsinA)	Diffuse cytoplasmic; ring-like inclusions (porcine torsinA)	Henriksen et al. ⁹³
Human neuroblastoma SH-SY5Y cells	Diffuse cytoplasmic (excluding nucleoplasm)	Perinuclear; inclusions	Granata et al. ³⁸
Human neuroblastoma SH-SY5Y cells	Diffuse cytoplasmic (colocalized with PDI); no inclusions (found in ~4% of cells)	Inclusions (found in ~100% of cells); minimally colocalized with PDI	Vulinovic et al. ⁹⁶
Human neuroblastoma SK-N-AS cells	Diffuse cytoplasmic; minimal inclusions (~15% of cells); minimal perinuclear	Inclusions (~90% of cells); perinuclear	Cheng et al. ⁹⁷
Human neuroblastoma BE(2)-C cells (undifferentiated and differentiated)	Diffuse cytoplasmic (colocalized with PDI)	Inclusions	Hettich et al. ⁷⁷
Mouse neuroblastoma N2A cells	Diffuse cytoplasmic	(-)	Armata et al. ⁷⁶
Mouse neural line CAD cells (differentiated)	Punctate cytoplasmic in cell body and neurite (colocalized with PDI, partially with VAMP2); not perinuclear	Inclusions (colocalized with PDI, but not with VAMP2 or GM130); not perinuclear; TEM shows torsinA and PDI in inclusions	Hewett et al. ²⁰
Mouse neural line CAD cells	Diffuse cytoplasmic in cell body and process	Inclusions (colocalized with KLC in 57% of cells)	Kamm et al. ³⁶
Mouse neural line CAD cells	(-)	Perinuclear; inclusions	Naismith et al. ¹⁸
Mouse neural line CAD cells	Diffuse cytoplasmic; minimal inclusions (<5% of cells)	Inclusions (~85% of cells); not perinuclear	Kock et al. ⁷⁹
Rat pheochromocytoma PC12 cells	Diffuse cytoplasmic (colocalized with PDI)	Perinuclear; inclusions (not colocalized with PDI)	Naismith et al. ¹⁸
Rat pheochromocytoma PC12 cells	Diffuse cytoplasmic	Inclusions	Torres et al. ⁸⁵
Rat pheochromocytoma PC6-3 cells (differentiated)	Diffuse cytoplasmic (colocalized with GRP78/BiP, calnexin)	Perinuclear (colocalized with laminA); inclusions (colocalized with laminA/C, laminB, emerin, etc.)	Gonzalez-Alegre and Paulson ⁶⁷
Rat pheochromocytoma PC6-3 cells	Diffuse cytoplasmic	Inclusions; overexpressed ΔE -torsinA recruits overexpressed WT-torsinA to inclusions	Gonzalez-Alegre et al. ⁶³

Table 1. Continued

Cell Type/Name	WT-torsinA	ΔE -torsinA	References
Rat pheochromocytoma PC6-3 cells	Diffuse cytoplasmic	Perinuclear; inclusions	Gordon and Gonzalez-Alegre, ²³ Gordon et al., ⁷³ Maric et al., ⁸⁹ Martin et al. ⁹⁸
Cultured neurons			
Mouse midbrain neurons (including substantia nigra, ventral tegmental area)	Diffuse cytoplasmic in soma and process; inclusions (~5% of all neurons, ~0% of TH-negative neurons, ~20% of TH-positive neurons)	Inclusions (54% of all neurons, 43% of TH-negative neurons, 84% of TH-positive neurons) (colocalized with TH, but not with PDI) (TH-positive neurons comprised 20–30% of all neurons)	O'Farrell et al. ⁶⁸
Mouse cerebral cortical neurons (7 DIV)	Diffuse cytoplasmic in soma and process	Perinuclear	Nery et al. ⁸⁸
Rat dorsal root ganglion neurons (3 DIV)	Diffuse cytoplasmic in soma; minimal perinuclear (4% of neurons); no inclusions (0% of neurons)	Diffuse cytoplasmic in soma; perinuclear (58%); inclusions (28%)	Kock et al. ⁹⁹
Neurons <i>in situ</i>			
Transgenic mouse, neurons in cerebral cortex	Diffuse cytoplasmic (colocalized with KDEL)	Perinuclear (colocalized with laminBI)	Goodchild and Dauer ¹⁰
Transgenic mouse, neurons in striatum	Diffuse/punctate cytoplasmic; no inclusions; not perinuclear; ~twofold increase in torsinA protein level in comparison to non-transgenic control mice	Same as in transgenic mice overexpressing WT-torsinA or non-transgenic control; ~twofold increase in torsinA protein level in comparison to non-transgenic control mice	Sharma et al. ¹⁴
Transgenic mouse, neurons in periaqueductal gray, pedunculopontine nucleus, pons	(–)	Inclusions (colocalized with lamin A/C, ubiquitin); these changes were absent in cerebral cortex, hippocampus, striatum, substantia nigra pars compacta, cerebellar cortex	Shashidharan et al. ¹⁰⁰

Table 1. Continued

Cell Type/Name	WT-torsinA	ΔE -torsinA	References
Transgenic mouse, neurons in substantia nigra, raphe nucleus, pedunculopontine nucleus, cerebellar cortex, deep cerebellar nuclei, gigantocellular reticular nucleus	Diffuse cytoplasmic in soma and dendrite; inclusions (colocalized with lamin A/C, ubiquitin-protein conjugates, but not with PDI) (inclusions not found in hippocampus, striatum, substantia nigra, cerebellar cortex); ~sixfold increase in human protein level from non-transgenic mice; high expression caused TEM abnormality of nuclear envelope (30% of neurons in striatum, 10–20% of brainstem neurons)	Similar to WT-torsinA overexpression; ~twofold increase in human protein level from non-transgenic mice; TEM nuclear envelope abnormality more frequent than in WT-torsinA overexpression (~50% of neurons in striatum, 30–40% of brainstem neurons)	Grundmann et al. ¹⁵
Transgenic mouse, neurons in cerebral cortex, striatum, substantia nigra pars compacta, pontine nuclei	Diffuse cytoplasmic in soma and proximal dendrite; no perinuclear; no inclusions; human torsinA transcript was not increased in comparison to control human brains; no TEM abnormality of nuclear envelope in striatum, pontine nuclei, cerebellar cortex, spinal cord	Same as WT-torsinA overexpression and non-transgenic control; human torsinA transcript was four-to-fivefold higher than in control human brains; no TEM abnormality of nuclear envelope in striatum, pontine nuclei, cerebellar cortex, spinal cord	Zhao et al. ²⁷ (same mice as reported in Sharma et al. ¹⁴)
Transgenic mouse, neurons in olfactory bulb	Diffuse cytoplasmic in soma and dendrite; inclusions in mitral cell; no staining in dentate gyrus of hippocampus or striatum	(–)	Regensburger et al. ¹⁰¹ (same mice as reported in Grundmann et al. ¹⁵)
Transgenic mouse, neurons in striatum (medium spiny neurons)	Diffuse/punctate cytoplasmic in soma and proximal dendrite; no staining in nucleoplasm, fibers	Diffuse/punctate cytoplasmic in soma and proximal dendrite; no staining in nucleoplasm or fibers.	Martella et al. ¹⁰² (same mice as reported in Sharma et al. ¹⁴)
Transgenic mouse, neurons in substantia nigra pars compacta (dopaminergic neurons; human torsinA was overexpressed only in dopaminergic neurons using TH promoter)	Diffuse cytoplasmic in soma and proximal dendrite	Perinuclear	Page et al. ²⁴
Transgenic mouse, neurons in striatum (cholinergic interneurons)	(–)	Same as in non-transgenic controls (diffuse cytoplasmic in soma and proximal dendrite)	Sciamanna et al. ⁷⁵ (same mice as reported in Sharma et al. ¹⁴)

Table 1. Continued

Cell Type/Name	WT-torsinA	ΔE -torsinA	References
Transgenic rat, neurons in olfactory bulb, cerebral cortex, hippocampus, striatum, substantia nigra	Diffuse cytoplasmic in soma and proximal dendrite (not colocalized with lamin A/C that showed continuous curve); normal TEM of nuclear envelope; protein expression level was not specified for morphological studies	Perinuclear in a discontinuous pattern (colocalized with lamin A/C); abnormal TEM of nuclear envelope; not expressed in pons, cerebellum, brainstem; torsinA protein expression level was ~sevenfold higher than the lowest expressing line	Grundmann et al. ¹⁰³

In this article, “perinuclear” indicates ring-like staining of the nuclear envelope completely surrounding the nucleoplasm. “Inclusions” indicate intracellular, cytoplasmic inclusion bodies, except where intranuclear inclusion is specifically mentioned in Table 3. The cytoplasmic inclusions were often located near cellular nuclei. (–) indicates not tested.

Table 1 includes results with human torsinA, except for one study that analyzed porcine torsinA (mentioned as such).

Table 1 and Table 2 include both controlled and uncontrolled expression of the exogenous transgene.

In Tables 1–3, the results are limited to the distributions of wild-type and ΔE -torsinA. They do not include other naturally occurring mutations or genetically engineered proteins. Results from experimental manipulations that could affect the torsinA distribution were not included. Such manipulations are, e.g., the co-transfection with non-torsinA transgene constructs, the induction of cellular stress and ischemic insults, and the pharmacological regulation of biological processes.

Abbreviations and comments in Tables 1–3: calbindin D-28K (Purkinje neuron marker); calnexin (ER marker); ChAT, Choline Acetyltransferase (cholinergic neuron marker); CSN4, COP9 signalosome complex subunit 4; DIV, Days *In Vitro*; EM, Electron Microscopy; emerin (nuclear envelope marker); ER, Endoplasmic Reticulum; GABA, γ -Aminobutyric Acid (inhibitory neurotransmitter); GFAP, Glial Fibrillary Acidic Protein (astrocyte marker); GMI130, Golgi Matrix protein of 130 kDa (Golgi apparatus marker); GRP78/BiP, Glucose-Regulated Protein of 78 kDa/Binding Immunoglobulin Protein, also known as heat shock 70 kDa protein 5 (HSPA5) (ER marker); KDEL, Lysine-Aspartic Acid-Glutamic Acid-Leucine Sequence (ER retention signal); KLC, Kinesin Light Chain (motor protein); lamins (nuclear envelope markers); NOS, Nitric Oxide Synthase (marker of a class of GABAergic neurons); NSE, Neuron-Specific Enolase (neuronal marker); nucleoporin (nuclear envelope marker); parvalbumin (marker of a class of GABAergic neurons); PDI, Protein Disulfide Isomerase (ER marker); SGI, Secretogranin I (secretory granule marker); snapin (SNAP25 [synaptosomal associated protein of 25 kDa]-binding protein); somatostatin (marker of a class of GABAergic neurons); TEM, Transmission Electron Microscopy; TH, Tyrosine Hydroxylase (dopaminergic/noradrenergic neuron marker); VAMP2, Vesicle-Associated Membrane Protein/Synaptobrevin 2 (synaptic vesicle/nerve terminal marker); VGAT, Vesicular GABA Transporter (GABAergic nerve terminal marker); VGLUT1 and 2, Vesicular Glutamate Transporters 1 and 2 (glutamatergic nerve terminal markers); VMAT2, Vesicular Monoamine Transporter 2 (monoaminergic marker); WT, wild type; ΔE , deletion of a single GAG codon in *TOR1A* or *Tor1a* gene (associated with DYT1 dystonia).

increase from the endogenous level).¹⁶ The fact that the expression level is a strong determinant of at least the ΔE -torsinA distribution pattern indicates that the localization would have to be extrapolated to estimate the distribution of torsinA proteins at the low, endogenous level. However, it is unclear how such extrapolation can be achieved and, even if it is possible, to what extent the extrapolated properties would reflect those of the endogenous proteins. As a side note, some exceptions to the consistent overexpression results have been reported. For example, ΔE -torsinA was mislocalized in the neuroblastoma cell line SH-SY5Y but not in the non-neuronal HeLa cell line²⁶ or in the neurons of transgenic mice overexpressing human ΔE -torsinA^{14,27} (Table 1). This phenomenon can be explained if the expression levels of wild-type and ΔE -torsinA were different in those studies.

Another disadvantage of the overexpression systems is their dependence on the properties of the promoters used to express the transgenes (promoters in transgenic animals have been reviewed in Tassone et al.,²⁸ Oleas et al.,²⁹ and Richter and Richter³⁰). For instance, the commonly used human cytomegalovirus (CMV) major immediate-early promoter/enhancer is considered strong, but its effect is influenced by neuronal activity, with depolarization increasing its efficiency more than 90-fold.³¹ Thus, expression will be low in

neurons with weak spontaneous activity, and such activity may be masked by high expression in more active neurons. Moreover, different promoters demonstrate different tissue specificities and developmental expression profiles.³²

In summary, the results obtained from overexpression systems are mostly clear and consistent, albeit with a few exceptions. However, caution is needed in interpreting them, especially with respect to the subcellular localization of torsinA proteins, until such outcomes are reliably replicated at endogenous levels of expression.

Inconsistent localization results for endogenous torsinA

Endogenous torsinA has been detected by indirect immunocytochemistry. The primary anti-torsinA antibody is detected using a secondary antibody conjugated with a fluorescent probe (fluorophore) or an enzyme that synthesizes colored or electron-dense product.³³ For a partial list of torsinA antibodies, see Xiao et al.³⁴ In sharp contrast to the outcomes for overexpression systems, the reported distribution of the endogenous torsinA protein is fairly inconsistent (Table 3).

In the case of *in vitro* studies, the cytoplasmic distribution of wild-type torsinA was typically diffuse, but it was punctate in a pattern consistent with vesicle staining in immature neurons and neuron-like tumor

Table 2. Subcellular Distributions of Human TorsinA Proteins at Different Overexpression Levels

Cell type/name	WT-torsinA	ΔE -torsinA	References
Human glioma Gli36 cells (expression level was actively controlled by tetracycline-regulated system)	Unchanged pattern (diffuse cytoplasmic; weak perinuclear), irrespective of expression levels	Perinuclear when expression level is low to moderate (two- to 10-fold increase from endogenous level); additional inclusions when the level is high (~20-fold increase from endogenous level)	Bragg et al. ¹⁶
Baby hamster kidney fibroblast BHK21 cells (expression level was monitored)	Unchanged pattern (diffuse cytoplasmic; weak perinuclear), irrespective of expression levels	Perinuclear when expression level is low to moderate; additional inclusions when the level is high	Goodchild and Dauer ¹⁰
Baby hamster kidney fibroblast BHK21 cells (expression level was monitored)	(-)	Perinuclear when the expression level is low; additional, diffuse cytoplasmic staining when the level is higher.	Goodchild and Dauer ⁹¹
Chinese hamster ovary epithelial-like CHO cells (expression level was monitored)	(-)	Perinuclear and diffuse cytoplasmic when expression level is low; additional inclusions when the level is high (e.g. >fourfold higher than the low expression)	Naismith et al. ¹⁸

cells.^{35–38} In addition, wild-type torsinA was present in neurites and growth cones of immature cells,^{35–38} whereas it was not detectable in axonal shafts or nerve terminals of more mature neurons.³⁹ In the fibroblasts of DYT1 dystonia patients, perinuclear staining was reported in one study¹⁰ but was absent in others.^{40,41} Notably, staining of cytoplasmic inclusions was absent in all cases, consistent with findings from studies in fibroblasts from control subjects.^{10,40,41}

In the case of neurons *in situ*, the results were more variable than those described above. The majority of studies found that wild-type torsinA was distributed diffusely throughout the cytoplasm of the somata and proximal dendrites. However, the following patterns were also described: strong staining of axons and nerve terminals;^{42–45} strong staining of the neuropil without staining of somata or dendrites;⁴⁶ staining of the proximal dendrites of some but not all neurons;⁴⁷ and staining of the nucleoplasm.^{47,48} Of interest is the notion that the torsinA-positive domain of the neuron does not completely overlap with the ER.⁴⁹ Even after nucleoplasmic staining was excluded, the torsinA staining covered only a portion of the cytoplasm,^{47,48} suggesting that a non-ER compartment could be stained. In the brains of DYT1 dystonia patients, the distribution of torsinA protein was the same as in those from control subjects, i.e., a lack of staining of inclusions or the nuclear envelope,^{44,49} although cytoplasmic inclusions were found in brainstem regions.⁵⁰

Approaches to filling the gap

The discrepancies in the distribution results for exogenous and endogenous torsinA proteins mainly arise from variation in the

reported distribution of endogenous torsinA. It is difficult to interpret the results of overexpression studies, given that the pattern or determinant of endogenous localization is not yet clear. The latter could reflect genuine variations in the biology of this protein, reflecting subtle differences and changes in torsinA expression. It is also possible that the subcellular distribution of torsinA differs by cell type, by brain region, by species, and/or developmental stage, and there may even be intrinsic variation among individuals. Furthermore, in the case of human brains, the levels of protein expression may be affected by how, and for how long, the subjects had experienced pathological stresses before death, because torsinA expression at both the mRNA⁵¹ and protein⁵² levels is regulated by insults.

Before discussing these interesting variations of biological importance, however, it should be stated that the more likely causes of variation are technical issues concerning specificity and efficiency in detecting endogenous torsinA. Identifying the sources of any technical complications will help explain the discrepancies in the observed distributions for exogenous and endogenous torsinA.

One potentially important challenge in comparing the data for the two types of expression is that the lower level of expression for endogenous torsinA will make it difficult to distinguish positive signals from background noise. Importantly, any factor that negatively influences the immunocytochemical detection procedures may affect interpretation. Thus, it will be particularly important to test whether each step in the immunocytochemical procedure is working properly and is optimized, in both *in vitro* and *in situ* studies, and at both the light and the electron microscopy levels. The following are ways that future

Table 3. Subcellular Localization of Endogenous TorsinA Proteins in Mammalian Systems

Cell type/name	WT-torsinA	ΔE -torsinA	References
Cultured non-neuronal cells			
Human fibroblasts	Diffuse/punctate cytoplasmic; minimal perinuclear	Strong perinuclear; weaker cytoplasmic; no inclusions; patients' cells (<i>TOR1A</i> ^{+/ΔE})	Goodchild and Dauer ¹⁰
Human fibroblasts	Diffuse cytoplasmic; not perinuclear	Same as WT-torsinA; no inclusions; patients' cells (<i>TOR1A</i> ^{+/ΔE})	Hewett et al. ⁴⁰
Human fibroblasts	Diffuse cytoplasmic; minimal perinuclear	Same as WT-torsinA; no inclusions; patients' cells (<i>TOR1A</i> ^{+/ΔE})	Nery et al. ⁴¹
Mouse embryonic fibroblasts	Diffuse cytoplasmic (partially colocalized with KDEL)	(-)	Giles et al. ²⁶
Cultured neuronal tumor cells			
Human neuroblastoma SH-SY5Y cells	Diffuse cytoplasmic in cell body; nucleoplasm	(-)	Hewett et al. ⁶⁶
Human neuroblastoma SH-SY5Y cells (differentiated)	Punctate cytoplasmic in cell body; punctate accumulation in neurite varicosity (colocalized with VAMP)	(-)	Ferrari-Toninelli et al. ³⁵
Human neuroblastoma SH-SY5Y cells	Diffuse cytoplasmic in cell body (colocalized with KDEL) (processes were not present)	(-)	Giles et al. ⁹⁵
Human neuroblastoma SH-SY5Y cells	Punctate cytoplasmic in cell body and process (colocalized with CSN4)	(-)	Granata et al. ³⁸
Human neuroglioma H4 cells	Diffuse cytoplasmic in cell body; no inclusions	(-)	McLean et al. ¹⁰⁴
Rat pheochromocytoma PC12 cells (differentiated)	Diffuse cytoplasmic in cell body and process (colocalized with PDI)	(-)	Hewett et al. ⁷²
Rat pheochromocytoma PC12 cells (differentiated)	Punctate accumulation in neurite tip (colocalized with snapin, SGI)	(-)	Granata et al. ³⁷
Cultured neurons			
Mouse cerebral cortical neurons	Diffuse cytoplasmic	(-)	Gonzalez-Alegre et al. ⁶³
Mouse cerebral cortical neurons (3–7 DIV)	Diffuse cytoplasmic (colocalized with KDEL); some perinuclear (more localized to nuclear envelope than in embryonic fibroblasts in the same study)	(-)	Giles et al. ²⁶
Mouse cerebral cortical neurons	Diffuse cytoplasmic	(-)	Kim et al. ¹⁰⁵

Table 3. Continued

Cell type/name	WT-torsinA	ΔE -torsinA	References
Mouse hippocampal neurons (4 DIV)	Punctate cytoplasmic in soma and process (colocalized with snapin)	(–)	Granata et al. ³⁷
Mouse cerebellar granule neurons (4–6 DIV)	Punctate cytoplasmic in soma and neurite (colocalized with CSN4)	(–)	Granata et al. ³⁸
Mouse neurons from cerebral cortex, hippocampus, striatum, ΔE -torsinA knock-in model (12–16 DIV)	Proximal dendrite; absent from axon and nerve terminal; soma was not evaluated	Same as in WT-torsinA; (<i>Tor1a</i> ^{+/ΔE} and <i>Tor1a</i> ^{ΔE/ΔE})	Koh et al. ³⁹
Rat cerebral cortical neurons (8 DIV)	Punctate cytoplasmic in soma, neurite, growth cone (colocalized with KLC1)	(–)	Kamm et al. ³⁶
Neurons <i>in situ</i>			
Human neurons in cerebral cortex, hippocampus, substantia nigra pars compacta	Diffuse cytoplasmic in soma, proximal dendrite; nucleoplasm; no staining of white matter	(–)	Shashidharan et al. ⁴⁸
Human neurons in cerebral cortex, substantia nigra pars compacta (in Parkinson's disease)	Positive staining in intracellular Lewy bodies (that are positive for α -synuclein)	(–)	Shashidharan et al. ⁶⁹
Human neurons in cerebral cortex, hippocampus, caudate-putamen, thalamus, substantia nigra pars compacta, oculomotor nucleus, cerebellar cortex, spinal cord	Diffuse/punctate cytoplasmic in soma, proximal dendrite; fibers in white matter, brachium of superior colliculus, etc; no staining of nucleoplasm	(–)	Konakova et al. ⁴²
Human neurons in hippocampus, substantia nigra (in diffuse Lewy body disease)	No staining of soma or dendrite; positive staining in Lewy bodies (colocalized with α -synuclein and ubiquitin, but closely associated with only α -synuclein at <30-nm resolution).	(–)	Sharma et al. ¹⁰⁶
Human neurons in substantia nigra (in dementia with Lewy body disease)	No staining of soma or dendrite; positive staining in Lewy bodies (colocalized with α -synuclein which in turn is colocalized with heat shock proteins)	(–)	McLean et al. ¹⁰⁴
Human neurons in cerebral cortex, hippocampus, caudate-putamen, substantia nigra pars compacta, oculomotor nucleus, cerebellar cortex	Punctate cytoplasmic in soma (limited colocalization with PDI); proximal dendrite; nucleoplasm (sparing nucleoli); punctate neuropil (putative nerve terminals); no inclusions; not perinuclear	Same as in WT-torsinA; patient's brain (<i>TOR1A</i> ^{+/ΔE})	Walker et al. ⁴⁹

Table 3. Continued

Cell type/name	WT-torsinA	ΔE -torsinA	References
Human neurons in cerebral cortex, hippocampus, oculomotor nucleus (in Huntington's disease, spinocerebellar ataxia type III, and Huntington's disease-like 2 which had previously been diagnosed as chorea-acanthocytosis ¹⁰⁷)	Positive in intranuclear inclusion bodies (that are positive for ubiquitin, expanded polyglutamine repeats)	(-)	Walker et al. ^{108,109}
Human neurons in hippocampus, caudate-putamen, nucleus basalis of Meynert, substantia nigra pars compacta, cerebellar cortex	Punctate cytoplasmic in soma, proximal dendrite; some axons and "pencil bundle fibers" (axons of medium spiny neurons in caudate-putamen); punctate neuropil (putative nerve terminals)	Same as in WT-torsinA; no inclusions; only caudate-putamen and substantia nigra pars compacta were analyzed in patients' brains (<i>TOR1A</i> ^{+/ΔE})	Rostasy et al. ⁴⁴
Human neurons in hippocampus, putamen, substantia nigra pars compacta, cerebellar cortex	No staining of soma or dendrite; punctate neuropil (putative axon, nerve terminals; vesicles in axons (immunocytochemistry)).	(-)	Augood et al. ⁴⁶
Human neurons in periaqueductal gray, pedunculopontine nucleus, cuneiform nucleus	No inclusions	Inclusions (colocalized with laminA/C, ubiquitin-protein conjugates, ubiquitin, but weakly with PDI); these inclusions were absent in cerebral cortex, hippocampus, striatum, substantia nigra pars compacta; patients' brains (<i>TOR1A</i> ^{+/ΔE})	McNaught et al. ⁵⁰
Human neurons in substantia nigra (in diffuse Lewy body disease)	Positive staining in Lewy bodies	(-)	O'Farrell et al. ⁸⁴
Human neurons in hippocampus, putamen, globus pallidus, substantia nigra pars compacta, oculomotor nucleus, superior colliculus, cerebellar cortex, dentate nucleus of deep cerebellar nuclei, during development	Diffuse/punctate cytoplasmic in soma, proximal dendrite; putative axon and nerve terminal; no nucleoplasm; no staining before postnatal 6 weeks	(-)	Siegert et al. ⁷⁰

Table 3. Continued

Cell type/name	WT-torsinA	ΔE -torsinA	References
Human neurons in pedunclopontine nucleus, cuneiform nucleus, pontine reticular formation (in normal subjects and patients with non-DYT1 dystonia)	No inclusions	(-)	Holton et al. ¹¹⁰
Patus monkey neurons in hippocampus	Nucleoplasm, axon, nerve terminal (immuno-EM); no other compartments were analyzed	(-)	Walker et al. ⁴³
Macaque monkey neurons in putamen	No staining of soma or dendrite; staining in neuropil; vesicles in nerve terminals (immuno-EM).	(-)	Augood et al. ⁴⁶
Mouse neurons in cerebral cortex, hippocampus, caudate-putamen, thalamus, substantia nigra pars compacta, motor nucleus of trigeminal nerve, cerebellar cortex	Strong staining of fibers in cerebral cortical white matter, corpus callosum, internal capsule, anterior commissure, cerebral peduncle, etc; diffuse/punctate cytoplasmic in soma, proximal dendrite	(-)	Konakova and Pulst ⁴⁵
Mouse neurons in peri-aqueductal gray, pedunclopontine nucleus, pontine nuclei	Diffuse cytoplasmic in soma (colocalized with PDI, but not with lamin A/C or ubiquitin)	(-)	Shashidharan et al. ¹⁰⁰
Mouse neurons in cerebral cortex, substantia nigra pars compacta, pontine nuclei of ΔE -torsinA knock-in model	Diffuse cytoplasmic in soma; no inclusions in brains of male or female mice	Inclusions only in pontine nuclei of male mice (colocalized with ubiquitin) (not found in female mice) (<i>Tor1a</i> ^{+/ΔE})	Dang et al. ¹¹¹
Mouse neurons in striatum (cholinergic interneurons)	Diffuse cytoplasmic in soma and proximal dendrite	(-)	Sciamanna et al. ⁷⁵
Mouse neurons in cerebellar cortex, deep cerebellar nuclei	Diffuse/punctate cytoplasmic in soma, dendrite, dendritic spine (partially colocalized with calbindin D-28K, parvalbumin), nerve terminal (partially colocalized with VGLUT1, VGLUT2, VGAT); nucleoplasm of neurons expressing high level of torsinA	(-)	Puglisi et al. ⁷¹
Mouse neurons in red nucleus, interstitial nucleus of Cajal, of brain-restricted nestin-Cre <i>Tor1a</i> ^{fllox/ΔE} model ("nestin selective knock-in" mouse)	Diffuse cytoplasmic (expression level was ~60% of <i>Tor1a</i> ^{+/ΔE} mice) (nestin-Cre control mouse)	Perinuclear (expression level was ~20% of <i>Tor1a</i> ^{+/ΔE} mice) (<i>Tor1a</i> ^{-/ΔE})	Liang et al. ⁶¹

Table 3. Continued

Cell type/name	WT-torsinA	ΔE -torsinA	References
Rat neurons in hippocampus, substantia nigra pars compacta, cerebellar cortex, deep cerebellar nuclei	Nucleoplasm; diffuse cytoplasmic in soma, proximal dendrite (partially colocalized with TH)	(-)	Shashidharan et al. ⁴⁸
Rat neurons in cerebral cortex, hippocampus, striatum, cerebellar cortex	Nucleoplasm (sparing nucleoli); diffuse cytoplasmic in soma (partially colocalized with NSE, NOS, parvalbumin, GABA or ChAT), proximal dendrites of some but not all neurons; punctate neuropil (putative nerve terminals)	(-)	Walker et al. ⁴⁷
Rat neurons in cerebral cortex, hippocampus, striatum, substantia nigra pars compacta, cerebellar cortex	Diffuse cytoplasmic in soma, dendrite; not perinuclear or nucleoplasmic; punctate “pencil fiber bundle” axons in young striatum	(-)	Xiao et al. ³⁴
Rat neurons in cerebral cortex, hippocampus, striatum, thalamus, during development	Cytoplasm in soma, processes; increased staining intensity during postnatal 7 to 28 days	(-)	Oberlin et al. ¹¹²
Rat neurons in striatum, globus pallidus, subthalamic nucleus	Nucleoplasm (sparing nucleoli); less strong in perikaryal cytoplasm and proximal dendrite	(-)	Yamada et al. ¹¹³
Rat neurons in hippocampus	Diffuse cytoplasmic in soma, proximal dendrite of pyramidal neurons and parvalbumin- or somatostatin-positive interneurons	(-)	Zhao et al. ⁵²
Rat neurons in dorsal root ganglion	Diffuse cytoplasmic in soma; axon	(-)	Zhao et al. ⁵²

Note that the cells of DYT1 dystonia patients are listed under “ ΔE -torsinA.” However, they are heterozygous for the mutant allele (*TOR1A*^{+/ ΔE}), i.e., both wild-type and ΔE -torsinA proteins will be present. Similarly, heterozygous ΔE -torsinA knock-in mice (*Tor1a*^{+/ ΔE}) will express two types of full-length proteins. In contrast, the homozygous ΔE -torsinA knock-in mice (*Tor1a* ^{$\Delta E/\Delta E$}) express only the ΔE -torsinA protein, and the brain-restricted nestin-Cre *Tor1a*^{fl ΔE / ΔE} mice express the ΔE -torsinA protein and exons 1 and 2 (out of exons 1–5) of wild-type protein (i.e., effectively *Tor1a*^{-/ ΔE}).

studies can achieve such optimization, as suggested for immunocytochemical reports more generally.^{33,53–55}

Firstly, it will be important to test primary antibodies for their specificity in binding to the antigen. The literature suggests at least five controls for general immunocytochemical procedures.^{53–55} The first control is to manipulate expression of the antigen. Suggested approaches are genetic deletion in knock-out animals or down-regulation by RNA interference. A variation of this test is to upregulate the antigen, for example, by overexpressing GFP-tagged antigen, and detecting this protein simultaneously by GFP fluorescence and immunocytochemistry using secondary antibody conjugated with a different fluorophore.⁵⁵ A drawback of this approach is that

predominance of the overexpressed antigen can mask the specificity of a weak antibody. The second control is to preadsorb the antibody with the immunizing peptide. The third control is to compare the specificity of immunostaining at the cellular level to mRNA expression as assessed by *in situ* hybridization, taking into consideration that the two signals may be detected in different subcellular compartments. The fourth control is to test the antibody for overlap of its signal with that from other antibodies raised against non-overlapping epitopes of the same protein. The feasibility of this approach depends on the repertoire of available antibodies. The fifth control is to test the ability of the antibody to detect a single protein entity by Western blotting. However, in this case it needs to be born in mind that the proteins are

in different states with respect to their secondary, tertiary and quaternary structures when recognized by immunocytochemistry vs. Western blotting (denatured by sodium dodecyl sulfate, heat or reducing condition), and thus this test could produce irrelevant information. Controls of these types are especially important for polyclonal antibodies, given that they identify multiple epitopes in the antigen (torsinA), some of which could be shared with non-related proteins. Such non-specific binding is less likely to occur with monoclonal antibodies, for which the epitopes are specific peptide sequences that are not shared with other proteins.

Secondly, it will likewise be vital to control secondary antibodies for their specificity in binding to a primary antibody of interest. A typical method is to omit the primary antibody, and examine whether the signal is eliminated.^{53,55} Omission of both the primary and secondary antibodies will establish to what extent the native tissue produces background noise, e.g., autofluorescence (“label control” in Burry⁵⁵).

Thirdly, other aspects of immunocytochemical procedures will also require careful optimization. These include: the concentration of the primary antibody (e.g., too high a concentration leads to non-specific staining³³); the type and duration of chemical fixation (e.g., one torsinA antibody was incompatible with procedures of cryo-immuno-electron microscopy³⁷); and the type of embedding medium (e.g., paraffin embedding under heat can reduce the antigenicity). These aspects are especially important for *in situ* studies, because the procedures typically take longer than those required for *in vitro* studies and are therefore more susceptible to artifacts caused by non-ideal conditions. For example, animal tissues are chemically fixed for a prolonged period of time, first by trans-cardiac perfusion with a fixative (perfusion fixation) for ~30 min, and second by immersion of the tissue in the same fixative (immersion post-fixation) for one to two days. In contrast, cultured cells are typically fixed (immersion fixation) for ~30 min only. If torsinA antigenicity is affected by the duration of fixation, the results can be variable depending on the protocols used in individual experiments.

Lastly, not only the staining of samples, but also the imaging of signals can be affected by numerous factors. The first is optical resolution along the focal axis (in the z-direction). Resolution is limited to ~1 μm even when optical sectioning is carried out by confocal microscopy, and it is worse in the case of widefield microscopy.⁵⁶ Therefore, when paraffin sections (e.g., 3- μm thickness^{57,58}) and floating sections (e.g., 50- μm thickness⁵⁹) are used, the signal detected at any focal plane will be affected by stray signal from adjacent structures in other focal planes. Some forms of super-resolution fluorescence imaging will suffer from the same problem in thick sections. This issue is best resolved when ultrathin sectioning is achieved physically (e.g., 50–200 nm).⁶⁰ Second, the level of the focal plane is important because of the poor z-resolution of light microscopy. Even a non-diffuse signal can look diffuse and be mistaken as ER-like if the focal plane is outside the target structure. Third, it is essential to keep the imaging parameters at the same values (e.g., intensity setting and exposure time).⁵⁵ Otherwise, increasing the detection sensitivity could inappropriately lead to the inclusion of background noise as

positive signal, and to an incorrect interpretation such as a diffuse ER-like pattern.

The influences of the technical aspects discussed above have not been extensively evaluated in the field of DYT1 dystonia, although considerable efforts have been made. These include using the torsinA knock-out tissue in immunostaining⁶¹ or Western blotting,^{61,62} using antisense knock-down in immunostaining^{35,63,64} or Western blotting,^{35,38,63,65} Western blotting using torsinA-overexpressing cells,^{15,17,20,21,23,40,46,48,66–68} antigen preadsorption test in cellular staining^{39,42,44,45,47,48,69–71} or Western blotting,^{20,42,45,72} secondary antibody controls,^{34,36,39,44,47,49,50,71} and the spatial range of signal spread in tissue.⁷¹

The key to definitive resolution of torsinA localization will be to evaluate these parameters for each antibody using each protocol, and using each tissue of interest in a given species. We understand that these efforts will be labor-intensive, and also that it will not be feasible to use such controls in studying human brains. However, systematic evaluation in at least the cellular and animal models will produce reliable immunocytochemical data that can be compared qualitatively to that produced in overexpression studies.

Of note, although species-specific antibodies are available,^{23,73} one of the current challenges is that no antibody available can distinguish between the wild-type and ΔE -torsinA proteins.^{11,13,39,74,75} The study of torsinA distribution will be significantly improved if antibodies or other tools capable of doing so are developed.

Conclusions

Knowledge of the subcellular distribution of torsinA will be critical for understanding how wild-type torsinA affects neuronal function, as well as how ΔE -torsinA leads to neuronal dysfunction. This information will help researchers narrow down the potential pathophysiological roles of torsinA in DYT1 dystonia. For this purpose, it will be important to reassess the prevailing model of torsinA distribution based on exogenous torsinA, after the endogenous distribution is reliably established. Such reassessments will be valuable regardless of the precise outcome. Should the endogenous and exogenous distributions match, torsinA mislocalization in the model will be reinforced as one of the key events in pathogenesis. Should they differ, it will indicate that ΔE -torsinA is mislocalized as a consequence of abnormally high expression. In this case the new results will be particularly important in providing novel insights into pathophysiological mechanisms. Even under such conditions, it should be emphasized that this article is not aimed at downplaying the value of overexpression studies. Such studies make it possible, for example, to modify the molecular structure of torsinA and to identify regulatory interacting partners. Nevertheless, at this point, it will be essential to establish whether overexpression studies truly reflect the roles of endogenous torsinA and the pathogenesis of DYT1 dystonia.

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