THE DYSTONIA-ASSOCIATED PROTEIN TORSINA MODULATES SYNAPTIC VESICLE RECYCLING

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Running title: TorsinA and neurotransmitter release
N. pages: 21

The loss of a glutamic acid residue in the AAA-ATPase (ATPases Associated with diverse cellular activities) torsinA is responsible for most cases of early-onset autosomal dominant primary dystonia. In this study, we found that snapin, which binds SNAP-25 (synaptosome-associated protein of 25,000 daltons) and enhances the association of the SNARE complex (soluble N-ethylmaleimide sensitive factor attachment protein receptor) with synaptotagmin, is an interacting partner for both wild-type (wt) and mutant torsinA. Snapin co-localised with endogenous torsinA on dense core granules in PC12 cells and is recruited to perinuclear inclusions containing mutant ΔE-torsinA in neuroblastoma SH-SY5Y cells. In view of these observations, synaptic vesicle recycling was analysed using the lipophilic dye FM1-43 and an antibody directed against an intravesicular epitope of synaptotagmin I. We found that over-expression of wt-torsinA negatively affects synaptic vesicle endocytosis. Conversely, over-expression of ΔE-torsinA in neuroblastoma cells increases FM1-43 uptake. Knock down of snapin and/or torsinA using siRNAs had a similar inhibitory effect on the exo-endocytic process. In addition, down-regulation of torsinA causes the persistence of synaptotagmin I on the plasma membrane, which closely resembles the effect observed by the over-expression of ΔE-torsinA mutant. Altogether, these findings suggest that torsinA plays a role together with snapin in regulated exocytosis and that ΔE-torsinA exerts its pathological effects through a loss of function mechanism. This may affect neuronal uptake of neurotransmitters, such as dopamine, playing a role in the development of dystonic movements.

The majority of cases of early-onset, primary torsion dystonia (PTD) are caused by a dominantly inherited mutation in the DYT1 (TOR1A) gene on chromosome 9q34 (1). DYT1 dystonia manifests in childhood, typically with dystonia in a limb which spreads to the trunk and other limbs, usually sparing cranio-cervical muscles (2,3). There is no evidence for neurodegeneration in DYT1 dystonia, implying that abnormal movements are caused by a functional neuronal defect (4). All cases of typical DYT1 dystonia are caused by an in-frame GAG deletion (ΔGAG302/303; ΔE) in DYT1 gene, resulting in the loss of a glutamic acid in the C-terminal region of the encoded protein, torsinA (1). TorsinA is a member of the AAA ATPase superfamily of chaperone-like proteins (5). In mammalian neuronal cells, torsinA is found throughout the cytoplasm, neurite processes and growth cones (6,7). TorsinA has also been found the lumen of endoplasmic reticulum (ER) and in the space between the inner and the outer membrane of the nuclear envelope (NE) (8-11). In contrast, in cells over-expressing the mutant (ΔE-torsinA), the protein is redistributed from ER to NE and accumulates in large perinuclear membranous inclusions, which appeared to arise from the nuclear envelope (7,12-14). TorsinA-positive inclusions have been found in the midbrain of DYT1 patients, suggesting they are relevant to the pathogenesis of DYT1 dystonia (15). In SH-SY5Y neuroblastoma cells, AE-enriched inclusions contain the vesicular monoamine transporter 2 (VMAT2), a membrane-associated protein involved in loading dopamine vesicles (14). Other
indirect evidence for abnormal dopaminergic function in DYT1 dystonia comes from cell models showing that torsinA affects the membrane distribution of the dopamine transporter (DAT) and influences the activation of dopaminergic D2 receptors in a transgenic mouse model (16,17). Consistent with these observations, torsinA is highly expressed in dopaminergic neurons of the substantia nigra (18,19). More recent work in a DYT1 transgenic mouse model has suggested that mutant torsinA impaired dopamine release (20).

To investigate the role of torsinA further, we performed a yeast two-hybrid screening using full-length wt-torsinA and ΔE-torsinA as baits. Snapin, a SNAP25 (synaptosomal associated protein of 25 kDa) binding protein (21), was identified and its interaction with both wild-type and mutant torsinA confirmed by in vitro and in vivo assays. Snapin is thought to promote the maturation/priming of synaptic vesicles (SVs) by interacting with components of the SNARE complex (21,22). In view of this observation, we investigated whether overexpression of wild-type or mutant torsinA affects SV recycling in SH-SY5Y cells. Additionally, to understand the functional link between torsinA and snapin, we examined the effects of siRNA-based knock-down of both proteins. Our findings suggest that overexpression of wt-torsinA as well as knock-down of the endogenous torsinA negatively affects SV turnover, whereas ΔE-torsinA appears to act as a loss of function mutant by enhancing synaptic membrane turnover.

**Experimental procedures**

**Chemicals and antibodies** - Reagents were from Sigma-Aldrich (St. Louis, MO), unless otherwise specified. The mouse monoclonal anti-haemagglutinin (HA) antibody is from the Cancer Research UK Monoclonal Antibody Service. Rabbit polyclonal Syt-163 antibody was raised against a peptide corresponding to the N-terminal 19 residues of rat synaptotagmin I (SytI). Rabbit polyclonal anti-snapin and anti-green fluorescent protein (GFP) antibodies were kind gifts from Dr. R. Jahn (Max Planck Institute, Göttingen, DE) (23) and Dr. T. Hunt (Cancer Research UK London Research Institute). The monoclonal anti-SytI M48 and anti-SGI LF19 antibodies were kindly provided by Dr. T.H. Sölter (University of Heidelberg, DE) and by Prof. H. Winler, (University of Innsbruck, Austria), respectively. The monoclonal anti-human torsinA antibody is from Cell Signaling Technology (Danvers, MA), the monoclonal anti-Protein Disulfide Isomerase (PDI) antibody from Stressgen (Victoria, Canada), the polyclonal anti-VAMP2 from Wako Chemical (Osaka, JP) and the rat torsinA from Abcam (Cambridge Science Park, Cambridge, UK). AlexaFluor®-488, -555 and -647 conjugated goat anti-rabbit and anti-mouse secondary antibodies were from Invitrogen (Paisley, UK). Horseradish peroxidase (HRP)-conjugated secondary antibodies were from DAKO UK (Ely, UK) and ECL was from GE Healthcare (Little Chalfont, UK).

**Cell culture** - SH-SY5Y cell lines stably transfected with pcDNA3.1, containing either wild-type DYT1, GAG-deleted DYT1 (DYT1-ΔE) or no insert (14) were grown in 1:1 mixture of EMEM (Promochem, Middlesex, UK), Ham’s F12 Nutrient Mixture (Gibco) and 10% fetal calf serum (FCS) at 37°C and 5% CO2 under selective conditions (0.4 mg/ml G418; Gibco).

**Yeast two-hybrid screening** - The Matchmaker yeast two-hybrid system 3 (Clontech, Mountain View, CA) was used according to manufacturer’s instructions. To generate the baits, human full-length DYT1 and DYT1-ΔE cDNAs were inserted in-frame into the HindIII and BamHI sites of the pGBK7 vector (Clontech). Baits were transformed into S. cerevisiae Y187 strain (MATa), which was then mated with AH109 yeast strain (MATa) pre-transformed with an adult human brain cDNA library. Positive clones were selected for growth on Ade-/His-/Trp-/Leu-/a-gal plates. Plasmid DNA was isolated and transformed in E. coli using pGEM® Easy Vector System (Promega, Madison, WI) and DNA sequencing was performed using automated methods. To confirm the specificity of the interactions, cDNA from positive colonies was rescued, retransformed in fresh yeast cells and tested for β-galactosidase activity, using the yeast β-galactosidase assay kit (Pierce Chemical, Rockford, IL) according to the manufacturer’s instructions.

**Expression and purification of recombinant proteins** - DYT1 and DYT1-ΔE full-length and their six deletion mutants (tors1-3) were amplified by PCR from pGBK7 and the C-terminal deletion mutant of human snapin from pSFV1-PV-IRES-GFP vector (a kind gift from Dr. J. Rett, Physiologisches Institut, Homburg, Germany) (22) and subcloned into EcoRI and Sall of PGE/T4-1 (GE Healthcare) as glutathione-S-transferase
(GST) fusion protein. A vector encoding full length snapin fused to GST was kindly provided by Dr. R. Jahn (23). Protein expression was induced in E. coli BL21 by the addition of 400 μM isopropyl-beta-D-thiogalactopyranoside (IPTG) for 4 h at 30°C (Lalli et al., 1999). Upon lysis of the bacteria in 20 mM Tris-HCl pH 7.5, 0.5% Tween, 2 mM EDTA, 0.1% 2-mercaptoethanol, 4 μg/ml pepstatin, 0.5 mM phenylmethylenesulphonylfuoride (PMSF), and EDTA-free protein inhibitors (Roche, Mannheim, DE), fusion proteins were purified on glutathione-Sepharose beads for 1 h at 4°C, washed three times with PBS containing 0.05% Tween, 0.5 M NaCl and eluted in 20 mM reduced glutathione, 50 mM Tris-HCl pH 8.0. Purified torsinA and its mutant were then dialysed against 20 mM Hepes-KOH pH 7.0, 200 mM KCl, 2 mM 2-mercaptoethanol, and 0.5 mM ATP, whereas snapin and its fragments were dialysed against PBS.

In vitro transcription-translation and pull-down assays. For pull-down assays, GST-fusion proteins containing torsinA, ΔE-torsinA, snapin and its deletion mutants were bound to glutathione-Sepharose in Hank’s buffer (20 mM Hepes-NaOH pH 7.4, 0.44 mM KH2PO4, 0.42 mM Na2HPO4, 5.36 mM KCl, 136 mM NaCl, 0.81 mM MgSO4, 1.26 mM CaCl2, 6.1 mM glucose) containing 0.1% BSA (Hank’s-BSA) for 1 h at 4°C. Beads were then blocked with 2% BSA in Hank’s buffer for 1 h at 4°C and washed three times with Hank’s. [35S]-labelled proteins were generated using TnT Quick coupled transcription/translation system (Promega), pre-cleared on glutathione-Sepharose beads for 1 h at 4°C and then incubated with either pre-bound GST-fusion proteins or GST alone for 1 h at 4°C in Hank’s-BSA. Glutathione-Sepharose beads were then washed with ice-cold Hank’s-BSA containing 250 mM NaCl, 1% Triton X-100 and resuspended in loading buffer. Eluted proteins were then analysed by autoradiography. The gel was stained with Coomassie Blue to visualise the GST fusion proteins. SH-SY5Y cell lines expressing wild-type HA-tagged fusion torsinA and ΔE-torsinA were washed in PBS, scraped and then lysed in lysis buffer (50 mM Hepes-NaOH pH 7.4, 0.1 mM EDTA, and protease inhibitors) containing 0.5% CHAPS (Calbiochem, Darmstadt, DE) for 30 min at 4°C under constant agitation. Pre-cleared cell extracts were incubated with immobilised GST-snapin or GST overnight at 4°C. After six washes with lysis buffer, bound proteins were eluted in loading buffer and analysed by Western blot.

Immunoprecipitation and Western Blot- Cell extracts from SH-SY5Y expressing HA-tagged wild-type DYT1, DYT1-ΔE (20 μg protein/lane), prepared as above, were incubated with anti-snapin antibodies (30 μg antibody/sample; 1 mg of antibody for 1 ml of resin) overnight at 4°C. As a negative control, cell lysates were mixed with an irrelevant antibody (anti-GFP). Protein A-Sepharose beads (GE Healthcare) were then added to each samples and incubated for 1 h at 4°C under constant stirring. After extensive washes with lysis buffer containing 0.5% CHAPS, beads were resuspended in loading buffer, boiled and analyzed in SDS-PAGE followed by Western blot. Immunoprecipitates were blotted with anti-HA (1:1000) and anti-snapin (1:250) antibodies. Lysates of SH-SY5Y cells expressing wild-type DYT1, DYT1-ΔE or the control vector pcDNA3.1 were resuspended in loading buffer, boiled and analyzed by Western blot (10 μg/lane). Nitrocellulose membranes were incubated with anti-VAMP2 (1:1000), anti-synaptotagmin I (M48, 1:200) or anti-actin (1:1000) antibodies, followed by HRP-conjugated secondary antibodies. Immunoreactive bands were revealed by ECL and quantified (n=3) using NIH Image 1.61 software (http://rsb.info.nih.gov/nih-image/).

Hippocampal neuron culture- E18 mouse embryos were dissected in PBS pH 7.4 containing 0.6% glucose (PBS-G). Once removed, hippocampi were kept on ice in 1 ml of PBS-G buffer. Trypsin was added to 0.025% final concentration and the tissues were incubated at 37°C for 15 min with constant stirring. Cells were centrifuged at 1,000 rpm for 5 min at room temperature and then resuspended in growth medium (DMEM, 10% horse serum, 2 mM glutamine, 4.5 g glucose, 1 mM Na-pyruvate, gentamicin, penicillin and streptomycin) prior to plating on coverslips pre-treated with poly-L-lysine (0.1 mg/ml) for 1 h at RT. The following day, the growth medium was replaced with differentiating medium (Neurobasal, 2% B27, 2 mM glutamine, penicillin and streptomycin). Cells were analysed for immunofluorescence after 4 days.

Immunofluorescence- SH-SY5Y cell lines were plated onto glass coverslips and allowed to grow overnight. PC12 cells were grown on poly-L-lysine-coated coverslips and differentiated with
100 ng/ml nerve grow factor (NGF) in DMEM for 72 h (24). Cells were fixed with 4% paraformaldehyde (PFA) in PBS for 20 min at room temperature. SH-SY5Y were washed with PBS and incubated with 50 mM NH4Cl for 5 min. Cells were rinsed and PBS containing 10% goat serum was added for 1 h at room temperature. PC12 cells were incubated in blocking solution buffer (10% goat serum, 2% BSA and 0.25% gelatin) for 1 h. Primary antibodies were diluted (anti-HA, 1:1000; anti-torsinA 1:200, anti-snapin, 1:250; anti-Syt1 M48, 1:250, anti-VAMP2, 1:500, anti-SGI 1:1000 and anti-PDI 1:500) in PBS containing 0.05% saponin and 10% goat serum and incubated for 1 h at room temperature. After rinsing with PBS three times for 10 min, AlexaFluor®-488, -555 and -647 conjugated secondary antibodies diluted in PBS (1:500) were applied for 1 h at room temperature. Coverslips were then washed and mounted with Mowiol 4-88 (EMD Bioscience, La Jolla, CA). Treatment with cycloheximide (10 µg/ml; Calbiochem) on control cells and cells expressing wt-torsinA was carried for 15 min, before fixing with 4% PFA and processing as described above. Images were acquired by confocal microscopy (Zeiss LSM510; Carl Zeiss, Jena, DE) with a 63x Plan-Apochromat oil immersion objective. The second strategy is based on the uptake and unloading of the styryl dye FM1-43 in SH-SY5Y cells stably expressing HA-tagged wild-type DYT1, DYT1-ΔE or control vector. SH-SY5Y plated on MatTek dishes (MatTek, Ashland, MA) were incubated with 10 µM FM1-43 dye (Invitrogen) in high potassium buffer (5 mM Hepes-NaOH pH 7.4, 37 mM NaCl, 100 mM KCl, 1 mM MgCl2, 2.5 mM CaCl2, 10 mM glucose) for 1 min, followed by 1 min incubation with Advasep-7 (1 mM, CyDex, Lenexa, KS) and by 2 washes with low potassium buffer (5 mM Hepes-NaOH pH 7.4, 132 mM NaCl, 5 mM KCl, 1 mM MgCl2, 2.5 mM CaCl2, 10 mM glucose) to remove the surface-bound dye. As negative control, FM1-43 was added to the cells in absence of calcium (5 mM Hepes-NaOH pH 7.4, 37 mM NaCl, 100 mM KCl, 3.5 mM MgCl2, 250 mM EGTA, 10 mM glucose) and in presence of 50 µM 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetraacetoxymethyl ester (BAPTA-AM, Invitrogen). Images were taken every 2 s with a Zeiss LSM 510 microscope equipped with a Nikon x63, 1.4 NA Plan Ph3 oil-immersion objective. The excitation was provided by a 488-nm argon laser and emitted light was collected using 560 filter set (Omega Optical, Brattleboro, VT). The mean fluorescence intensity of five
individual cells for each sample in three independent experiments was measured using Zeiss LSM 510 software version 3.2.

**RNA interference** - On-target plus Smartpool of siRNAs for human torsinA (60 nM) and human snapin (20 nM, Dharmacon, Chicago, IL) were used to knock-down gene expression. siRNAs were transfected into SHSY-5Y cells using 1-2 μl of Lipofectamit 2000 (Invitrogen) in OptiMEM (Gibco), 5 h after transfection, the medium was replaced with EMEM:F12, 10% FCS and cells were used at 72 h after transfections. Cells transfected with 20-60 nM scrambled oligonucleotides were used as control. RNAi-mediated knock-down of torsinA and/or snapin was verified by immunoblot and immunofluorescence analysis using monoclonal anti-torsinA and rabbit anti-snapin antibodies as previously described.

**Statistical analysis** - Student's t-test analysis was assessed using Kaleidagraph version 4 (Synergy Software, PA).

**RESULTS**

**Identification of wild-type and mutant torsinA interacting proteins** - We undertook a yeast two-hybrid screening to identify proteins that interact with both wild-type torsinA (wt-torsinA) and mutant torsinA (ΔE-torsinA). Human full-length DYT1 (DYT1-wt) and its GAG-deleted mutant (DYT1-ΔE) were used as baits to screen an adult human brain cDNA library under high stringency conditions. As a result, eight independent clones were isolated using DYT1-ΔE and five using DYT1-wt. Three clones obtained using ΔE-torsinA as a bait encoded for the C-terminal region (residues 112-300) of snapin, a synaptic protein previously involved in the regulation of neurotransmitter release at central synapses (21).

The interaction of wt-torsinA and ΔE-torsinA with snapin was first verified by an independent yeast two-hybrid analysis (Fig. S1). The β-galactosidase activity of single colonies was measured within 2 h and compared to that of a yeast strain co-transformed with wt-torsinA and its previously-identified binding partner kinesin light chain 1 (KLC1) (6). As shown in Fig. 1A, the strength of the interaction of wt-torsinA with full-length snapin and KLC1 was comparable under our experimental conditions. No significant difference was found between the binding of snapin with wt-torsinA and ΔE-torsinA.

These interactions were confirmed in GST binding assays: wt-torsinA and ΔE-torsinA expressed as GST recombinant fusion proteins were able to bind in vitro translated snapin (Fig. 1B). These results confirmed the specificity of the binding, since no interaction of snapin with GST alone was observed. To identify the specific domains involved in this interaction, a series of truncated mutants of wt-torsinA, ΔE-torsinA and snapin were generated and tested in pull down assays (Fig. 1B). Deletion clones for wt-torsinA and ΔE-torsinA, corresponding to its first 181 residues and including the ATP-binding domain (tors-1), or only spanning the C-terminal coiled-coil region (residues 251-332; tors-3) were unable to bind snapin. In contrast, a fragment containing both ATP-binding and coiled-coil domains (residues 91-332; tors-2) showed the same intensity of binding of the full-length proteins (Fig. 1B). This finding indicates that the binding site is situated in this region and the N-terminus is not required for the interaction with snapin. In agreement with the results shown in Fig. 1A, no significant difference was observed in snapin binding between wt-torsinA and its mutant (Fig. 1B).

In a parallel experiment, full-length snapin and its C-terminal fragment (residues 83-136; CC-snapin), expressed as GST recombinant proteins, were equally able to bind both wt-torsinA and ΔE-torsinA (Fig. 1C). In contrast, no binding to immobilised GST was detected. This result indicates that the coiled-coil region of snapin alone is sufficient to mediate the interaction with both wild type and mutant torsinA.

A pull-down experiment was also performed using detergent extracts derived from stably transfected SH-SY5Y cells expressing wt and ΔE-torsinA tagged with an HA epitope. Specific interaction of GST-snapin with both wild type and mutant torsinA was revealed using an anti-HA antibody (Fig. 1D). Snapin appeared to bind equally ΔE-torsinA and wt-torsinA, whereas no binding was detected with GST alone. The ability of the endogenous snapin to interact with wt-torsinA and ΔE-torsin was also analysed by coimmunoprecipitation from extracts derived from SH-SY5Y cells expressing HA-wt-torsinA and HA-ΔE-torsinA (Fig. 1E). Consistent with the pull-down results shown in Fig. 1D, snapin was able to bind both wt and mutant torsinA. In contrast, no specific binding was detected using a polyclonal anti-GFP antibody (Fig. 1E).
Snapin partially co-localises with mutant torsinA in SH-SY5Y cells- In order to gain further insights into the interaction between torsinA and snapin in living cells, we performed an immunofluorescence analysis in SH-SY5Y cells over-expressing torsinA using antibodies against endogenous snapin and the HA tag of torsinA. At the cell periphery, snapin co-localises with synaptotagmin I (SytI), a specific marker of SVs and secretory granules, and this co-distribution is not grossly altered by the overexpression of ΔE-torsinA (Fig. S3). However, in these cells, endogenous snapin also accumulates in the typical torsinA-positive inclusions (Fig. S4J,N). As expected, the average in the cytosol (Fig. S5J,N). Cells were then washed, fixed, and these sections were post-embedded and the inclusions were visualised by cryoimmuno electron microscopy (Fig. 2G). Snapin showed little co-localisation with torsinA on the NE (Fig. 2A-C), a previously described site of accumulation of ΔE-torsinA (28,29).

We also analysed the distribution of snapin in SH-SY5Y overexpressing wt-torsinA. The diffuse cellular distribution of both snapin and wt-torsinA prevented the identification of any specific site of co-localisation (data not shown). Therefore, to assess if the overlapping between these proteins could occur in the ER where a pool torsinA resides (8,9,30), we co-stained with the ER marker protein disulphide isomerase (PDI) (Fig. S4B,F). This approach revealed a level of co-localisation of torsinA and snapin in this subcellular compartment (Fig. S4D,H; in white). However, the inhibition of protein synthesis by cycloheximide caused the loss of co-localisation between snapin and PDI without altering the overlap between snapin and wt-torsinA (Fig. S4I,P; in cyan) or the overall distribution of PDI (Fig. S4J,N; in red). These observations suggest that in SH-SY5Y cells, wt-torsinA and snapin are co-localised both in the secretory pathway and in a peripheral compartment depleted of ER proteins and containing exocytic markers, such as SytI.

Snapin and endogenous torsinA overlap on secretory organelles in PC12- To identify this compartment, we analysed the distribution of endogenous torsinA and snapin using specific antibodies in PC12 cells differentiated for 72 h with NGF (Fig. 3). Both torsinA and snapin showed a punctate pattern in the cytosol (Fig. 3A,C,E) with accumulation at the neurite tips (Fig. 3B,D), where extensive co-localisation was detected (Fig. 3F). Similarly, the two endogenous proteins showed similar distribution in primary hippocampal neurons with extensive overlap in both the cell bodies and the neural processes (Fig. S5). Cryo-sections of differentiated PC12 cells obtained using a flat-embedding technique allowed us to preserve the in situ orientation of the neurites (26) and localise endogenous snapin by immunolabelling with anti-snapin antibody decorated with 10 nm protein A gold (PAG). Numerous gold particles were associated to a subset of dense-core granules at the neurite tips (Fig. 3G,H). No gold label was detected on dense-core granules incubated only with 10 nm PAG on the control sections (data not shown). Although the anti-torsinA antibody was able to detect endogenous torsinA by immunofluorescence (Fig. 3A,B), the antibody was not compatible with the processing required for cryo-immuno EM (data not shown).

To verify that the co-localisation of torsinA and snapin was restricted to these organelles, PC12 cells were co-stained for secretogranin I (SGI), a secretory granule marker. As shown in Fig. 4, several SGI-positive organelles contained torsinA and snapin (Fig. 4G,H, in white); however, other SGI-containing vesicles were positive only for snapin or torsinA, suggesting that neurite tips hold a heterogeneous population of granules, which may contain either one or both proteins.

TorsinA affects SV recycling- In the view of previous studies suggesting the involvement of torsinA in dopamine transport (16) and the putative role of snapin in neurotransmitter release (21) (31), we tested whether torsinA could play a role in SV trafficking. To this end, we performed an exoendocytosis assay in SH-SY5Y cells, using an antibody against the intravesicular domain of SytI (Syt-163). This antibody is internalised upon fusion of the SV with the presynaptic membrane, which determines the exposure of the luminal domain of SytI on the cell surface (27,32). Therefore, the signal detected with this antibody in resting conditions and upon stimulation reflects the level of SytI exposed on the plasma membrane at steady-state, or upon SV exocytosis and recycling, respectively. SH-SY5Y cells stably transfected with wt and ΔE-torsinA were incubated with the Syt-163 antibody in resting (5 mM KCl; Fig. 5A,C,E) or depolarising conditions (100 mM KCl; Fig. 5B,D,F). Cells were then washed, fixed, permeabilised and finally stained with a fluorescently-conjugated secondary antibody, prior to immunofluorescence analysis. Mean fluorescence intensity of random fields was quantified (Fig. 5G). As expected, the average level of SytI detected on the surface of control cells
under depolarising conditions was significantly higher (Fig. 5B) than in resting cells (Fig. 5A). In SH-SY5Y cells over-expressing wt-torsinA, SytI labelling on the plasma membrane on stimulation was reduced compared with the control cells (Fig. 5D), suggesting that overexpression of torsinA severely impairs SV turnover. In contrast, cells overexpressing ΔE-torsinA showed a significant accumulation of Syt-163 signal on the plasma membrane in resting conditions (Fig. 5E), which did not increase upon depolarisation (Fig. 5F). Therefore, overexpression of ΔE-torsinA appears to affect the rate of vesicle endocytosis in SH-SY5Y cells, resulting in an accumulation of SytI on the plasma membrane and alteration of membrane identity.

The different efficiency of membrane recycling seen in wt-torsinA and ΔE-torsinA expressing cells may be dependent on an altered rate of SV biogenesis, which could be downregulated by over-expression of wt-torsinA and increased by ΔE-torsinA. We therefore quantified the total amount of SytI and VAMP2, another marker of SVs and secretory granules, in control SH-SY5Y cells and in cells expressing wt and ΔE-torsinA (Fig. 6). Whereas the concentration of SytI remained constant in the three cell lines both in immunofluorescence and immunoblotting (Fig. 6A-C,G), VAMP2 expression was higher in ΔE-torsinA-positive cells (Fig. 6F) than control cells, and was further reduced in wt-torsinA-expressing cells (Fig. 6E). These findings suggest that torsinA is unlikely to control the biogenesis of the vesicles; however it seems to tightly regulate the expression of specific vesicle proteins, such as VAMP2, or a subpopulation of secretory vesicles expressing VAMP2.

In the light of these results, we sought to test SV exo-endocytosis using FM1-43 uptake (33,34). This styryl dye selectively labels organelles undergoing exocytosis and endocytosis in living cells, providing a means of monitoring these processes in real time. In our experimental protocol, SH-SY5Y cells bearing a control plasmid or expressing either wt-torsinA or ΔE-torsinA, were exposed for 1 min to the dye in high potassium conditions (100 mM KCl) prior to rapid washing in control medium and imaging (5 mM KCl; Fig. S6A,D,G; see Materials and Methods). The experiment was repeated three times and the quantitative analysis of the uptake is shown in Fig. 7A. We found that FM1-43 uptake is higher in ΔE-torsinA expressing cells (140 ± 10; p< 0.01) and lower in wt-torsinA cells (77 ± 3; p< 0.05) compared to control SH-SY5Y cells (105 ± 6). To monitor the rate of SV exocytosis, FM1-43 loaded cells were treated after 30 s with 100 mM KCl, resulting in a strong depolarisation and fluorescence loss (Fig. S6B,E,H). Unstimulated cells were used as control (baseline). After stimulation, the residual fluorescence was higher in the mutant and lower in the wt-torsinA SH-SY5Y cells than the control (Fig. 7B). However, once the signal was normalised for the different loading, the amount and overall kinetics of FM1-43 release was equal in control, wt and mutant torsinA expressing cells (Fig. 7C), suggesting that exocytosis is not affected by wt- and ΔE-torsinA overexpression. To confirm that FM1-43 release was due to bona-fide Ca²⁺-dependent exocytosis, we repeated the experiment in the presence of extracellular and intracellular Ca²⁺ chelators. This treatment completely blocked FM1-43 release, demonstrating that exo-endocytosis in SH-SY5Y cells is strictly Ca²⁺ dependent (Fig. 7D).

Effect of the knock-down of torsinA and snapin on membrane turnover and SV recycling. In order to further understand the role of torsinA and snapin in the regulation of exo-endocytosis, an RNA interference study was performed. Control SH-SY5Y cells were transfected with a pool of siRNA for torsinA or snapin or a combination of both, and the efficiency of the knock-down was checked after 3 d by immunofluorescence and immunoblotting (Fig. S7). Both torsinA and snapin expression was reduced by 90% upon introduction of the siRNA (Fig. S7D,H). In the double knock-down, the expression of both snapin and torsinA was reduced by 70% (Fig. S7J,K). As a control, cells were transfected with scrambled RNA and no effect on either torsinA or snapin expression was observed (Fig. S7A,B). Exo-endocytosis in cells treated with siRNAs was analysed according to the two methods described previously. As shown in Fig. 8, SytI labelling of cells transfected with snapin siRNA showed a low signal (78 ± 2, p< 0.05) in response to depolarisation compared to the control (set to 100%; Fig. 8C,D). In contrast, cells treated with torsinA siRNA showed a higher labelling for SytI on the surface prior to stimulation (110 ± 3), which did not increase on depolarisation (107 ± 4, p< 0.05; Fig. 8E,F). Interestingly, this phenotype resembled SH-SY5Y cells overexpressing mutant torsinA (Fig. 5E,F), strongly suggesting that ΔE-
torsinA is a loss of function mutant. Treatment with both siRNAs had an inhibitory effect, resulting in a decreased exposure of SytI on the membrane upon stimulation (85± 3, p< 0.05), similar to the snapin knock-down (Fig. 8G,H). This suggests that snapin is likely to act upstream torsinA in the pathway of regulation of the SV turnover.

Cells treated with one or both siRNA for torsinA and snapin were also analysed for FM1-43 uptake in resting (Fig. S6J,M,P) and depolarising conditions (Fig. S6K,N,Q). The results shown in Fig. 8J indicate that treatment with both single torsinA or snapin siRNA and double (torsinA + snapin siRNA) negatively affected the uptake of FM1-43 with similar efficiency. This suggests that torsinA and snapin act on the same pathway and their presence is required to ensure a normal level of endocytosis. Furthermore, knocking down torsinA, snapin or both proteins resulted in a reduced level of FM1-43 release compared with control cells, after the signal was normalised for the different loading (Fig. 8K), indicating that endogenous levels of torsinA and snapin are also required for regulated secretion.

**DISCUSSION**

In the present study, we used yeast two-hybrid analysis to identify a new binding partner of torsinA, the SNARE-associated protein snapin. We have reported that snapin shows a robust interaction with wild type and mutant torsinA. Previously, it has been shown that the ATP-binding domain is essential for torsinA function and cellular distribution (6,13). Here, we have demonstrated that this portion of torsinA, and/or the adjacent linker region, has the additional role of recruiting snapin. This mode of binding is different from that described for KLC1, which only requires the coiled-coil region of torsinA for maximal interaction (6).

Other binding partners have been described for torsinA, including lamina-associated polypeptide 1 (LAP1) in the NE, and a novel transmembrane protein (Lull1) in the ER (35). These multiple interactions suggest that torsinA may have distinct roles in different cellular compartments. Our immunofluorescence studies showed that endogenous torsinA co-localises with snapin on secretory granules at neurites tips. In cells over-expressing ΔE-torsinA, accumulation of snapin was also seen in ΔE-positive perinuclear membrane whorls.

Snapin is a ubiquitously expressed protein, which was initially found to be associated with SVs, where it binds the SNARE complex by a direct interaction with SNAP25. The recruitment of snapin to the SNARE complex is thought to be required to enhance the interaction between the SNAREs and SytI (21). This association is a crucial step in the mechanism of exocytosis, which leads to the fusion of SVs with the plasma membrane triggered by Ca^{2+} influx (36). Protein kinase A modulates neurotransmitter release by targeting snapin for phosphorylation in neurons (22,37). Despite conflicting evidence about the role of snapin in neurotransmitter release (23), recent analysis of a snapin knockout mouse model supports a role for this protein in Ca^{2+}-dependent neurosecretion (31). In the same study, snapin was also shown to be associated with SVs along with other markers, such as VAMP2, Syt1 and VMAT2. Several other proteins have been identified as interacting partners of snapin in both neuronal and non-neuronal cells (38). Interestingly, some are involved in membrane or cargo sorting: BLOC-1 is involved in the biogenesis of endosomal-lysosomal organelles; EBAG9 acts as inhibitor of large dense-core vesicles exocytosis in PC12 cells; and the vanilloid receptor interacts with snapin during its transfer from carrier vesicles to the plasma membrane (39-41). Consistent with these findings, our data indicates that snapin knock-down has a negative effect on the exo-endocytic pathway with consequent reduction in vesicle turnover (SytI labelling) and decrease uptake and release of FM1-43. The interaction reported here of torsinA with snapin supports the hypothesis that torsinA may be involved in, or influence, SV dynamics in neurons.

Analysis of the effects of torsinA on neurosecretion in SH-SY5Y stably transfected with wild-type DYT1 and DYT1-ΔE showed that over-expressing wt-torsinA negatively affects SV recycling, as demonstrated by the impairment of FM1-43 uptake following depolarisation. In contrast, ΔE-torsinA appeared to enhance membrane recycling at the expense of the accuracy of the retrieval of secretory vesicle proteins, such as SytI, from the plasma membrane. Knock-down of torsinA via RNA interference, shows a similar phenotype to snapin, resulting in a general inhibitory effect on the exo-endocytic pathway. Moreover, the ablation of both proteins mirrors the effect of single knock-downs, consistent with the idea that torsinA and snapin
play a role in the same pathway. Interestingly, the effect of torsinA knock-down resembles the phenotype caused by ΔE-torsinA overexpression. To our knowledge, this is the first direct demonstration that ΔE-torsinA is a loss of function mutant, thereby providing evidence of how the AE mutation may affect torsinA function and cause its pathological effects. Our findings indicate that torsinA plays a role in the recycling of SV membrane, which occurs upon release of neurotransmitters. This strengthens the link between torsinA and abnormal neurotransmission, which may affect the dopamine pathway. Interestingly, Torres et al. (2004) proposed that wt-torsinA is able to regulate the distribution of membrane-associated proteins, such as the dopamine transporter receptor (DAT), affecting the re-uptake of dopamine. Moreover, Misbahuddin et al. (2005) showed that ΔE–torsinA enriched inclusions contain VMAT2, which is crucial for maintaining physiological levels of dopamine release.

Dopaminergic pathways have been implicated in the pathogenesis of dystonic movements from study of other forms of dystonias, including L-DOPA-responsive dystonia with diurnal fluctuations (Segawa’s dystonia) (42), and hereditary juvenile dystonia-parkinsonism (43). In addition, studies in a transgenic mouse model of DYT1 dystonia, found an abnormal balance between the dopaminergic and the cholinergic signalling (17), and abnormal dopamine release has been reported in another DYT1 transgenic mouse (20). Finally, it is tantalising that, in human brain, torsinA is highly expressed in dopaminergic neurons of the substantia nigra (18).

Although our data suggest that torsinA and snapin play a role in neurosecretion, the molecular mechanism responsible for torsinA-dependent regulation of neurotransmitter release is not completely understood. One hypothesis is that torsinA may have a chaperone-like function (44,45), regulating the folding of proteins involved in SV trafficking, such as snapin. Interestingly, torsinA displays sequence homology with N-ethylmaleimide sensitive fusion protein (NSF) (5), an AAA-ATPase essential for neurosecretion that is responsible for SNARE recycling (46). Alternatively, torsinA may be able to modulate snapin phosphorylation, which is critical for its function (22). Finally, snapin may mediate the association of torsinA with SV and secretory granules. This is consistent with results obtained in human brain showing association of torsinA with SVs in the striatum (18) and with our findings that torsinA and snapin co-distribute with the secretory granules maker SGI in PC12 cells. In addition to these possibilities, which are not mutually exclusive, the effects of torsinA on SytI localisation suggests that it may also influence events of membrane sorting not directly dependent on snapin function.

The molecular pathogenesis of ΔE-torsinA remains unclear. The formation of membranous whorls caused by expression of ΔE-torsinA is evident in CAD and SH-SY5Y transfected cells (7,14), but has been also observed in other non-neuronal cell lines overexpressing ΔE-torsinA (data not shown). These inclusions appear to originate from the NE (47) and have been detected in DYT1 brain, supporting a role in the pathogenesis of dystonia (15). Perinuclear inclusions have also been detected in mice expressing ΔE-torsinA (48,49). In addition, the fact that the phenotype of torsinA knockout is similar to the DYT1-ΔE knock-in argues that the loss of torsinA function play an important part in the pathology (50). This is supported by our observation that overexpression of ΔE-torsinA and knock-down of endogenous torsinA give rise to the same altered distribution of SytI on the plasma membrane.

In conclusion, our data suggests that torsinA has a regulatory role in vesicle recycling. The ΔE mutation impairs torsinA function, uncoupling endocytosis from membrane depolarisation, and causing the mislocalisation of SV proteins on the cell surface. This in turn may disrupt the neuronal pathways involved in the control of movement.

The mechanism by which ΔE-torsinA exerts its dominant-negative effect could involve mis-targeting of proteins involved in SV trafficking, such as snapin and VMAT-2, into the membranous inclusion bodies. Alternatively, ΔE-torsinA might lead to the sequestration of torsinA from other cellular compartments, interfering with its normal functions. One of these functions, highlighted in this paper, may be the maintenance of a functional secretory pathway in neurons.

REFERENCES

FOOTNOTES
Key words: dystonia; DYT1; FM1-43; synaptotagmin 1; snapin; torsinA.
This work has been supported by a grant from the Wellcome Trust (A.G.) and Cancer Research UK (L.C. and G.S).

Acknowledgements
We thank A. Weston for preliminary Electron Microscopy analysis and S. Tabrizi and S. Salinas for critical reading of the manuscript.

FIGURE LEGENDS
Figure 1. A) Snapin interacts specifically with torsinA and its ΔE mutant. The strength of interaction of wt-torsinA and ΔE-torsinA with snapin was quantified by a β-galactosidase assay on single yeast colonies as shown in Fig. S1. The β-galactosidase activity of wt-torsinA and ΔE-torsinA cotransformed with snapin was comparable with the positive control (wt-torsinA and KLC1), which was set to 100%. No colonies were observed for single transformation with wt-torsinA, ΔE-torsinA and snapin alone. n=3 colonies were measured for each sample. Error bars represent S.E.M. B) Schematic representation of the full-length and truncated versions of wild-type and mutant torsinA; SP, sequence peptide; CC, coiled-coil domain. The filled region (91-181) denotes the ATPase domain. ΔGAG302/303 indicates the position of the 3bp deletion. Wild type and mutant GST-torsinA bound [35S]-labelled snapin in an in vitro pull-down assay. The corresponding Coomassie blue staining of the SDS-PAGE gel is shown in Fig. S2A. C) Schematic representation of full-length and truncated C-terminal snapin (CC-snapin); HS, hydrophobic sequence; CC, coiled-coil domain. Both snapin and CC-snapin interact with [35S]-labelled wt- and ΔE-torsinA. The Coomassie blue staining corresponding to this experiment is shown in Fig. S2B. Lanes Tₛ, TₛΔ and TₛΔE show 1/10th of the starting material for snapin, wild type and mutant torsinA, respectively. D) SH-SY5Y cells lines
expressing HA-wt-torsinA and HA-ΔE-torsinA were incubated with GST-snapin and GST pre-bound to glutathione beads. The bound material was analysed by Western blot using an anti-HA antibody. Wt and mutant torsinA bind snapin with equal efficiency. The Ponceau staining of the nitrocellulose membranes corresponding to this experiment is shown in Fig. S2C. E) Endogenous snapin forms a complex with wt and mutant torsinA in SH-SY5Y extracts. Lysates were incubated with either anti-snapin or anti-GFP antibodies, and the immunoprecipitated material was analysed by Western blot with an anti-HA antibody. Wt- and ΔE-torsinA bind endogenous snapin with equal strength. Lanes Twt and TΔE show 1/10th of the starting material. Snapin bound to the beads was detected by Western blot as shown in Fig. S2D.

Figure 2. ΔE-torsinA partially co-localises with endogenous snapin in SH-SY5Y cells. Cells overexpressing ΔE-torsinA (A, D, green) were stained with a monoclonal anti-HA antibody. Endogenous snapin was revealed with a rabbit polyclonal anti-snapin antibody (B, E, red) and analysed by confocal microscopy._merged pictures are shown in C, F. Scale bars=5 μm (A-C) and 2 μm (D-F). G) Cryosections of ΔE-torsinA expressing SH-SY5Y cells were immunolabeled with a polyclonal anti-snapin antibody followed by 10 nm protein-A gold and imaged by transmission electron microscopy. Gold particles were concentrated in membrane whorls. Scale bar=200 nm.

Figure 3. Endogenous torsinA co-localises with snapin in PC12 cells. Differentiated PC12 cells were stained for endogenous torsinA (A, B, red) and for endogenous snapin (C, D, green). The merged images (E, F, in yellow) show high level of co-localisation in the neurite tips. Scale bars=5 μm (A, C, E) and 2 μm (B, D, F). G) An overview of the architecture of differentiated PC12 cells by electron microscopy of cryosections using the flat-embedding technique. A neurite tip packed with dense-core granules lies next to the body of another cell. Areas of fluorescence co-localisation in neurite tips (E-F) are likely to correspond to the dense-core granules, which are labelled with snapin immunogold (10 nm). H shows a higher magnification of the boxed area in G. Scale bar= 2.5 μm (G) and 250 nm (H).

Figure 4. Endogenous torsinA and snapin overlap with secretogranin I (SGI) in PC12 cells. PC12 after 72 h of differentiation in presence of NGF, were fixed and stained for SGI with a specific monoclonal mouse antibody (A, B, green), endogenous torsinA (C, D, red) and endogenous snapin (E, F, blue). The merged images (G, H, in white) show a population of granules triple-positive for SGI, snapin and torsinA. Scale bars=5 μm (A, C, E, G) and 2 μm (B, D, F, H).

Figure 5. Synaptotagmin (SytI) is accumulated on the surface of SH-SY5Y cells expressing mutant torsinA. SH-SY5Y cells stably transfected with control vector (A, B), wt-torsinA (C, D) and ΔE-torsinA (E, F) were incubated in resting (5 mM K⁺; A, C, E) and depolarising conditions (100 mM K⁺; B, D, F). In control cells, the intensity of SytI labelling increases in response to 100 mM K⁺ (B) compared to resting cells (A). Cells expressing wt-torsinA show a strongly reduced response to depolarisation (D), whereas ΔE-torsinA cells display an increased surface accumulation of SytI in resting conditions (E), which is not significantly changed upon stimulation (F). Scale bar=10 μm. G) Quantification of the anti-SytI antibody uptake. Empty columns refer to resting conditions (5 mM K⁺), whereas filled columns show the extent of SytI staining in depolarising conditions (100 mM K⁺) (n=3). Error bars represent S.E.M. Asterisks indicate p < 0.05 in Student’s t test.

Figure 6. TorsinA regulates selectively the expression of VAMP2 in SH-SY5Y cells. Control (A, D), wt-torsinA (B, E) and ΔE-torsinA (C, F) SH-SY5Y expressing cells were stained for SytI and VAMP2 in immunofluorescence (A-F) and Western blot (G). ΔE-torsinA expressing cells display an increase of VAMP2 expression (150% ± 3.5 SD, n= 3; F) as compared to control cells (set to 100%; D). In contrast, cells expressing wt-torsinA (E) show a reduction of VAMP2 levels to 50% ± 4.0 SD of the control. Actin was used as loading control in G. The level of expression of SytI remains constant in all cell lines. The intensity of the bands was quantified as described in Materials and Methods.
Figure 7. Effects of wt-torsinA and ΔE-torsinA on FM1-43 uptake and release in SH-SY5Y cells. A) FM1-43 uptake in control, wt-torsinA and ΔE-torsinA expressing cells after 1 min incubation under depolarising conditions. FM1-43 uptake in ΔE-torsinA expressing cells is higher than control cells, and is further decreased in SH-SY5Y expressing wt-torsinA (see also Fig. S6A,D,G). B) FM1-43 release upon stimulation with 100 mM K⁺ in control, wt- and ΔE-torsinA expressing cells. The amount of FM1-43 in ΔE-torsinA cells is indicated as baseline. The arrowhead indicates the addition of 100 mM KCl. C) Percentage of FM1-43 dye released in control, wt- and ΔE-torsinA cells after normalisation for the different loading. D) The release of FM1-43 is blocked by the Ca²⁺ chelators EGTA and BAPTA-AM. Error bars represent S.E.M. Single asterisk indicates p < 0.05 and double asterisks indicate p < 0.01 in Student’s t test.

Figure 8. Knock-down of torsinA and snapin affect SytI exposure on the plasma membrane and reduce FM1-43 uptake and release. SH-SY5Y cells treated with control (scramble, A,B), snapin (C,D) torsinA (E,F) and double (snapin + torsinA; G,H) siRNAs were analysed for SytI labelling on the plasma membrane in resting condition (A,C,E,G) and upon stimulation with 100 mM KCl (B,D,F,H). Knock-down of snapin and snapin+torsinA decrease the intensity of SytI signal (D,H,I). In contrast, higher levels of SytI were observed on the plasma membrane in torsinA knock-down cells in resting and stimulated condition (E,F,J) (in I, empty columns, 5 mM KCl; filled columns, 100 mM KCl). J) FM1-43 uptake in snapin (59% ± 3), torsinA (62% ± 2) and snapin + torsinA (57% ± 4.6) knock-down cells is reduced comparing with the control cells (scramble siRNA; set to 100% ± 5). K) FM1-43 release is reduced in knock-down cells for snapin (30% ± 3), torsinA (26% ± 2) and snapin + torsinA (24% ± 3) compared to control cells after normalisation for the different loading (54% ± 8) (n=3). Error bars represent S.E.M. Asterisks indicate p < 0.05 in Student’s t test.
Fig. 1
Fig. 2
Fig 5
Fig. 6
Fig 8
Online Supplemental Material

Figure S1. The yeast strain AH109 was co-transformed with prey plasmid pACT2-snapin and pGBK7-wt-torsinA and pGBK7-ΔE-torsinA baits. Yeast colonies were grown on selective plates containing X-gal. Co-transformation of wt-torsinA with KLC1 was used as positive control. pGBK7-wt-torsinA, -ΔE-torsinA and -snapin alone and the pGKT7 vector were used as negative controls.

Figure S2. A) Coomassie blue staining of the SDS-PAGE gels corresponding to the GST-pull down in Fig. 1B. Black arrows indicate wt- and mutant torsinA; black arrowheads indicate truncated versions tors-1; white arrows indicate tors-2 and white arrowheads indicate tors-3. B) Coomassie blue staining of the SDS-PAGE gels corresponding to the GST-pull down in Fig. 1C. C) Ponceau staining of the nitrocellulose membranes corresponding to the pull down assay in Fig. 1D. The position of GST (arrowheads) and GST-snapin (arrows) is shown. D) Snapin used in the immunoprecipitation assay in Fig. 1E was revealed with anti-snapin antibody (arrows).

Figure S3. The co-localisation of snapin (C,D, red) and SytI (A,B, in green) in ΔE-torsinA cells is shown (E,F, in yellow). Scale bars=5 μm (A,C,E) and 2 μm (B,D,F).

Figure S4. Endogenous snapin partially co-localises with overexpressed wt-torsinA and the ER marker PDI in SH-SY5Y cells. Cells overexpressing wt-torsinA were stained with an anti-HA antibody (A, E, green). PDI was revealed with a monoclonal anti-PDI antibody (B, F, red) and a rabbit polyclonal antibody was used to detect endogenous snapin (C, G, blue). Merged pictures are shown in D, H (white). Upon treatment with cycloheximide, snapin (K, O, blue) co-distributed with HA-wt-torsinA (I, M, green) in a PDI-negative compartment (J, N) as shown in the merge panels L, P (cyan). Scale bars=5 μm (A-D, I-L) and 2 μm (E-H, M-P).

Figure S5. Endogenous torsinA and snapin co-localise in mouse hippocampal neurons. Primary culture of hippocampal neurons from E18 mouse embryos were immunostained for torsinA (A,E,I, red), and snapin (B,F,J, green). Merge pictures show the overlap between torsinA and snapin in the cell body (C,G, in yellow) and neuronal processes (C,K, in yellow).

Figure S6. FM1-43 staining of control, wt-torsinA, ΔE-torsinA and untransfected SH-SY5Y cells treated with siRNA. High K induced loading of FM1-43 is shown in A,D,G for control and cells overexpressing wt mutant torsinA, respectively. J,M,P show FM1-43 staining in cells transfected with siRNA for snapin, torsinA and both proteins. High K stimulated destaining of FM1-43 is shown in B,E,H for control and cells overexpressing wt and ΔE-torsinA, and in K,N,Q for snapin, torsinA and double RNA-based knock-down cells.

Figure S7. The efficiency of SiRNA-induced knock-down of torsinA and snapin was assessed by immunofluorescence (A-L) and Western blot (M) analyses. Control cells were transfected with a scramble oligonucleotide (A,B). The effectiveness of single knock-down for torsinA and snapin is 90% ± 5 SD and for double knock-down is 70% ± 9 SD. Quantification was performed as described in Materials and Methods.
Fig. S2
Fig. S4
Fig. S5
Fig. S7
The dystonia-associated protein torsina modulates synaptic vesicle recycling
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J. Biol. Chem. published online December 31, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M704097200

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