A Close Association of TorsinA and α -Synuclein in Lewy Bodies

A Fluorescence Resonance Energy Transfer Study

Nutan Sharma,* Jeffrey Hewett,[†]
Laurie J. Ozelius,[‡] Vijaya Ramesh,[‡]
Pamela J. McLean,* Xandra O. Breakefield,[†] and
Bradley T. Hyman*

From the Department of Neurology,* Alzheimer's Disease Research Unit, and the Department of Neurology,[†] Molecular Neurogenetics Unit, Massachusetts General Hospital East, Charlestown, Massachusetts; and the Department of Molecular Genetics,[‡] Albert Einstein College of Medicine, Bronx, New York

TorsinA, a novel protein in which a mutation causes dominant, early onset torsion dystonia, may serve as a chaperone for misfolded proteins that require refolding or degradation. It has been hypothesized that misfolded α -synuclein, a protein in which two mutations cause autosomal dominantly inherited Parkinson's disease, serves as a nidus for the development of a Lewy body. We hypothesized that torsinA plays a role in the cellular processing of α -synuclein. We demonstrate that anti-torsin antibodies stain Lewy bodies and Lewy neurites in the substantia nigra and cortex. Using sensitive fluorescent resonance energy transfer (FRET) techniques, we find evidence of a close association between torsinA and α -synuclein in Lewy bodies. (*Am J Pathol* 2001, 159:339–344)

TorsinA is a novel protein in which loss of a single glutamic acid residue has been identified as the cause of >60% of cases of dominantly inherited, early onset torsion dystonia.^{1,2} Highest levels of torsinA mRNA in human brain are found in dopaminergic neurons of the substantia nigra pars compacta.³ TorsinA and the homologous proteins, torsinB, torp1, and torp2, contain an ATP-binding site and are thought to be related to the AAA+ family of ATPases. This family performs chaperone-like functions, assisting in protein trafficking and membrane fusion.^{4,5} One possible role for torsinA is as a chaperone for folding of proteins, also serving to identify abnormal proteins for refolding or degradation.

Lewy bodies (LBs) are eosinophilic cytoplasmic inclusions that are found in human neurons in a spectrum of neurodegenerative diseases, including diffuse LB disease (DLBD) and idiopathic Parkinson's disease.⁶ α -Synuclein

is a prominent component of LBs.^{7–9} Mutant forms of α -synuclein are associated with a rare, autosomal-dominant form of Parkinson's disease.^{10,11} It has been hypothesized that α -synuclein changes conformation, in response to exogenous insults, and condenses to form the nidus of a subsequent LB.¹² The intracellular accumulation of LBs is indicative of vulnerable neurons, that are unable to maintain the normal housekeeping functions essential for prolonged survival. Thus, the accumulation of α -synuclein within LBs of dopaminergic cells of the substantia nigra is likely an essential element in the nigra-specific pathology seen in LB diseases.

We found that torsinA immunostains LBs using multiple antibodies directed against different epitopes of torsinA. We also used highly sensitive fluorescence resonance energy transfer (FRET) approaches to test the hypothesis that there is an association between α -synuclein and torsinA in LBs. We observed strong FRET signals, indicating a close intermolecular association, between torsinA and α -synuclein. In contrast we did not find evidence of a close intermolecular association between torsinA and ubiquitin, another component of LBs. These data indicate that, in LBs, α -synuclein aggregates interact with the putative chaperone protein torsinA.

Materials and Methods

Human Brain Tissue

Fresh-frozen substantia nigra and hippocampal tissues from five patients, with a pathological diagnosis of DLBD, and five control cases were obtained from the Harvard Brain Tissue Resource Center. Fresh-frozen hippocampal tissues from three patients with a pathological diagnosis of Alzheimer's disease were obtained from the Massachusetts Alzheimer's Disease Research Center Brain Bank. The tissue was fixed with 4% paraformaldehyde and stored at -20°C as free-floating, 40- μm -thick coronal sections. The sections were permeabilized with 0.5%

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Address reprint requests to Bradley T. Hyman, M.D., Ph.D., Alzheimer's Disease Research Unit, Department of Neurology, Massachusetts General Hospital East, 149 13th St., Charlestown, MA 02129. E-mail: b_hyman@helix.mgh.harvard.edu.

Table 1. Antipeptide Antibodies Used in Immunohistochemistry and the Region Against Which Each Is Directed

Antibody	Protein	Region in protein	Species raised in	Secondary antibody used
TAB1	Torsin	a.a. 299–312	Rabbit	BODIPY goat anti-rabbit
TAB3	Torsin	a.a. 222–239	Rabbit	BODIPY goat anti-rabbit
D-MG10	Torsin	a.a. 208–249	Mouse	Cy3 donkey anti-mouse
H3C	Alpha-synuclein	a.a. 128–140	Mouse	Cy3 donkey anti-mouse
SC-7012	Alpha-synuclein	a.a. 1–19	Goat	Cy3 donkey anti-goat

a.a., amino acids.

Triton X-100 in Tris-buffered saline, pH 7.4, blocked with 1.5% normal donkey serum (Jackson ImmunoResearch, West Grove, PA), and probed with antibodies directed against various proteins, as described below.

Immunohistochemistry

Information regarding the primary and secondary antibodies used is summarized in Table 1. The anti- α -synuclein goat polyclonal antibody, SC-7012 (Santa Cruz Biotechnology, Santa Cruz, CA), is directed against amino acids 1 to 19. The SC-7012 antibody was used at a dilution of 1:100. The anti- α -synuclein mouse monoclonal antibody, H3C, was a gift of Dr. David Clayton, University of Illinois.¹³ H3C is directed against the carboxy terminal, amino acids 128 to 140. The H3C antibody was used at a dilution of 1:5000. The rabbit anti-ubiquitin antibody (DAKO, Carpinteria, CA) was used at a dilution of 1:100. A panel of anti-torsin antibodies was used. The rabbit polyclonal antibody, TAB1, is directed against amino acids 299 to 312 of torsinA and was affinity purified as previously described.¹⁴ A second anti-torsin rabbit polyclonal antibody, TAB3, is directed against amino acids 222 to 239 of torsinA and was also affinity purified as previously described.¹⁴ Both affinity-purified antibodies were used without dilution. The mouse monoclonal antibody, D-MG10, is directed against amino acids 208 to 249 of torsinA and used at a dilution of 1:1000.¹⁴ BODIPY- and cy3-conjugated secondary antibodies were used at a dilution of 1:200 (Molecular Probes, Eugene, OR, and Jackson ImmunoResearch, West Grove PA). Each secondary antibody was incubated separately with the tissue sections. In those sections in which an anti-goat secondary antibody was used, the anti-goat antibody was incubated first to avoid the binding of one secondary antibody to another. The tissue was then washed extensively before incubation with the remaining secondary antibody.

Microscopy

TorsinA, α -synuclein, and ubiquitin immunoreactivity in tissue sections was examined systematically under a $\times 40$ objective. Confocal microscope images were obtained with the MRC-1024 Bio-Rad laser confocal imaging system mounted on a Nikon TE300 microscope (Bio-Rad, Hercules, CA). An excitation wavelength of 568 nm and an emission wavelength of 605 nm was used for cy3.

An excitation wavelength of 488 nm and an emission wavelength of 522 nm was used for BODIPY.

Fluorescence Resonance Energy Transfer (FRET)

FRET was performed and quantified using an acceptor photobleaching method that was developed for laser-scanning confocal microscopy.¹⁵ The amount of FRET is dependent on the inverse sixth power of the intermolecular separation of the fluorophores. FRET demonstrates coupling between fluorophores that are within ~ 10 nm of one another. Using an indirect immunofluorescent approach, two epitopes that are labeled with distinct fluorophores and produce a FRET signal are within, at most, 30 nm of each other.¹⁵ This calculation takes into consideration the maximal distance between four antibody molecules, each having a diameter of 5 nm, and the maximal distance of 10 nm between fluorophores for a FRET signal to be seen. Thus, FRET provides substantially higher resolution than standard optical techniques (0.5 to 1.0 μ m). Although the exact distance between antigens cannot be calculated with certainty using this technique, relative distances can be inferred from the relative strength of the FRET signal, provided that the concentration of fluorophores and antibodies remains unchanged.

The experimental paradigm assessed the extent of FRET by measuring the donor fluorescence before (D_A) and after (D) photobleaching of the acceptor. An initial scan was obtained at low energy using the 488-nm line of the krypton-argon laser to record the BODIPY signal (emission maxima close to 522 nm). A second scan was performed with the 568 line (emission maxima close to 605 nm) to record the cy3 signal. A small section of the double-stained region (5 μ m $\times$ 5 μ m) was then photobleached with intense 568 nm light to destroy the acceptor molecule, cy3. The region was then rescanned using 488 nm light. An increase in the BODIPY signal, within the confines of the area in which the cy3 signal has been photobleached, is a measure of the extent of FRET between two proteins. The amount of energy transfer was calculated as the ratio of donor fluorescence in the presence or absence of the acceptor.

$$\text{Energy transfer} = \frac{D}{D_A}$$

D_A is the fluorescence intensity of the donor (BODIPY) in the presence of the acceptor (ie, before photobleaching)

Figure 1. Immunostaining of LBs in the substantia nigra with antibodies directed against α -synuclein, torsin, and ubiquitin. **a:** α -Synuclein immunoreactivity with H3C. **b:** Torsin immunoreactivity with TAB1. **c:** Torsin immunoreactivity with D-MG10. **d:** α -Synuclein immunoreactivity with SC-7012. **e:** Torsin immunoreactivity with TAB3. **f:** Ubiquitin immunoreactivity with anti-ubiquitin antibody. Scale bar, 27 μ m.

and D is the fluorescence intensity of the donor alone (ie, after photobleaching). The ratio of D/D_A equals 1.0 in the absence of FRET. If D/D_A is >1.0 , the fluorophores must be within 10 nm of each other and the antigens must be within 30 nm of each other. The magnitude of the D/D_A ratio that is >1.0 is proportional to the proximity of the fluorophores.

Statistical Analyses

The ratio of D/D_A was compared to the null hypothesis value of 1.0 by one group *t*-tests.

Results

We studied brain tissue from five cases of pathologically confirmed DLBD and five control cases. The tissue was simultaneously examined by double immunofluorescence for the presence of two of the following proteins: torsinA, α -synuclein, and ubiquitin. Sections of both substantia nigra and hippocampus were stained with antibodies against two different proteins. LBs that were immunoreactive for both antibodies were analyzed using the FRET technique. Sections of both substantia nigra and hippocampus were also stained with the secondary

antibodies alone. No immunostaining of LBs was observed in those sections (data not shown).

LBs were identified by their morphology and immunoreactivity for α -synuclein. LBs in both the substantia nigra and hippocampus immunostain for torsinA, the amino and carboxy termini of α -synuclein and ubiquitin (Figure 1). When the anti-torsinA antibody, TAB1, was preabsorbed with the peptide against which it was raised, no staining of LBs was seen (data not shown). Lewy neurites, which are thought to be the neuronal processes of vulnerable neurons, also immunostain for both α -synuclein and torsinA. There was no difference among the three different torsinA antibodies with regard to the pattern of LB or Lewy neurite immunostaining. All LBs that were immunoreactive for α -synuclein were also immunoreactive for torsinA ($n = 48$). No TAB1 immunostaining was observed in the neuronal cell bodies of the substantia nigra in either DLBD or control cases. Similarly, no TAB1 immunostaining was observed in the hippocampal formation in either Alzheimer's disease or control cases, reflecting low levels of protein expression.

The FRET technique was used to test the hypothesis that torsinA and α -synuclein are closely associated within LBs. Initially, the antibody against the amino terminus of α -synuclein (SC-7012) and the TAB1 antibody were

Figure 2. FRET occurs between α -synuclein and torsinA in LBs of the substantia nigra. The TAB1 antibody was used for torsin immunostaining. The SC-7012 antibody was used for α -synuclein immunostaining. **a:** BODIPY signal (torsin) following excitation at 488 nm. **b:** cy3 signal (α -synuclein) following excitation at 568 nm. **c:** Increase in donor fluorescence is observed within the discrete area of the cell that was photobleached. **d:** Discrete area of cy3 ($10 \mu\text{mol/L} \times 10 \mu\text{mol/L}$) was photobleached using intense 568 nm light. Scale bar, $27 \mu\text{m}$.

used. A strong FRET signal ($D/D_A = 1.50 \pm 0.19$, $n = 8$, $P < 0.0005$) was observed between α -synuclein and torsinA in LBs (Figure 2). This observation was confirmed using another antibody that recognizes α -synuclein, H3C. H3C is directed against the carboxy terminus of α -synuclein, which is ~ 120 amino acids downstream from the amino-terminal region recognized by the SC-7012 antibody. A strong FRET signal ($D/D_A = 1.25 \pm 0.16$, $n = 14$, $P < 0.0005$) was also observed in LBs using the antibodies H3C and TAB1.

Two other anti-torsinA antibodies, directed against different epitopes of the protein, were used to confirm the result observed using anti- α -synuclein antibodies and TAB1. LBs were stained with the anti- α -synuclein antibody SC-7012 and TAB3. TAB3 is directed against amino acids 222 to 239 of torsinA. A strong FRET signal ($D/D_A = 1.35 \pm 0.10$, $n = 6$, $P < 0.005$) was observed between

α -synuclein (SC-7012) and torsinA (TAB3). These data support the hypothesis of a close relationship between torsinA and α -synuclein in LBs. The association between torsinA and α -synuclein was further confirmed using the monoclonal anti-torsin antibody D-MG10, directed against amino acids 208 to 249, and SC-7012 (FRET, $D/D_A = 1.30 \pm 0.10$, $n = 10$, $P < 0.0005$). Taken together, these results demonstrate that the carboxy terminus of torsinA has a close association with α -synuclein.

To determine whether the relationship between α -synuclein and torsinA is specific to LBs in the substantia nigra or is detectable in LBs in other brain regions, sections of hippocampus from cases of DLBD were stained for both α -synuclein and torsinA. Sections were stained with a combination of either SC-7012 and TAB1 or H3C and TAB1. Intracytoplasmic inclusions, immunoreactive for both α -synuclein and torsinA, were seen in

Table 2. Quantification of FRET Analysis

Antibodies	Tissue	D/D _A	Standard deviation	Sample size	P
TAB1 + SC-7012	Substantia nigra	1.50	± 0.19	8	<0.0005
TAB1 + H3C	Substantia nigra	1.25	± 0.16	14	<0.0005
TAB3 + SC-7012	Substantia nigra	1.35	± 0.10	6	<0.005
D-MG10 + SC-7012	Substantia nigra	1.30	± 0.10	10	<0.0005
TAB1 + SC-7012	Hippocampus	1.17	± 0.12	9	<0.005
TAB1 + H3C	Hippocampus	1.15	± 0.04	8	<0.0005
D-MG10 + anti-Ubiquitin	Substantia nigra	1.02	± 0.05	9	NS

TAB1, TAB3, and D-MG10 antibodies are directed against torsin. H3C and SC-7012 antibodies are directed against alpha-synuclein. D/D_A, amount of energy transfer; Std dev, standard deviation of the sample; NS, not significant.

hippocampal pyramidal neurons. A detectable FRET signal ($D/D_A = 1.17 \pm 0.12$, $n = 9$, $P < 0.005$) was observed using the antibodies SC-7012 and TAB1. A FRET signal ($D/D_A = 1.15 \pm 0.04$, $n = 8$, $P < 0.0005$) was also observed using the antibodies H3C and TAB1.

Additional experiments were undertaken to study the association between torsinA and ubiquitin within LBs of the substantia nigra. Sections of substantia nigra were stained with both D-MG10 and the anti-ubiquitin antibody described in the Materials and Methods section. At the light level, torsinA and ubiquitin appear to co-localize in LBs. No significant FRET signal was seen ($D/D_A = 1.02 \pm 0.05$, $n = 9$, not significant). These data indicate that, despite the presence of numerous proteins within a LB, nonspecific FRET signals are not generated by indirect immunofluorescence. The FRET signal seen between torsinA and α -synuclein indicates that these proteins are closely and specifically associated with one another.

Discussion

TorsinA staining of LBs was seen using a panel of three antibodies directed against distinct epitopes of torsinA. The anti-torsin antibody, TAB1, is directed against a region that has 76% homology to a related protein, torsinB. However, *in situ* hybridization analysis of control human brain has revealed that torsinA is expressed at high levels in the dopamine neurons of the substantia nigra pars compacta, as well as in hippocampus, whereas torsinB is not expressed at appreciable levels in any region of normal adult human brain.³ Finally, the pattern of staining with TAB1 was identical to the pattern observed with two other torsin antibodies, directed against different epitopes, suggesting that TAB1 immunostaining reflects the presence of torsinA. Moreover, another report using a torsin antibody directed against amino acids 323 to 332, revealed immunostaining of LBs.¹⁶ Thus, torsinA seems to be a component of LBs. In contrast, the hippocampal formation of both control and Alzheimer's cases was not immunoreactive for torsin. These data indicate that torsinA is not ubiquitously expressed throughout the central nervous system and its presence in LBs may reflect its function as a chaperone protein.

The strength of the FRET signals suggest that torsinA has a specific, close association with α -synuclein within LBs. This is in contrast to FRET data obtained on α -synuclein and α -B crystallin, a heat-shock protein

found in 10 to 15% of LBs. In parallel studies, we did not detect FRET between α -synuclein and α -B crystallin in LBs,¹⁷ but did observe a strong FRET signal that has been observed between the amino terminus of α -synuclein and ubiquitin.¹⁷ These data indicate that the three-dimensional structure of α -synuclein, torsinA, ubiquitin, and α -B crystallin within the LB reflects specific interactions rather than a random packing in of various proteins.

The current data highlight the utility of FRET in the examination of intermolecular interactions in cells and tissues. The FRET technique used is an adaptation of a well established tool used in biophysical studies.¹⁸ An indirect immunofluorescent FRET technique has been used to demonstrate a tight intermolecular association between kinases and phospho-epitopes on tau in neurofibrillary tangles,¹⁵ between dystrophin and actin in skeletal muscle tissue sections,¹⁹ between heterodimer subunits of somatostatin receptors,²⁰ and between tumor necrosis factor- α receptors.²¹ We have detected robust FRET between α -synuclein and ubiquitin in LBs¹⁷ and now extend this work to examine the relationship between the putative chaperone protein, torsinA, and α -synuclein in LBs. Co-localization at the light level provides resolution on the order of magnitude of the wavelength of the light used, which in this case is $\sim 0.5 \mu\text{m}$. The resolution using FRET is more than 10-fold higher than that seen in standard immunofluorescence. Moreover, FRET data are inherently quantitative. Comparisons between FRET pairs in different experimental circumstances can be used to gain insight into biophysical processes. For example, the stronger FRET signal between TAB1 and SC-7012 in classic LBs in the nigra compared to that seen in the hippocampus (Table 2) likely reflects a looser three-dimensional organization of the LB in the hippocampus compared to the nigra, supporting a conclusion previously based solely on morphological grounds.²²

In summary, these data demonstrate directly that torsinA is present in LBs where it is closely associated with α -synuclein. Although the function of torsinA remains speculative, sequence homology indicates that it is part of a family of putative chaperone proteins, and our data support the putative role of torsinA as a chaperone protein. The presence of torsinA in LBs may be a consequence of its role in identifying and interacting with abnormal proteins that must be cleared by the cell.

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References

- Ozelius LJ, Hewett JW, Page CE, Bressman SB, Kramer PL, Shalish C, de Leon D, Brin MF, Raymond D, Corey DP, Fahn S, Risch NJ, Buckler AJ, Gusella JF, Breakefield XO: The early-onset torsion dystonia gene (DYT1) encodes an ATP-binding protein. *Nat Genet* 1997, 17:40–48
- Klein C, Friedman J, Bressman S, Vieregge P, Brin MF, Pramstaller PP, De Leon D, Hagenah J, Sieberer M, Fleet C, Kiely R, Xin W, Breakefield XO, Ozelius LJ, Sims KB: Genetic testing for early-onset torsion dystonia (DYT1): introduction of a simple screening method, experiences from testing of a large patient cohort, and ethical aspects. *Genet Test* 1999, 3:323–328
- Augood SJ, Martin DM, Ozelius LJ, Breakefield XO, Penney Jr JB, Standaert DG: Distribution of the mRNAs encoding torsinA and torsinB in the normal adult human brain. *Ann Neurol* 1999, 46:761–769
- Neuwald A, Aravind L, Spouge J, Koonin EV: AAA+: a class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. *Genome Res* 1999, 9:27–43
- Ozelius L, Hewett J, Page CE, Bressman SB, Kramer PL, Shalish C, De Leon D, Brin MF, Raymond D, Jacoby D, Penney J, Risch NJ, Fahn S, Gusella JF, Breakefield XO: The gene (DYT1) for early-onset torsion dystonia encodes a novel protein related to the Clp protease/heat shock family. *Adv Neurol* 1998, 78:93–105
- Pollanen MS, Dickson DW, Bergeron C: Pathology and biology of the Lewy Body. *J Neuropathol Exp Neurol* 1993, 52:183–191
- Irizarry MC, Growdon W, Gomez-Isla T, Newell K, George JM, Clayton DF, Hyman BT: Nigral and cortical Lewy bodies and dystrophic nigral neurites in Parkinson's disease and cortical Lewy body disease contain alpha synuclein immunoreactivity. *J Neuropathol Exp Neurol* 1998, 57:334–337
- Takeda A, Mallory M, Sundsmo M, Honer W, Hansen L, Masliah E: Abnormal accumulation of NACP/alpha-synuclein in neurodegenerative disorders. *Am J Pathol* 1998, 152:367–372
- Wakabayashi K, Matsumoto K, Takayama K, Yoshimoto M, Takahashi H: NACP, a presynaptic protein, immunoreactivity in Lewy bodies in Parkinson's disease. *Neurosci Lett* 1997, 239:38–45
- Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, Pike B, Root H, Rubenstein J, Boyer R, Stenroos ES, Chandrasekharappa S, Athanassiadou A, Papapetropoulos T, Johnson WG, Lazzarini AM, Duvoisin RC, Di Iorio G, Golbe LI, Nussbaum RL: Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science* 1997, 276:2045–2047
- Kruger R, Kuhn W, Muller T, Woitalla D, Graeber M, Kosel S, Przuntek H, Epplen JT, Schols L, Riess O: Ala30Pro mutation in the gene encoding alpha synuclein in Parkinson's disease. *Nat Genet* 1998, 18:106–108
- El-Agnaf OMA, Jakes R, Curran MD, Middleton D, Ingenito R, Bianchi E, Pessi A, Neill D, Wallace A: Aggregates from mutant and wild-type alpha-synuclein proteins and NAC peptide induce apoptotic cell death in human neuroblastoma cells by formation of beta-sheet and amyloid-like filaments. *FEBS Lett* 1998, 440:71–75
- Withers GS, George JM, Banker GA, Clayton DF: Delayed localization of synelfin (synuclein, NACP) to presynaptic terminals in cultured rat hippocampal neurons. *Dev Brain Res* 1997, 99:87–94
- Knowles RB, Chin J, Ruff CT, Hyman BT: Demonstration by fluorescence resonance energy transfer of a close association between activated MAP kinase and neurofibrillary tangles: implications for MAP kinase activation in Alzheimer disease. *J Neuropathol Exp Neurol* 1999, 58:1090–1098
- Hewett J, Gonzalez-Agosti C, Slater D, Ziefer P, Li S, Bergeron D, Jacoby D, Ozelius LJ, Ramesh V, Breakefield XO: Mutant torsinA, responsible for early onset torsion dystonia, forms membrane inclusions in cultured neural cells. *Hum Mol Genet* 2000, 9:1403–1413
- Shashidharan P, Good PF, Hsu A, Perl DB, Brin MF, Olanow CW: Torsin-A accumulation in Lewy bodies in sporadic Parkinson's disease brain. *Brain Res* 2000, 877:379–381
- Sharma N, McLean PJ, Kawamata H, Irizarry MC, Hyman BT: Alpha synuclein has an altered conformation and shows a tight intermolecular interaction with ubiquitin in Lewy bodies. *Acta Neuropathol* (in press)
- Gordon GW, Berry G, Liang XH, Levine B, Herman B: Quantitative fluorescence resonance energy transfer measurements using fluorescence microscopy. *Biophys J* 1998, 74:2702–2718
- Root DD: In situ molecular association of dystrophin with actin revealed by sensitized emission immuno-resonance energy transfer. *Proc Natl Acad Sci USA* 1997, 94:5685–5690
- Rocheville M, Lange DC, Kumar U, Sasi R, Patel RC, Patel YC: Subtypes of the somatostatin receptor assemble as functional homo- and heterodimers. *J Biol Chem* 2000, 275:7862–7869
- Chan FK, Chun HJ, Zheng L, Siegel RM, Bui KL, Lenardo MJ: A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. *Science* 2000, 288:2351–2354
- Gomez-Tortosa E, Newell K, Irizarry MC, Sanders JL, Hyman BT: Alpha synuclein immunoreactivity in dementia with Lewy bodies: morphological staging and comparison with ubiquitin immunostaining. *Acta Neuropathol* 2000, 99:352–357