

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/11609681>

Surguchov A, Palazzo RE, Surgucheva I. Gamma synuclein: subcellular localization in neuronal and non-neuronal cells and effect on signal transduction. Cell Motil Cytoskeleton 49: 2...

ARTICLE *in* CELL MOTILITY AND THE CYTOSKELETON · AUGUST 2001

Impact Factor: 4.19 · DOI: 10.1002/cm.1035 · Source: PubMed

CITATIONS

52

READS

24

3 AUTHORS, INCLUDING:



[Andrei Surguchov](#)

University of Kansas

99 PUBLICATIONS 1,776 CITATIONS

[SEE PROFILE](#)



[Irina Surgucheva](#)

University of Kansas

52 PUBLICATIONS 1,331 CITATIONS

[SEE PROFILE](#)

Synucleins in Glaucoma: Implication of γ -Synuclein in Glaucomatous Alterations in the Optic Nerve

Irina Surgucheva,¹ Belinda McMahan,¹ Farid Ahmed,² Stanislav Tomarev,² Martin B. Wax,¹ and Andrei Surguchov^{1*}

¹Department of Ophthalmology and Visual Sciences, Washington University, St. Louis, Missouri

²Laboratory of Molecular and Developmental Biology, National Eye Institute, National Institutes of Health, Bethesda, Maryland

Synucleins are small proteins associated with neurodegenerative diseases and some forms of cancer. They are studied predominantly in the brain; information about their presence and functions in ocular tissues is scarce. Here we describe the localization of three members of the synuclein family in the optic nerve of donors with different types of glaucoma compared with control samples from donors without ocular diseases. We did not find significant differences in the localization of α - and β -synucleins in the optic nerve or retina of glaucoma patients compared with controls, whereas considerable redistribution of γ -synuclein occurred in the glaucomatous optic nerve compared with control eye without glaucoma. In the optic nerve from control and glaucomatous individuals, nerve bundles are immunopositive for γ -synuclein; however, a strong γ -synuclein-immunopositive staining in a subset of glial cells was observed in the lamina and postlamina cribrosa regions of the optic nerve only in glaucoma patients. In the optic nerve of rats with episcleral vein cauterization used as an animal model of glaucoma, the quantity of both γ -synuclein mRNA and protein was decreased compared with the optic nerves of control animals. Incubation of rat astrocyte culture at elevated hydrostatic pressure reduced the amount of γ -synuclein but did not affect the quantities of actin and glial fibrillary acidic protein. These data suggest that significant changes in the pattern of expression and/or localization occur in the glaucomatous optic nerve for γ -synuclein but not for α - and β -members of the synuclein family. © 2002 Wiley-Liss, Inc.

Key words: astrocytes; cell models; nucleus; centrosomes; optic nerve; γ -synuclein

The synucleins are a family of soluble proteins that are widely expressed in neurons and other cell types of the central nervous system. Recent studies suggest that synucleins contribute to the pathophysiology of severe human illnesses, including neuronal degenerations. α -Synuclein is known to be a component of the filaments in Lewy bodies in Parkinson's disease (PD), dementia with

Lewy bodies (Spillantini et al., 1997, 1998; Baba et al., 1998), and glial cytoplasmic inclusions in multiple system atrophy (Tu et al., 1998; Dickson et al., 1999). An Ala53Thr mutation in the α -synuclein gene has been proved to cause a familial type of PD (Polymeropoulos et al., 1997). In addition, a second type of missense mutation, Ala30Pro, has been reported in familial PD (Krüger et al., 1998).

The synucleinopathies are a diverse group of neurodegenerative diseases with a similar pathologic lesion composed of aggregates of insoluble α -synuclein protein in selectively vulnerable populations of neurons and glia. Growing evidence links the formation of abnormal filamentous aggregates to the onset and progression of clinical symptoms and the characteristic lesions in affected brain regions of patients with neurodegenerative disorders.

α -Synuclein is a small acidic protein composed of 140 amino acid residues. It has seven incomplete repeats of 11 amino acids with a core of KTKEGV, whereas the C-terminal portion has no known structural elements. β -Synuclein originally isolated from the bovine brain is highly homologous to α -synuclein. Because of the similar localization of α - and β -synucleins proteins, predominantly in the presynaptic terminals of neurons, it has been speculated that they are involved in synaptic function. γ -Synuclein was isolated from breast cancer tissue and initially termed "breast cancer-specific gene 1" (BCSG1; Ji et al., 1997). γ -Synuclein is expressed in the brain (Galvin

Contract grant sponsor: NJH NEI; Contract grant number: EY 13784; Contract grant sponsor: The Glaucoma Foundation; Contract grant number: QB42308; Contract grant sponsor: Carl Marshall Reeves and Mildred Almen Reeves Foundation; Contract grant sponsor: ADRC; Contract grant number: 99-6403.

*Correspondence to: Andrei Surguchov at his current address, 4004 Haworth Hall, Kansas University, Lawrence, KS 66045.
E-mail: apsurguchov@yahoo.com

Received 29 October 2001; Revised 26 December 2001; Accepted 28 December 2001

Published online 6 March 2002 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.10198

TABLE I. Clinical Data on Postmortem Glaucomatous Eyes

Patient number	Age (years)	Diagnosis	IOP
Control group			Normal
1	84	No glaucoma symptoms	
2	66	No glaucoma symptoms	
3	68	No glaucoma symptoms	
4	74	No glaucoma symptoms	
5	85	No glaucoma symptoms	
6	74	No glaucoma symptoms	
Primary open-angle glaucoma			Elevated
7	72	POAG	
8	70	POAG	
9	61	POAG	
10	70	POAG	
Chronic-angle-closure glaucoma			Elevated
11	67	CACG	
Normal-tension glaucoma			
12	78	NTG	Normal
Glaucoma, no history			
13	87	No history	Unknown
14	79	No history	
15	79	No history	
Pseudoexfoliative form			
16	85		Elevated

et al., 1999) and spinal cord, in addition to breast tissue (Lia et al., 1999), but is most abundant in the peripheral nervous system (Buchman et al., 1998a; Ninkina et al., 1998, 1999; Lavedan et al., 1998).

In spite of the growing number of publications on synucleins and their established involvement in neurodegenerative diseases, their presence in ocular cells and tissues and possible role in eye diseases was not studied until recently (Surguchov et al., 1999, 2001a). We have shown that all three members of the synuclein family (α , β , and γ) are differentially expressed in the retina and optic nerve and that the expression pattern for some members of the family changes significantly in the retinas of Alzheimer's disease (AD) patients and in transgenic mice overexpressing α -synuclein (Surguchov et al., 2001a).

Glaucoma, the second leading cause of permanent vision loss in the world (Quigley, 1996), is a group of ocular disorders that are responsible for the excavation and atrophy of the optic nerve, loss of retinal ganglion cells, and gradual loss of visual field (Quigley, 1993; Friedman and Walter, 1999). The pathogenesis of this disease is far from being completely understood. Glaucoma is usually considered as a neurodegenerative disease, and there are some studies proposing possible common mechanisms and/or common components with neurodegenerative diseases of the brain, e.g., AD (Vickers et al., 1995a, b; 1997). Other investigators, although accepting common symptoms and mediators of toxicity for these two diseases, consider that they have distinct temporal, subcellular, and signal-transduction features (Schwartz et al., 1999). In addition, glaucoma is usually, but not always, associated with elevated intraocular pressure (IOP).

Here we demonstrate considerable changes in the localization of γ -synuclein in the optic nerve of glaucoma

patients and in an animal model of glaucoma. In addition, incubation of astrocytes at elevated hydrostatic pressure causes a reduction in the amount of γ -synuclein. These data suggest that γ -synuclein may be implicated in the glaucomatous changes that occur in the optic nerve.

MATERIALS AND METHODS

Tissue Preparation

Sagittal paraffin-embedded 6 μm human tissue sections were a gift of Dr. M.R. Hernandez (Washington University, St. Louis, MO). The sections were prepared as described earlier (Agapova et al., 2001). Postmortem human eyes from 16 donors were analyzed; among the donors, 10 had been diagnosed with different forms of glaucoma. Age of the ocular tissue donors ranged from 61 to 87 years (Table I). Six human donor eyes without history of eye disease or neurodegenerative disease were used as age-matched controls.

The eyes were obtained from the Glaucoma Research Foundation (San Francisco, CA), from the Mid-America Eye Bank (St. Louis, MO), and from Dr. Martin B. Wax (Washington University, St. Louis, MO). Clinical findings of the glaucoma patients were well documented during 5–13 years of follow-up period, including IOP readings and optic disc and visual field changes. There was no diabetes, collagen vascular disease, infection, or sepsis in any of the donors. The cause of death for all of the donors was acute myocardial infarction or cardiopulmonary failure. The eyes were enucleated within 2–4 hr after death and processed and fixed within 6–12 hr in either 10% buffered formaldehyde or 4% paraformaldehyde. The posterior poles were dissected free of surrounding tissues, washed extensively in 0.2% glycine in phosphate-buffered saline (PBS) at pH 7.4, embedded in paraffin, and oriented sagittally for 5 μm sections.

Antibodies

Antibody to γ -synuclein (G-syn AB01) was raised and affinity purified by reacting with immunizing peptide immobilized on cyanogen bromide-activated Sepharose 4B as described previously (Surguchov et al., 2001a). This antibody was diluted 1:3,000–1:4,000 for immunohistochemical (IHC) staining. In control experiments with peptide antigen, no staining was observed. For experiments with rat samples, we also used commercial antibody to γ -synuclein raised against a peptide corresponding to amino acid 114–127 of the human γ -synuclein (ABCAM U.K.). This antibody cross reacts with rat γ -synuclein. It was diluted 1:750 for Western blot and 1:3,000 for IHC staining. We used, as antibody for α -synuclein, rabbit or sheep polyclonal antiserum produced by Chemicon International Inc. (Temecula, CA), dilution 1:1,000 for IHC staining. Affinity-purified polyclonal rabbit anti- β -synuclein antibody was purchased from Chemicon International Inc. and was diluted 1:5,000 for IHC staining. Biotinylated secondary antibody was identified by reacting with the streptavidin-peroxidase conjugate (Vector Laboratories, Burlingame, CA). For Western blot, secondary antibodies were conjugated with peroxidase, dilution 1:50,000 (Amersham, Buckinghamshire, United Kingdom). For α -tubulin, we used monoclonal anti- α -tubulin DM 1A antibody (Sigma, St. Louis, MO) diluted 1:5,000 and, for actin, monoclonal anti-actinN350 antibody (Amersham) in dilution 1:2,500.

Immunohistochemical Staining of the Optic Nerve

Human or rat eye tissues were paraffin embedded, and slices of 6 μ m were cut and placed on silane-coated slides. Before immunostaining, slides were deparaffinized and incubated for 1 hr in PBS glycine at room temperature to reduce nonspecific binding. Heat-induced epitope retrieval procedure was used for both human and rat slices. We followed the protocol of epitope retrieval described by Zymed (South San Francisco, CA). Slides were preincubated with 5% milk for 30 min, rinsed, and incubated with primary antibodies for 30 min. The slides were washed in PBS/Triton X-100 three times for 5 min each, with a subsequent wash in PBS for 5 min. Biotinylated secondary antibody was placed on the sections and incubated for 30 min, washed with PBS, and reacted with the streptavidin-peroxidase conjugate (Vector Laboratories) for 30 min. The bound antibody-peroxidase complexes on the sections were visualized using a 3,3-diaminobenzidine tetrahydrochloride (DAB) substrate solution consisting of 1.5 mg DAB and 50 μ l 30% hydrogen peroxide, in 10 ml 0.1 M Tris, pH 7.6. The sections were incubated in the dark until brown staining appeared, washed in PBS, counterstained with hematoxylin, dehydrated, and coverslipped with Permount. Control sections were run in parallel, omitting only the primary antibody.

A7 Astrocytes From Rat Optic Nerve

We used immortalized rat central nervous system cells of primary cultures of rat optic nerve A7 (gift of Dr. H. Geller). The culture was immortalized with murine leukemia virus psi-2, SV-40-6, which is defective in assembly and contains the SV-40 large T antigen and neomycin-resistance genes, as described earlier (Geller and Dubois-Dalcq, 1988). This stable immortalized clonal cell line expressed nuclear SV-40 large T cells and the

astrocyte-specific marker glial fibrillary acidic protein (GFAP). The cells were grown as described previously (Geller and Dubois-Dalcq, 1988).

Use of Hydrostatic Pressure

A pressure chamber equipped with a manometer was used according to the protocol described previously (Ricard et al., 2000). Briefly, the A7 cells were split on the glass coverslips in 35 mm Petri dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). The cells were grown at 37°C in 5% CO₂ until they reached 40% confluence. Then, the experimental plates were put in the chamber, where the hydrostatic pressure was raised to 60 mm Hg above ambient pressure. The chambers were placed in a tissue culture incubator and maintained at 37°C. Water was placed inside the chamber to maintain 97% relative humidity. The control plates were cultured under similar conditions at normal atmospheric pressure.

Immunocytochemical Staining

For single and double staining of primary culture of rat astrocytes, the cells were split on glass coverslips at ~50–60% confluence and kept at 37°C in 5% CO₂. Cells were washed in PBS and fixed with 100% methanol for 10 min at -20°C, then with a methanol:acetone mixture (1:1) for 4 min at -20°C. After intensive washing in PBS, the samples were blocked for 1 hr in 1% bovine serum albumin (BSA) in PBS with 0.1% Triton X-100 at room temperature. For single or double staining, the coverslips were placed facing downward on a drop of primary antibody (20–40 μ l) diluted in blocking solution and left in a humidified chamber at 4°C overnight. After washing with PBS three times for 7 min each, coverslips were incubated with goat anti-rabbit Oregon green-conjugated or goat anti-mouse rhodamine red-conjugated secondary antibody. After the final wash, coverslips were mounted in Vectashield (Vector Laboratories). Fluorescent images were taken using an Olympus BH-2 fluorescence microscope (Olympus Japan) equipped with a 568 nm filter for rhodamine red and a 488 nm filter for Oregon green. Images were recorded by digital photography (Spot Diagnostic Instruments) and stored as computer files.

Rat Model of Glaucoma

All experiments complied with the guidelines published in the NIH Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 8523) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Twelve animals were used in these experiments. The experiments were performed with interval of two months. With the animal under anesthesia (mixture of 45 mg/kg of ketamine and 9 mg/kg of xylazine), IOP was elevated in the left eye of the adult female albino Wistar rats by cauterizing three episcleral veins as previously described (Shareef et al., 1995). The right eye served as a sham-operated control. The IOP value was measured with a precalibrated Mentor pneumatonometer (Bio-Rad, Hercules, CA). Those rats in which IOP returned to normal after 2–4 weeks were excluded from the study. The remaining rats were sacrificed approximately 6 weeks after the operation and used for preparation of optic nerve samples for IHC staining and Western blot analysis.

Semiquantitative RT-PCR

One microgram of total RNA was used for cDNA synthesis using Superscript reverse transcriptase (Gibco BRL, Grand Island, NY) and oligo(dT)-primer. cDNA samples were diluted to provide a linear range of PCR. After adjustment of cDNA concentration for each individual pair of samples from the control and experimental eyes of the same animal, relative abundances of mRNA for γ -synuclein were estimated. The following primers were used: for γ -Synuclein, forward primer 5'-ggaggccaaagcagaagg-3' and reverse primer 5'-agcgctctggaaagggtgatccgaa-3', product size 282 bp; primers for γ -synuclein were located in different exons; for cyclophilin, forward primer 5'-tcctccttccacagaattttcc-3' and reverse primer 5'aatttagtgttccacagtgg-3', product size 345 bp. PCRs were performed in a PTC-200 Thermal Cycler (MJ Research, Watertown, MA) using AmpliTaq polymerase (Perkin-Elmer, Oak Brook, IL) as described previously (Ahmed et al., 2001). Each PCR was repeated at least twice. After initial denaturation for 90 sec at 94°C, the following conditions were used for amplification: 30 cycles of 30 sec at 94°C, 90 sec at 59°C, 60 sec at 72°C, and a final incubation for 5 min at 72°C. PCR products were analyzed by agarose gel electrophoresis. Gels were stained by ethidium bromide, and the intensity of DNA bands was estimated using Chemilimager 4000 software (Alpha Innotech Inc., San Leandro, CA). Our previous experiments with the *Myoc/Tigr* gene demonstrated that, under the conditions used in our experiments, semiquantitative RT-PCR provided reliable estimates of the changes in the level of analyzed mRNA that are confirmed by real-time PCR and northern blot hybridization (Ahmed et al., 2001).

Western Blots

Total protein was measured by the Bradford method (Pierce, Rockford, IL). Rat brain was a gift of Dr. C. Romano (Department of Ophthalmology, Washington University, St. Louis, MO). Total protein extract (15–20 μ g) was loaded on a 12% polyacrylamide gel. After electrophoresis, proteins were transferred onto nitrocellulose membrane at 0.45 μ M (Bio-Rad). Nonspecific binding sites were blocked by immersing the membrane in 5% blocking reagent in Tris-buffered saline Tween (TBS-T) for 1 hr at room temperature on an orbital shaker. Membranes were washed, incubated with antibody, and exposed to the film as described by the manufacturers of ECL Western blotting detection reagents (Amersham Pharmacia Biotech). To quantitate the squares of the peaks, the films were scanned by gel scanner AlphaImager 2200 (Alpha Innotech Inc.) using the software AlphaEase v5.5.

RESULTS

Synuclein Localization in Human Retina and Optic Nerve of Glaucoma Patients

We analyzed the distribution of three members of the synuclein family in ocular tissues of donors with different forms of glaucoma. No significant differences in the localization of α - and β -synuclein between normal and glaucomatous patients were found (not shown). For all samples, we observed that the pattern of immunopositive staining for these two members of the synuclein family was

identical or similar to the pattern described in our previous publication (Surguchov et al., 2001a). However, the localization of γ -synuclein in glaucomatous optic nerve changes significantly both in the area of lamina cribrosa and in the postlamina area. As shown in Figure 1A,B, γ -synuclein is present in nerve bundles and practically absent in glial cells in the area of lamina cribrosa in control eyes. In the optic nerves of donors with primary open-angle glaucoma (POAG), chronic-angle-closure glaucoma (COAG), or normal-tension glaucoma (NTG) and some glaucoma patients without history of the disease, in addition to nerve bundles, a strong immunopositive staining of glial cells was seen (Fig. 1C–F, arrows). Similar changes were found in post lamina area of the optic nerve (Fig. 2). Strong γ -synuclein-immunopositive staining of inclusions in glial cells accompanied by a reduction in the staining of nerve bundles was also observed for a patient with pseudo-exfoliative form of glaucoma (Table I, patient 16; not shown). In more advanced cases of disease, when nerve bundles are heavily disorganized, the appearance of strong γ -synuclein-immunopositive inclusions becomes more evident.

We also observed some differences in the staining pattern of synucleins in retinas of glaucoma patients compared with age-matched controls. For example, weak staining by γ -synuclein of the inner plexiform layer was observed, whereas both layers were immunonegative for γ -synuclein in control samples (not shown). To gain a deeper insight into the effect of elevated pressure on the localization of γ -synuclein in the optic nerve, we used an animal model of glaucoma.

IHC Staining of the Optic Nerve of Rats Used as a Model of Glaucoma

In the optic nerves of both control and experimental animals, nerve bundles are immunopositive for α - and β -synucleins throughout the optic nerve (not shown). The staining of some processes of glial cells is also observed. This pattern of staining is similar to the pattern described previously for human optic nerve (Surguchov et al., 2001a). We did not observe significant changes in α - and β -synuclein immunoreactivity under experimental animals compared with controls.

The majority of γ -synuclein in the optic nerve of control eyes is also localized in the nerve bundles (Fig. 3A, arrows). At the same time, in the optic nerve of experimental rats with high IOP, γ -synuclein-immunopositive glial inclusions were observed, whereas the intensity of nerve bundle staining was reduced (Fig. 3B).

Effect of Experimental IOP Elevation on γ -Synuclein mRNA Level in Rat Optic Nerve Head

To gain insight into mechanisms involved in the regulation of γ -synuclein after experimental induction of elevated IOP, levels of γ -synuclein mRNA were evaluated in the optic nerve of experimental and control animals by semiquantitative PCR (Fig. 4). In the optic nerve of experimental animals with elevated IOP, the level of γ -synuclein mRNA is slightly reduced [for example, com-

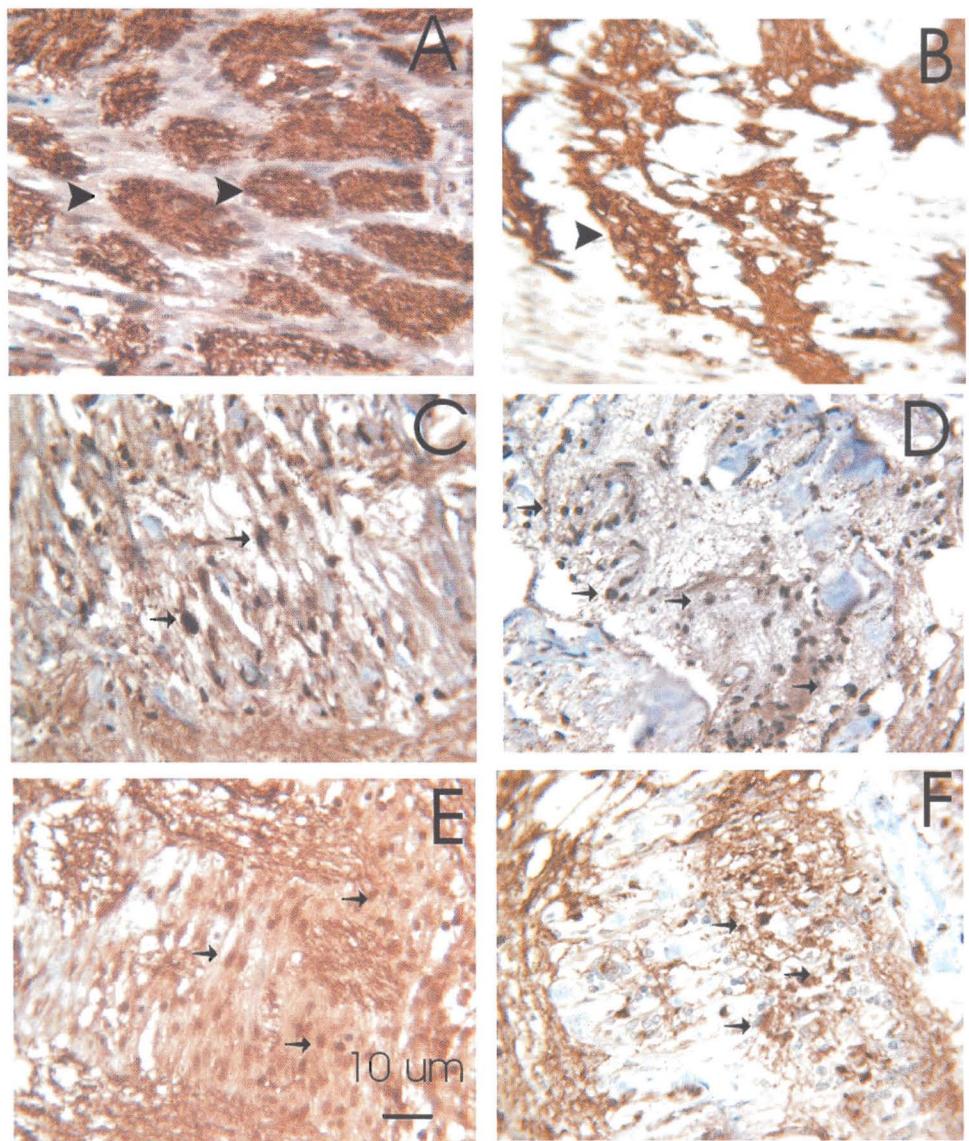


Fig. 1. Sections of the human optic nerve in the area lamina cribrosa. γ -Synuclein was detected using DAB reagent (peroxidase, brown); samples were counterstained with hematoxylin (blue). Sections were stained with G-synAB-01 antibody as described in Materials and Methods. **A, B:** Samples from control individuals (samples 1 and 2, respectively, in Table I). **C:** Sample from a patient with CACG (patient 11, Table I). **D:** Sample from a patient with NTG (patient 12, Table I). **E:** Sample from a patient with POAG (patient 7, Table I). **F:** Patient with glaucoma without history of disease (patient 14, Table I). Arrowheads in A and B indicate nerve bundles, and arrows in C–F show immunopositive staining in glial cells. Scale bar = 10 μ m.

pare Fig. 4, lane 3 (control), with lane 4 (experimental eye) and lane 5 (control) with lane 6 (experimental eye)]. The most significant reduction was observed 4 weeks after operation (reduction to 73% compared with controls). The difference between control and experimental samples was statistically significant ($P = 0.032$). No significant changes in the level of cyclophilin mRNA used as a control were observed in experimental animals (Fig. 4, bottom).

Effect of Elevated Pressure on the Amount of γ -Synuclein in Neural Ocular Tissues

According to Western blot analysis, the amount of γ -synuclein was reduced in the optic nerve of experimental rats ($59\% \pm 4\%$ compared with control samples, average of three experiments; Fig. 5A, lanes 4, 5), whereas no

significant changes were found in the retina of experimental animals compared with control samples (Fig. 5A, lanes 2, 3). At the same time, elevated IOP and elevated hydrostatic pressure did not affect the amount of actin in the optic nerve of experimental animals (Fig. 5C, lanes 4, 5) or in astrocytes (Fig. 5C, lanes 7, 8) but reduced the amount of α -tubulin both in the optic nerve of experimental animals (Fig. 5B, lanes 4, 5) and in astrocytes incubated at elevated hydrostatic pressure (Fig. 5B, lanes 6, 7). Unlike the case with the optic nerve and astrocytes, no effect of elevated IOP on the amount of γ -synuclein, α -tubulin, and actin in the retina was found (Fig. 5A, lanes 2, 3, for γ -synuclein; Fig. 5B, lanes 2, 3, for α -tubulin; Fig. 5C, lanes 4, 5, for actin).

The reduction of γ -synuclein was also observed after immunohistochemical staining of astrocytes incubated un-

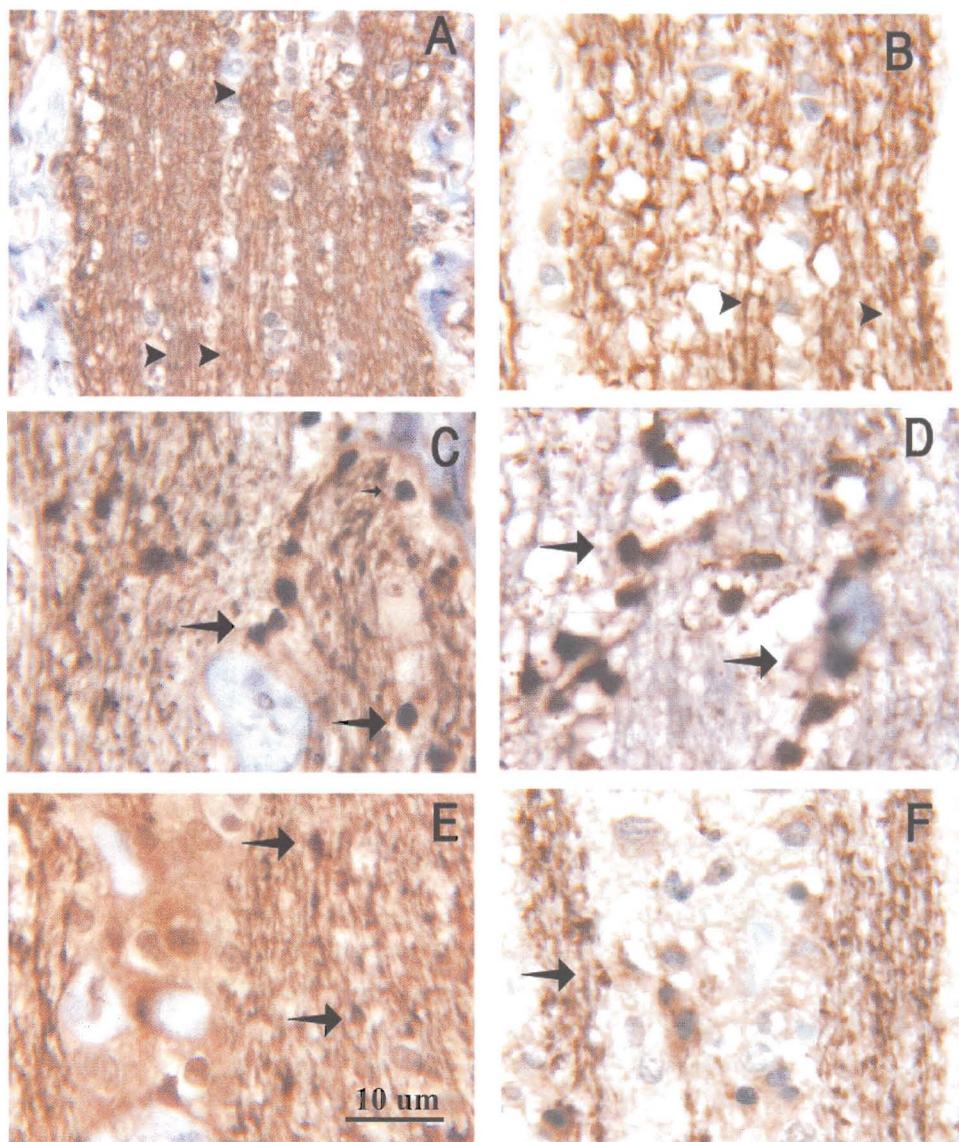


Fig. 2. Longitudinal sections of the human postlamina part of the optic nerve. γ -Synuclein was detected using DAB reagent (peroxidase, brown); samples were counterstained with hematoxylin (blue) as described in Materials and Methods. Sections were stained with G-synAB-01 antibody. **A,B:** Samples from control individuals (samples 1 and 2, respectively, in Table I). **C:** Sample from a patient with CACG (patient 11, Table I). **D:** Sample from a patient with NTG (patient 12, Table I). **E:** Sample from a patient with POAG (patient 7, Table I). **F:** Patient with glaucoma without history of disease (patient 14, Table I). Arrowheads in A and B indicate nerve bundles, and arrows in C–F show immunopositive staining in glial cells. Scale bar = 10 μ m.

der elevated hydrostatic pressure (Fig. 6A,B). The majority of γ -synuclein-immunopositive staining in astrocytes was located in the nucleus, and a fraction of it was associated with intracellular structures located near the nucleus, which were previously identified as centrosomes (Surguchov et al., 2001b; Fig. 6, arrows).

DISCUSSION

The synucleinopathies are a diverse group of neurodegenerative disorders that share a common pathologic lesion composed of aggregates of insoluble synucleins in selectively vulnerable populations of neurons and glia (Galvin et al., 2001). Most papers describing synuclein-related pathologies concern α -synuclein. The role of synucleins in neurodegenerative diseases is well established for brain tissues but has not been characterized in detail for

different ocular pathologies in the retina and optic nerve. This is the first study to characterize the expression of three members of the synuclein family of proteins in tissues and cells of ocular origin in connection with glaucoma. As shown in Figures 1 and 2, we found abnormal IHC staining for γ -synuclein in the glaucomatous optic nerve tissues. For patients with different types of glaucoma, we observed γ -synuclein-positive staining in a subset of glial cells, presumably reactive astrocytes, whereas, in the optic nerves of control individuals, the staining of nerve bundles prevailed. However, we have not seen noticeable changes in the pattern of α - and β -synuclein staining in glaucomatous optic nerves compared with controls.

To clarify whether elevated pressure may be a factor regulating the level of γ -synuclein, we used two model systems, and animal model in which elevated IOP in the

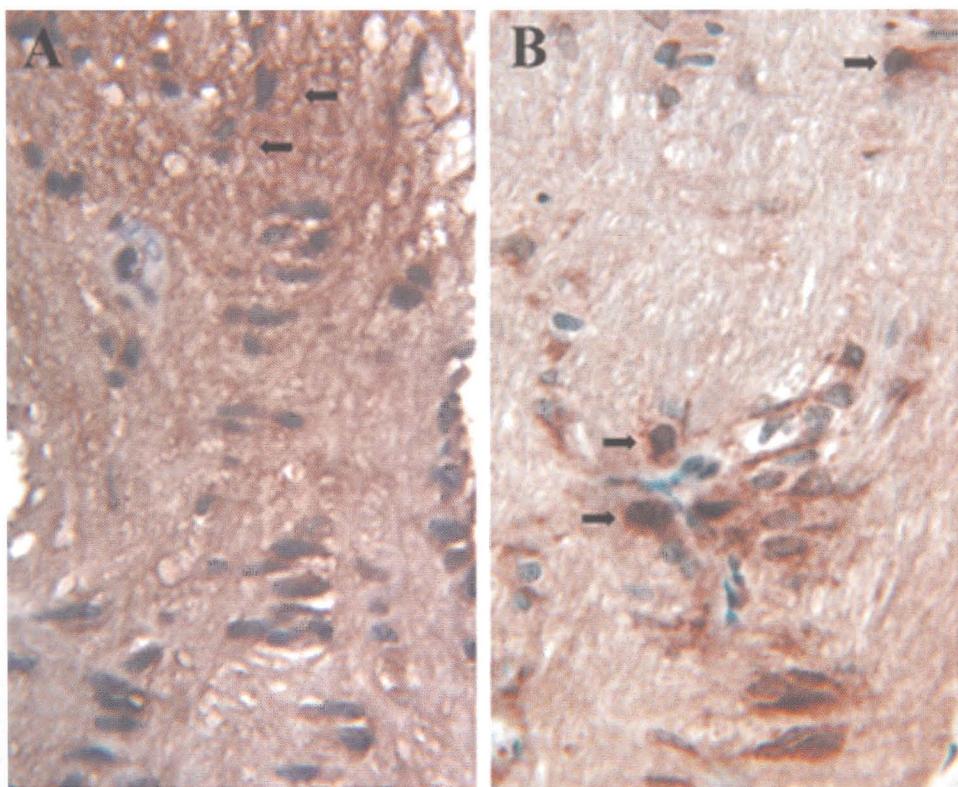


Fig. 3. Longitudinal section of the rat optic nerve. **A:** Sample from a control eye; arrows show nerve bundles. **B:** Sample from an experimental eye; arrows show γ -synuclein-immunopositive glial inclusions. γ -Synuclein was detected using DAB reagent (peroxidase, brown); samples were counterstained with hematoxylin (blue). Sections were stained with commercial antibody (see Materials and Methods).

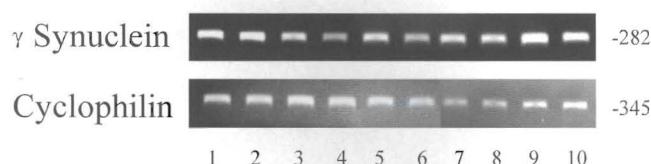


Fig. 4. mRNA level for γ -synuclein (top) and cyclophilin (bottom) measured by semiquantitative RT-PCR in the optic nerve of rats after elevation of IOP. **Lanes 1–4:** Four weeks after cauterization. **Lanes 5–10:** Five weeks after cauterization of three episcleral veins. Lanes 1, 5: RNA isolated from a control eye C1; lanes 2, 6: RNA isolated from experimental eye E1; lanes 3, 7: RNA isolated from control eye C2; lanes 4, 8: RNA isolated from experimental eye E2; lanes 9, 10: RNA isolated from control eye C3 and experimental eye E3, respectively. RT-PCR was carried out as described in Materials and Methods and in a previous paper (Ahmed et al., 2001).

eye was generated by a cauterization of episcleral veins (Shareef et al., 1995) and a cell model in which the effect of hydrostatic pressure on astrocytes was analyzed in a pressurized chamber (Ricard et al., 2000). According to Western blot analysis, elevated hydrostatic pressure caused a reduction of γ -synuclein level in the optic nerve and astrocytes but did not affect its amount in the retina. The reduction of γ -synuclein as a result of elevated pressure shows that it does not behave as the majority of stress proteins or heat shock proteins, the expression of which is up-regulated as a result of stress. At the same time, characteristic lesions were observed both in human glaucoma-

tous optic nerve (Figs. 1, 2) and in the optic nerve of rats with high IOP (Fig. 3) as glial inclusions immunopositive for γ -synuclein.

The changes in γ -synuclein immunoreactivity in the optic nerve that we observed in the animal model in response to elevated IOP are slow compared with the reaction of glial cells to elevation of IOP described in rat retina by Wang and coworkers (2000). These authors observed rapid and synchronous reactivity of glial cells within hours after elevation of IOP, which is supposedly linked to neuronal degeneration.

The appearance of glial inclusions immunopositive for γ -synuclein in the optic nerve of glaucoma patients and in an animal model of glaucoma (Figs. 1–3) may be considered as a histopathological hallmark of glaucomatous alterations. Glial cells are key elements in the dynamic environment of neurons, forming a functional unit involved in homeostasis, plasticity, and neurotransmission (Ridet et al., 1997). Astrocytes, the major cell type in the optic nerve head, are most intimately involved in the reaction to different stresses in the optic nerve, including IOP and ischemia. One of the roles of reactive astrocytes is the preservation of neural tissue integrity from different injuries. Astrocytes of the optic nerve head have been shown to be reactive in glaucomatous eyes (Rao and Lund, 1993; Hernandez and Pena, 1997). Activated astrocytes and microglia alter the microenvironment of neurons. Reactive astrocytes are responsible for generating a glial scar that limits the area of damage. At the same time,

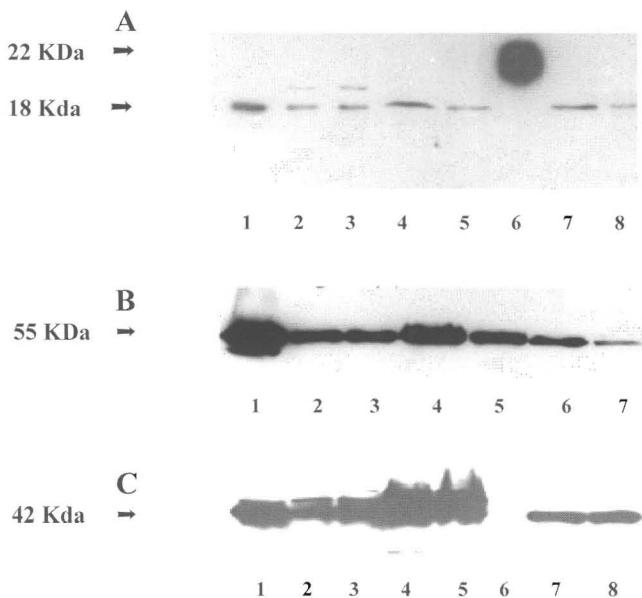


Fig. 5. Effect of elevated pressure on the amount of γ -synuclein (A), α -tubulin (B), and actin (C) in rat tissue samples analyzed by Western blotting. **A:** Extracts from rat brain (lane 1), retina (lanes 2, 3) or optic nerve (lanes 4, 5) were prepared as described previously (Surguchov et al., 2001a,b) and subjected to electrophoresis in 12% PAGE, Western blotted, and probed with antibody to γ -synuclein (ABCAM U.K.). Lane 2: Retinal extract from control rats. Lane 3: Retinal extract from experimental rats. Lane 4: Extract from the optic nerve of control rats. Lane 5: Extract from the optic nerve of experimental rats. Lane 6: Recombinant γ -synuclein was expressed using pTrcHis expression vector (Invitrogen, La Jolla, CA) and purified according to the manufacturer's recommendations. Purified γ -synuclein contains six histidine residues attached to the N-terminus of the protein. Lane 7: Extract from control A7 rat astrocytes. Lane 8: Extract from A7 rat astrocytes subjected to elevated continuous hydrostatic pressure as described in Materials and Methods. **B:** Extracts from rat brain (lane 1), retina (lanes 2, 3), or optic nerve (lanes 4, 5) were prepared as described previously (Surguchov et al., 2001a) and subjected to electrophoresis in 12% PAGE, Western blotted, and probed with antibody to α -tubulin. Lane 2: Retinal extract from control rats. Lane 3: Retinal extract from experimental rats. Lane 4: Extract from the optic nerve of control rats. Lane 5: Extract from the optic nerve of experimental rats. Lane 6: Extract from control A7 rat astrocytes. Lane 7: Extract from A7 rat astrocytes subjected to elevated continuous hydrostatic pressure as described in Materials and Methods. **C:** Extracts from rat brain (lane 1), retina (lanes 2, 3), or optic nerve (lanes 4, 5) were prepared as described previously (Surguchov et al., 2001a) and subjected to electrophoresis in 12% PAGE, Western blotted, and probed with antibody to actin. Lane 2: Retinal extract from control rats. Lane 3: Retinal extract from experimental rats. Lane 4: Extract from the optic nerve of control rats. Lane 5: Extract from the optic nerve of experimental rats. Lane 6: Recombinant γ -synuclein. Lane 7: Extract from control A7 rat astrocytes. Lane 8: Extract from A7 rat astrocytes subjected to elevated continuous hydrostatic pressure as described in Materials and Methods.

reactive astrocytes may damage axons of retinal ganglion cells through the release of neurotoxic agents and mediators (Hernandez and Pena, 1997). In spite of extensive studies of astrocytes and microglia during the past several

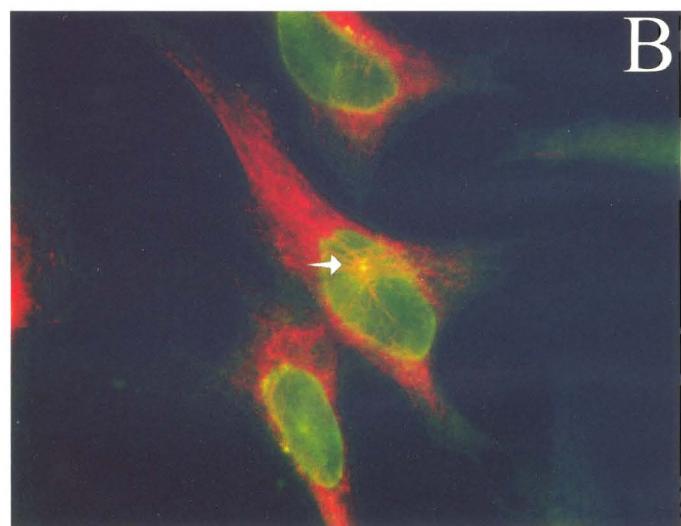
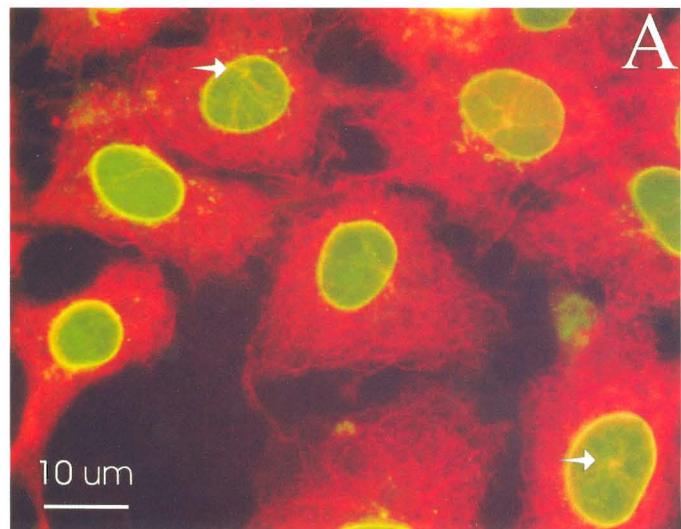


Fig. 6. γ -Synuclein localization in a rat A7 astrocyte culture. **A:** Control astrocyte culture. **B:** Astrocyte culture incubated at elevated hydrostatic pressure. Nuclear localization and association with centrosomes (arrows) are shown. Red, microtubules; green, γ -synuclein.

years, their role in normal eye tissues and in glaucomatous optic nerve is still under debate.

The changes in the localization of γ -synuclein immunoreactivity that we have found in human glaucomatous optic nerve and in the optic nerve of experimental animal model are most probably the consequences rather than a cause of pathological changes that occur in glaucoma. However, we cannot exclude a more direct role for γ -synuclein in the etiology of this disease. Several putative mechanisms may be involved. 1) This role may be connected with the γ -synuclein effect on neurofilament network integrity described recently. Injection of γ -synuclein-expressing vector in cultured sensory neurons caused a dramatic decrease in triplet neurofilament protein

staining (Buchman et al., 1998b). On the other hand, distortion of the neurofilament network is common in neurodegenerative diseases in general and in glaucoma in particular (Vickers et al., 1995b). 2) Another hypothetical mechanism of γ -synuclein involvement in glaucomatous alterations in the optic nerve is related to its role in the signal transduction pathway. γ -Synuclein activates Elk-1 and MAPK (Surguchov et al., 1999, 2001b), and thus its accumulation in glial inclusions described here may cause interruption of such signaling. 3) According to our preliminary results, γ -synuclein activates several types of matrix metalloproteinases (MMPs), enzymes that play an important role in remodeling of the extracellular matrix and possibly are involved in glaucomatous changes in the optic nerve (Agapova et al., 2001).

The data on the role of synucleins in neurodegeneration and their effect on cell survival are controversial. They concern almost exclusively α -synuclein, and in the majority of studies a toxic effect of this member of the synuclein family has been seen (El-Agnaf et al., 1998; El-Agnaf and Irvine, 2000; Iwata et al., 2001). The level of toxicity depends on the aggregation of α -synuclein and formation of fibrils (El-Agnaf and Irvine, 2000) and is increased in the presence of two point mutations described for this protein (Kanda et al., 2000). In other studies, α -synuclein supposedly is not involved in neurodegeneration and apoptotic death but rather is implicated in the compensatory response with concomitant survival (Kholodilov et al., 1999), chaperon activity (Ostrerova et al., 1999; Souza et al., 2000a), or even a protective effect (O'Malley and Jensen, 2000). The level of synuclein toxicity and its effect on cell viability most probably depend not on a single factor but on a combination of several factors, modifying the structure, i.e., 1) posttranslational modifications, including nitrosylation (Souza et al., 2000b); 2) the level of dimerization-oligomerization (El-Agnaf et al., 1998); and 3) the presence of mutations (El-Agnaf et al., 1998). For some of the heat shock proteins it has been shown that their dimerization strongly diminishes their chaperone activity (van de Klundert et al., 1998).

In the majority of previous publications concerning the role of synucleins in neurodegenerative diseases, an important role of α -synuclein has been documented. Here, we suggest a possible γ -synuclein involvement in glaucomatous alterations in the optic nerve, and in a previous publication we demonstrated a redistribution of γ -synuclein immunoreactivity in the retina of AD patients (Surguchov et al., 2001a).

The results presented here do not point to elevated pressure as a major factor mediating the role of γ -synuclein in the changes observed in the glaucomatous optic nerve. Thus, pressure-independent factors not yet identified may account for these changes. Indeed, similar alterations in the pattern of γ -synuclein staining were observed in patients with elevated and normal IOP (compare, for example, panel D with panels C, E, F in Figs. 1 and 2). In addition, we did not find up-regulation of γ -synuclein as a result of

elevated pressure (as one would anticipate for a stress protein or a protein possessing chaperone activity). On the contrary, a reduction of its immunoreactivity was found both in the optic nerve and in astrocytes incubated under elevated pressure. These data may reflect the fact that elevated pressure is only one of several important factors in the etiology of glaucoma. However, the similarity of changes in immunoreactivity observed in different groups of glaucoma patients suggests that γ -synuclein may potentially be considered as a marker of glaucomatous changes in the optic nerve. Overall, these findings point to the importance of further studies of the γ -synuclein role in ocular diseases and other pathologies.

ACKNOWLEDGMENTS

We thank Dr. M.R. Hernandez for providing us with an experimental pressure chamber and for human ocular tissue sections. We express our gratitude to Dr. H. Geller for the A7 astrocyte culture.

REFERENCES

- Agapova OA, Ricard CS, Salvador-Silva M, Hernandez MR. 2001. Expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in human optic nerve head astrocytes. *Glia* 33:205–216.
- Ahmed S, Torrado M, Johnson E, Morrison J, Tomarev S. 2001. Changes in mRNA levels for the *Myoc/Tigr* gene in the rat eye after experimental elevation of intraocular pressure or optic nerve transection. *Invest Ophthalmol Vis Sci* 42:3165–3172.
- Baba M, Nakajo S, Tu PH, Tomita T, Nakaya K, Lee VM, Trojanowski JQ, Iwatsubo T. 1998. Aggregation of alpha-synuclein in Lewy bodies of sporadic Parkinson's disease and dementia with Lewy bodies. *Am J Pathol* 152:879–884.
- Buchman VL, Hunter HJ, Pinon LG, Thompson J, Privalova EM, Ninkina NN, Davies AM. 1998a. Persyn, a member of the synuclein family, has a distinct pattern of expression in the developing nervous system. *J Neurosci* 18:9335–9341.
- Buchman VL, Adu J, Pinon LG, Ninkina NN, Davies AM. 1998b. Persyn, a member of the synuclein family, influences neurofilament network integrity. *Nat Neurosci* 1:101–103.
- Dickson DW, Liu W, Hardy J, Farrer M, Mehta N, Uitti R, Mark M, Zimmerman T, Golbe L, Sage J, Sima A, D'Amato C, Albin R, Gilman S, Yen SH. 1999. Widespread alterations of alpha-synuclein in multiple system atrophy. *Am J Pathol* 155:1241–1251.
- El-Agnaf OM, Irvine GB. 2000. Review: formation and properties of amyloid-like fibrils derived from alpha-synuclein and related proteins. *J Struct Biol* 130:300–309.
- El-Agnaf OM, Jakes R, Curran MD, Middleton D, Ingenito R, Bianchi E, Pessi A, Neill D, Wallace A. 1998. 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* 440:71–75.
- Friedman JS, Walter MA. 1999. Glaucoma genetics, present and future. *Clin Genet* 55:71–79.
- Galvin JE, Uryu K, Lee VMY, Trojanowski JQ. 1999. Axon pathology in Parkinson's disease and Lewy body dementia hippocampus contains α -, β - and γ -synuclein. *Proc Natl Acad Sci USA* 96:13450–13455.
- Galvin JE, Lee VMY, Trojanowski JQ. 2001. Synucleinopathies: clinical and pathological implications. *Arch Neurol* 58:186–190.
- Geller HM, Dubois-Dalcq M. 1988. Antigenic and functional characterization of a rat central nervous system-derived cell line immortalized by a retroviral vector. *J Cell Biol* 107:1977–1986.
- Hernandez MR, Pena JD. 1997. The optic nerve head in glaucomatous optic neuropathy. *Arch Ophthalmol* 115:389–395.

- Iwata A, Maruyama M, Kanazawa I, Nukina N. 2001. Alpha synuclein affects the MAP kinase pathway and accelerates cell death. *J Biol Chem* 276:45320–45329.
- Ji H, Liu YE, Jia T, Wang M, Liu J, Xiao G, Joseph BK, Rosen C, Shi YE. 1997. Identification of a breast cancer-specific gene, BCSG1, by direct differential cDNA sequencing. *Cancer Res* 57:759–764.
- Jia T, Liu YE, Liu J, Shi YE. 1999. Stimulation of breast cancer invasion and metastasis by synuclein gamma. *Cancer Res* 59:742–747.
- Kanda S, Bishop JF, Eglitis MA, Yang Y, Mouradian MM. 2000. Enhanced vulnerability to oxidative stress by alpha-synuclein mutations and C-terminal truncation. *Neuroscience* 97:279–284.
- Kholodilov NG, Meystat M, Oo TF, Lo SE, Larsen KE, Sulzer D, Burke RE. 1999. Increased expression of rat synuclein in the substantia nigra pars compacta identified by mRNA different display in a model of developmental target injury. *J Neurochem* 73:2586–2599.
- Krüger R, Kuhn W, Müller T, Woitalla D, Graeber M, Kosel S, Przuntek H, Epplen JT, Schols L, Riess O. 1998. Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. *Nat Genet* 18:106–108.
- Lavedan C, Leroy E, Dehejia A, Buchholtz S, Dutra A, Nussbaum RL, Polymeropoulos MH. 1998. Identification, localization and characterization of the human gamma-synuclein gene. *Hum Genet* 3:106–112.
- Ninkina NN, Alimova-Kost MV, Paterson JW, Delaney L, Cohen BB, Imreh S, Gnuchev NV, Davies AM, Buchman VL. 1998. Organization, expression and polymorphism of the human persyn gene. *Hum Mol Genet* 7:1417–1424.
- Ninkina NN, Privalova EM, Pinon LG, Davies AM, Buchman VL. 1999. Developmentally regulated expression of persyn, a member of the synuclein family, in skin. *Exp Cell Res* 246:308–311.
- O'Malley K, Jensen P. 2000. Overexpressing α -synuclein in a dopameric cell line attenuates MPP⁺ and 6-OHDA neurotoxicity but not A-beta induced cell death. Abstracts of the Society for Neuroscience, New Orleans 1:44.
- Ostrerova N, Petrucci L, Farrer M, Mehta N, Choi P, Hardy J, Wolozin B. 1999. Alpha synuclein shares physical and functional homology with 14-3-3 proteins. *J Neurosci* 19:5782–5791.
- Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, Pike B, Root H, Rubenstein J, Boyer R, Stenoos ES, Chandrasekharappa S, Athanasiadou A, Papapetropoulos T, Johnson WG, Lazzarini AM, Duvoisin RC, Di Iorio G, Golbe LI, Nussbaum RL. 1997. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science* 276:2045–2047.
- Quigley HA. 1993. Open-angle glaucoma. *N Engl J Med* 328:1097–1106.
- Quigley HA. 1996. Number of people with glaucoma worldwide. *Br J Ophthalmol* 80:385–387.
- Rao K, Lund RD. 1993. Optic nerve degeneration induces the expression of MHC antigens in the rat visual system. *J Comp Neurol* 336:613–627.
- Ricard CS, Kobayashi S, Pena J, Salvador-Silva M, Agapova O, Hernandez MR. 2000. Selective expression of neural cell adhesion molecule (NCAM)-180 in optic nerve head astrocytes exposed to elevated hydrostatic pressure in vitro. *Mol Brain Res* 81:62–79.
- Ridet JL, Malhotra SK, Privat A, Gage FH. 1997. Reactive astrocytes: cellular and molecular cues to biological function. *Trends Neurosci* 20:570–577.
- Schwartz M, Yoles E, Levin LA. 1999. "Axogenic" and "somagenic" neurodegenerative diseases: definitions and therapeutic implications. *Mol Med Today* 5:470–473.
- Shareef SR, Garcia-Valenzuela E, Saliero A, Walsh J, Sharma SC. 1995. Chronic ocular hypertension following episcleral venous occlusion in rats. *Exp Eye Res* 61:379–382.
- Souza JM, Giasson BI, Lee VM, Ischiropoulos H. 2000a. Chaperone-like activity of synucleins. *FEBS Lett* 474:116–119.
- Souza JM, Giasson BI, Chen Q, Lee VM, Ischiropoulos H. 2000b. Dityrosine cross-linking promotes formation of stable alpha-synuclein polymers. Implication of nitritative and oxidative stress in the pathogenesis of neurodegenerative synucleinopathies. *J Biol Chem* 275:18344–18349.
- Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M. 1997. Alpha-synuclein in Lewy bodies. *Nature* 388:839–840.
- Spillantini MG, Crowther RA, Jakes R, Hasegawa M, Goedert M. 1998. Alpha-synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with Lewy bodies. *Proc Natl Acad Sci USA* 95:6469–6473.
- Surguchov A, Surgucheva I, Solessio E, Baehr W. 1999. Synoretin—a new protein belonging to the synuclein family. *Mol Cell Neurosci* 13:95–103.
- Surguchov A, McMahan B, Maslia E, Irina Surgucheva. 2001a. Synucleins in ocular tissues. *J Neurosci Res* 65:68–77.
- Surguchov A, Palazzo RE, Surgucheva I. 2001b. Gamma synuclein: subcellular localization in neuronal and non-neuronal cells and effect on signal transduction. *Cell Motil Cytoskel* 49:218–228.
- Tu PH, Galvin JE, Baba M, Giasson B, Tomita T, Leight S, Nakajo S, Iwatsubo T, Trojanowski JQ, Lee VM. 1998. Glial cytoplasmic inclusions in white matter oligodendrocytes of multiple system atrophy brains contain insoluble alpha-synuclein. *Ann Neurol* 44:415–422.
- Van de Klundert FA, Smulders RH, Gijssen ML, Lindner RA, Jaenike R, Carver JA, de Jong WW. 1998. The mammalian small heat-shock protein Hsp20 forms dimers and is a poor chaperone. *Eur J Biochem* 258:1014–1021.
- Vickers JC. 1997. The cellular mechanism underlying neuronal degeneration in glaucoma: parallels with Alzheimer's disease. *Aust NZ J Ophthalmol* 25:105–109.
- Vickers JC, Schumer RA, Podos SM, Wang RF, Riederer BM, Morrison JH. 1995a. Differential vulnerability of neurochemically identified subpopulations of retinal neurons in a monkey model of glaucoma. *Brain Res* 680:23–35.
- Vickers JC, Lazzarini RA, Riederer BM, Morrison JH. 1995b. Intrapereikaryal neurofilamentous accumulations in a subset of retinal ganglion cells in aged mice that express a human neurofilament gene. *Exp Neurol* 136:266–269.
- Wang X, Tay SSW, Ng YK. 2000. An immunohistochemical study of neuronal and glial cell reactions in retinae of rats with experimental glaucoma. *Exp Brain Res* 132:476–484.