Subcellular Localization of Wild-Type and Parkinson’s Disease-Associated Mutant α-Synuclein in Human and Transgenic Mouse Brain

Philipp J. Kahle,1 Manuela Neumann,2 Laurence Ozmen,3 Veronika Müller,1 Helmut Jacobsen,3 Alice Schindzielorz,1 Masayasu Okochi,1 Uwe Leimer,1 Herman van der Putten,4 Alphonse Probst,5 Elisabeth Kremmer,6 Hans A. Kretzschmar,2 and Christian Haass1

1Adolf Butenandt Institute, Department of Biochemistry, Ludwig Maximilians University, 80336 Munich, Germany, 2Institute of Neuropathology, University of Göttingen, 37075 Göttingen, Germany, 3Pharma Research, Gene Technology, F Hoffmann-La Roche Ltd., 4070 Basel, Switzerland, 4Nervous System, Novartis Pharma Ltd., 4002 Basel, Switzerland, 5Institute of Pathology, University of Basel, 4003 Basel, Switzerland, and 6Gesellschaft für Strahlenschutzforschung Institute of Molecular Immunology, 81377 Munich, Germany

Mutations in the α-synuclein (αSYN) gene are associated with rare cases of familial Parkinson’s disease, and αSYN is a major component of Lewy bodies and Lewy neurites. Here we have investigated the localization of wild-type and mutant [A30P]αSYN as well as βSYN at the cellular and subcellular level. Our direct comparative study demonstrates extensive synaptic colocalization of αSYN and βSYN in human and mouse brain. In a sucrose gradient equilibrium centrifugation assay, a portion of βSYN floated into lower density fractions, which also contained the synaptic vesicle marker synaptophysin. Likewise, wild-type and [A30P]αSYN were found in floating fractions. Subcellular fractionation of mouse brain revealed that both αSYN and βSYN were present in synaptosomes. In contrast to synaptophysin, βSYN and αSYN were recovered from the soluble fraction upon isolation of the synaptosomes. Synaptic colocalization of αSYN and βSYN was directly visualized by confocal microscopy of double-stained human brain sections. The Parkinson’s disease-associated human mutant [A30P]αSYN was found to colocalize with βSYN and synaptophysin in synapses of transgenic mouse brain. However, in addition to their normal presynaptic localization, transgenic wild-type and [A30P]αSYN abnormally accumulated in neuronal cell bodies and neurites throughout the brain. Thus, mutant [A30P]αSYN does not fail to be transported to synapses, but its transgenic overexpression apparently leads to abnormal cellular accumulations.

Key words: synuclein; synaptophysin; brain; synapse; Parkinson’s disease; Lewy body

Synucleins (SYNs) are ~14 kDa phosphoproteins predominantly expressed in brain. Members of the synuclein family include αSYN, βSYN, γSYN (Lavedan, 1998), and synoretin (Surguchov et al., 1999). The central domain of αSYN had been originally identified as the non-amyloid β-protein component (NAC) of Alzheimer’s disease plaques (Uéda et al., 1993). Full-length αSYN has been subsequently found in Lewy bodies (LBs), pale bodies, and Lewy neurites of patients with Parkinson’s disease (PD) and dementia with LBs, as well as in cytoplasmic inclusions characteristic for multiple system atrophy (Spillantini et al., 1997; Arima et al., 1998; Baba et al., 1998; Spillantini et al., 1998; Takeda et al., 1998a; Tu et al., 1998; Wakabayashi et al., 1998; Culvenor et al., 1999). LBs were αSYN-positive in LB variant of Alzheimer’s disease, familial Alzheimer’s disease, and Down’s syndrome (Lippa et al., 1998, 1999; Takeda et al., 1998b), as well as in neurodegeneration with brain iron accumulation type 1 (formerly known as Hallervorden–Spatz disease) (Arawaka et al., 1998; Wakabayashi et al., 1999).

Two missense mutations in the αSYN gene have been linked to familial PD (Polymeropoulos et al., 1997; Krüger et al., 1998). Both mutations accelerated the intrinsic property of αSYN to self-aggregate into fibrils that were morphologically similar to those isolated from LBs (Conway et al., 1998; Giasson et al., 1999; Narhi et al., 1999). Therefore, similar to most of the mutations associated with other familial forms of neurodegenerative disorders, αSYN mutations lead to the abnormal generation of an amyloidogenic variant, which is deposited in the disease-specific lesion (Hardy and Gh-Ow-Hardy, 1998; Lansbury, 1999; Selkoe, 1999).

The physiological function of synucleins is unknown. Targeted disruption of the αSYN gene in mice caused a subtle perturbation in dopaminergic neurotransmission (Abeliovich et al., 2000). The identification of αSYN binding proteins has pointed to potential roles in signal transduction, perhaps in the context of axonal transport (Jen et al., 1998; Engelder et al., 1999; Jensen et al., 1999; Osterrova et al., 1999). Another link to signal transduction events may be indicated by the fact that both αSYN and βSYN are phosphorylated (Nakajo et al., 1993; Okochi et al., 2000).

Previous immunohistochemical studies suggested an enrichment of αSYN and βSYN in presynaptic terminals, and subcellular fractionation studies revealed a potential synaptoosomal localization of αSYN and βSYN (Maroteaux and Scheller, 1991; Shibayama-Imazu et al., 1993; Jakes et al., 1994; George et al., 1995; Iwai et al., 1995; Irizarry et al., 1996). Here we have directly compared the cellular expression of wild-type and the PD-associated mutant [A30P]αSYN to that of βSYN. The integral membrane protein synaptophysin was used as a marker for synaptic vesicles. Our direct comparative study demonstrates extensive synaptic colocalization of αSYN and βSYN in human and mouse brain. In brains of transgenic mice expressing human mutant [A30P]αSYN, a synaptic colocalization with βSYN was found, suggesting that the mutation does not interfere with anterograde transport of αSYN to synapses. However, in addition to the presynaptic localization, both transgenic wild-type and mutant [A30P]αSYN abnormally accumulated in neuronal cell bodies and neurites throughout the brain.
Thus, transgenic αSYN did not fail to be transported to synapses, but overexpression apparently caused pathological accumulations in neurons.

MATERIALS AND METHODS

Antibodies. Synthetic βSYN(87–101) peptide EEFPDLLKPEEOAV linked to keyhole limpet hemocyanin via an N-terminal cysteine residue, and keyhole limpet hemocyanin-coupled αSYN(125–140) peptide TEMPS-SEEEDYDPEA and mouse αSYN(116–131) peptide MPVDPS-SEAYEMSEE were used to immunize rabbits (Eurogentec, Seraing, Belgium). The resulting antisera against βSYN (6485), αSYN (6482), and mouse αSYN (7544) were used for Western probing at working dilution 1:300. Both antisera (Mc 15) against human αSYN(117–131) peptide MPVDPNDNEAYEMSEE was produced in cooperation with Connex (Munich, Germany). The peptide was synthesized, directly coupled to keyhole limpet hemocyanin using 1-ethyl-3(3-dimethylaminopropyl)carbodiimide and used to immunize rabbits (St. Louis, MO).

Antibody characterization. Purified recombinant human synucleins expressed in SF-9 cells were donated by A. Jentsch. Anti-αSYN (Centurion, Abingdon, UK) and anti-βSYN purified from human brain (Baba et al., 1998) were kindly provided by T. Iwatsubo (University of Tokyo, Tokyo, Japan). Two micrograms of purified synucleins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane (0.45 µM Immobilon-P; Millipore, Bedford, MA). Western blots were blocked with 5% skimmed milk and probed with the above antibodies as indicated. Chemiluminescence was generated with SuperSignal (Pierce, Rockford, IL) unless otherwise stated and visualized on X-Omat film (Eastman Kodak, Rochester, NY).

Heat-stable cytosolic fractions were prepared from human (temporal cortex, epilepsy lobotomy of epilepsy patients and kept refrigerated after surgery. Brain tissue was homogenized by eight Dounce strokes in 4–5 vol of homogenization buffer, and postnuclear fractions were prepared (Jensen et al., 1998). Subcellular fractionation and enrichment of synaptosomal compartments were performed as described (Huttner et al., 1983; George et al., 1995). Proteins from the membranous pellets and the dilute soluble synaptic contents (LP2) were precipitated with trichloroacetic acid. Precipitates were collected by 16,000 × g centrifugation, washed with 70% acetone, neutralized, and redissolved in Laemmli’s buffer. Samples were resolved by 12.5% SDS-PAGE, and the corresponding Western blots were sequentially probed for αSYN and synaptophysin. When necessary, weak signals were detected with enhanced chemiluminescence ECLplus (Amersham BioScience, Little Chalfont, UK).

Sucrose gradient fractionation assays (Jensen et al., 1998) were performed with material prepared from wild-type and transgenic mouse and human brain. Briefly, samples were brought to 55% sucrose and overlaid with 1.5 ml. 1 hr at room temperature. Detection of antibody binding was done by the alkaline-phosphatase anti-alkaline phosphatase system according to the instructions of the manufacturer (Dako, High Wycombe, UK) using Neuf- tins as chromogen.

Double-immunolabeling of βSYN with either αSYN or synaptophysin was performed with two different fluorochrome-conjugated secondary antibodies. After incubation with the first primary antibody against βSYN for 1 hr, sections were washed (10 min) and incubated with goat-antirabbit antibody (Dako, High Wycombe, UK) at a dilution of 1:200) was kindly provided by W. W. Franke and R. E. Leube (Deutsches Forschungszentrum, Heidelberg, Germany). Horse anti-peroxidase-conjugated secondary antibodies (working dilution 1:5000) were purchased from Sigma (St. Louis, MO).

Antibody characterization. Purified recombinant human synucleins expressed in SF-9 cells were donated by A. Jentsch. Anti-αSYN (Centurion, Abingdon, UK) and anti-βSYN purified from human brain (Baba et al., 1998) were kindly provided by T. Iwatsubo (University of Tokyo, Tokyo, Japan). Two micrograms of purified synucleins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane (0.45 µM Immobilon-P; Millipore, Bedford, MA). Western blots were blocked with 5% skimmed milk and probed with the above antibodies as indicated. Chemiluminescence was generated with SuperSignal (Pierce, Rockford, IL) unless otherwise stated and visualized on X-Omat film (Eastman Kodak, Rochester, NY).

RNA isolation and Northern blot analysis. RNA was isolated from total brain of mice at 2, 5, 10, 16, and 20 d after birth. Organs were homogenized with beads in 0.9 ml of RNAlater–0.1 ml of chlorpromazine (in a FP120 (Bio 101) and chlorpromazine was removed by phenol/chloroform extraction and ethanol precipitation.

RESULTS

Selective detection of αSYN and βSYN

Amino acids 87–101 located in close C-terminal apposition to the NAC domain are absolutely conserved in all the mammalian βSYN sequences reported to date (Lavedan, 1998). To generate βSYN antibodies that do not cross-react with other members of the synuclein family, rabbits were immunized with the βSYN peptide 87–101 to generate antisera 6485. Antibody 6485 strongly reacted on Western blots with purified as well as recombinant βSYN (Fig. 1A, B). Human and mouse brain βSYN were both recognized (Fig. 1C). Preabsorption of antisera 6485 with 1 µg/ml immunizing peptide completely abolished immunoreactivity on Western blots (results not shown). Antiserum 6485 showed no cross-reactivity with αSYN and γSYN (Fig. 1A, B). Moreover, anti-βSYN 6485 immunoprecipitates were not detected with the αSYN-specific antibody Mc42 (Transduction Laboratories) and vice versa (data not shown). Therefore, antiserum 6485 can be used to specifically identify βSYN in human and mouse tissue.

Because we attempted to compare the cellular distribution of overexpressed human A30PaSYN with endogenous αSYN and βSYN in transgenic mice, we searched for antibodies that allow the exclusive identification of human αSYN. A double amino acid substitution is present close to the C termini of human and mouse.
αSYN. In the human sequence, amino acids 121–122 are Asp-Gln, whereas in mouse, the corresponding amino acids are Gly-Ser. The Mc15G7 raised against a peptide (human αSYN residues 116–131) encompassing this region was indeed specific for human αSYN, whereas in mouse, the corresponding amino acids are Gly-Ser. The Mc15G7 raised against a peptide (human αSYN residues 116–131) was found in the highest density fractions but was rather smeared throughout the lower density fractions (Fig. 2B).

When the floatation assay was performed with a 16,000 x g supernatant of the mouse brain postnuclear fraction, both αSYN and βSYN were depleted from the floating fractions and found exclusively in the highest density fractions (Fig. 2C). However, synaptophysin was detectable in the lower density fractions of 16,000 x g supernatants, as observed in floatation assays from 1000 x g supernatants. The synaptophysin-positive floating material in the 16,000 x g supernatant might represent free synaptic vesicles. Synaptosomes are pelleted at 16,000 x g (Huttner et al., 1983). Thus, the synuclein-positive material in the floating fractions of 1000 x g supernatants might represent synaptosomes, because the signal is lost upon centrifugation with 16,000 x g.

To further prove this possibility, systematic subcellular fractionation (Huttner et al., 1983) (Fig. 2D) was performed with mouse brain (Fig. 2E). Abundant αSYN immunoreactivity was detected in the postnuclear (1000 x g) supernatant (S1) and in the 12,500 x g supernatant (S2). The S2 fraction was subsequently subjected to 100,000 x g centrifugation. After this step, αSYN was detected in the supernatant (S3, the cytosolic fraction). A significant fraction of αSYN was also present in the 12,500 x g pellet (P2). This crude synaptosomal fraction was washed, lysed hypotonically, cleared by 25,000 x g centrifugation, and subjected to 100,000 x g centrifugation. After centrifugation, αSYN was found in the supernatant (LS2, the soluble content of synaptosomes). Reprobing the same membrane with antisera 6485 revealed a similar subcellular distribution of βSYN. The same subcellular distribution of αSYN was found in the human epilepsy control brain (Fig. 2F). Synaptophysin was found in the 100,000 x g pellets (P3 and L2) and was particularly enriched in the synaptosomal preparations (L2).

**Colocalization of αSYN and βSYN in normal brain**

Our direct comparative biochemical experiments revealed that αSYN and βSYN were reproducibly found in the same subcellular fractions. To confirm colocalization of αSYN and βSYN, we performed immunohistochemical experiments on human brain sections of temporal and cerebellar cortex. Immunostaining of temporal cortices revealed a strong synaptic neuropil staining throughout all cortical layers (data not shown). In the cerebellum, an intense staining was seen in the molecular layer, in discrete areas in the granule cell layer resembling cerebellar glomeruli, and around the somata of Purkinje cells (Fig. 3A, B). The cytoplasm of neuronal and glial cells, as well as the white matter, were immunonegative. The staining pattern was similar to that obtained with antibodies against synaptophysin (Fig. 3C). There were no different staining patterns in human and mouse brains.

To ascertain the colocalization of βSYN with αSYN as well as with synaptophysin, double-labeled immunofluorescent confocal laser scanning microscopy was performed. The βSYN-positive structures showed a marked overlap with αSYN and synaptophysin-positive synaptic structures enriched in the cerebellar glomeruli (Fig. 3D–F). Likewise, a virtually complete overlap was observed in the molecular layer (results not shown).

**Expression of [A30P]αSYN in transgenic mice**

Patients heterozygous for the A30P mutation in the αSYN gene developed an aggressive, early-onset form of PD (Krüger et al., 1998). To study the in vivo consequences of expression of [A30P]αSYN in brain, transgenic mice were generated. Oozytes were microinjected with a construct that harbored the coding region of the human αSYN cDNA bearing the A30P missense mutation (Fig. 4A). Expression of [A30P]αSYN from this construct was driven by the brain neuron-specific Thy-1 promoter (Kollia et al., 1987). Transgenic offspring was backcrossed into the C57BL/6 mouse strain.

Northern and Western blot analysis revealed strong expression of transgenic human [A30P]αSYN mRNA and protein, respectively, in whole brain (Fig. 4). There was no difference in the expression and localization of synucleins between males and females.

**Detection of αSYN and βSYN in synaptosomal fractions**

A sucrose gradient floatation assay has been used recently to describe synaptic vesicle association of αSYN in rat brain (Jensen et al., 1998). After equilibrium centrifugation of a postnuclear fraction from mouse brain, βSYN was found in the highest density fraction 8 and also floated into the lower density fraction 6 (Fig. 2A). A very similar profile was observed for αSYN (Fig. 2A). We also analyzed the distribution of the synaptic vesicle marker synaptophysin (Wiedenmann and Franke, 1985). Synaptophysin was exclusively identified in the floating fractions (Fig. 2A). As for the fresh mouse brain, αSYN from rapidly processed human temporal cortex of an epilepsy patient after lobotomy was present in the highest density fraction 13, as well as in the floating lower density fractions (Fig. 2B). Again, synaptophysin was not found in the lowest density fraction but was rather smeared throughout the lower density fractions (Fig. 2B).
males (data not shown). Lines 18 and 31 consistently expressed higher levels of transgenic protein (2–4 times more) compared with lines 8, 9, and 14. Judging from the Western blot probed with Mc42 that detected human and mouse aSYN equally well (see Fig. 1C), transgenic overexpression in lines 18 and 31 was estimated to be approximately twofold relative to the endogenous aSYN level. Small amounts of higher molecular weight species immunoreactive with anti-aSYN were occasionally observed (Fig. 4E, F). The mobility of this double band was consistent with that of an aSYN dimer. [A30P]aSYN expression was upregulated in the first postnatal month and remained high into old age (data not shown). This time course of expression approximately paralleled that of endogenous aSYN in wild-type and transgenic mice (data not shown).

Synaptosomal localization of [A30P]αSYN in transgenic mice

Mutant [A30P]αSYN was shown previously to associate less efficiently with cellular vesicles in in vitro assays (Jensen et al., 1998). Thus, a deficiency in axonal transport was suggested for [A30P]αSYN. To prove this possibility in vivo, whole brain homogenates of [A30P]αSYN-expressing mice were subjected to the sucrose gradient flotation assay. Immunoblotting with the human-specific antibody 3400 revealed [A30P]αSYN in both the highest density fraction 9 and the synaptophysin-containing floating fractions (Fig. 5). In the same animal, a very similar distribution was shown by Mc42, which detected both endogenous mouse αSYN...
and transgenic human [A30P]αSYN. The high-expressing lines 18 and 31 and the low-expressing lines 8, 9, and 14 showed the same distribution of [A30P]αSYN in the sucrose gradient (Fig. 5, and data not shown), thus excluding the possibility that high expression levels may overcome a partial loss of vesicle binding activity. These results suggest that [A30P]αSYN is anterogradely transported to synapses in vivo (also see below).

Abnormal accumulation of [A30P]αSYN in neuronal cell bodies and neurites in transgenic mouse brain

Immunohistochemistry using the human-specific antibody 15G7 was performed to visualize the subcellular localization of αSYN in transgenic mouse brain sections (Fig. 6A–C). Transgenic mice expressing similar levels of human wild-type αSYN were used as control. Normal neuropil and presynaptic staining of [A30P]αSYN and wild-type αSYN was evident throughout the brain, supporting our conclusion that both forms of αSYN are anterogradely transported. In addition to the normal presynaptic localization, the human transgene-specific antibody 15G7 revealed a strong, diffuse cytosolic immunostaining in neuronal cell bodies (Fig. 6B, C). In contrast, endogenous αSYN was not observed in somal compartments with the mouse-specific antiseraum 7544 (Fig. 6G). Moreover, abnormal αSYN-positive neurites were frequently observed (Fig. 7). Affected neurites contained diffuse αSYN immunoreactivity and sometimes bulged into single or multiple varicosities over a stretch of several micrometers (Fig. 7A–F). Abnormal αSYN-positive neurites were occasionally seen to emanate from a neuronal cell body with accumulated αSYN (Fig. 7A, B). Abnormal αSYN-positive profiles were observed in most brain areas, including the cerebellar Purkinje cells (Fig. 6B, C) and nucleus dentatus (Fig. 7E–G), substantia nigra and striatum, hippocampus, neocortex, and brainstem. Somal and neuritic accumulation of [A30P]αSYN was similar in all five transgenic mouse lines and was observed in half-year-old and 1-year-old animals. In contrast, the staining patterns of antibodies against the endogenous mouse αSYN (Fig. 7D), βSYN (Fig. 7C), and synaptophysin (results not shown) did not differ between nontransgenic and αSYN transgenic

Figure 3. Colocalization of αSYN and βSYN in human cerebellum. Antibodies 15G7 anti-αSYN (A), 6485 anti-βSYN (B), and anti-synaptophysin (C) all stained a punctate pattern in the molecular layer. The immunoreactivity in the cerebellar granule cell layer showed a patchy distribution corresponding to labeling of cerebellar glomeruli. Scale bar: 100 μm. Double-labeled immunofluorescent confocal microscopy revealed a colocalization of αSYN (D) and βSYN (E) in the cerebellar glomeruli of the granule cell layer, which resulted in a yellow signal in the superimposed digital picture (F). A similar colocalization is seen with synaptophysin (G) and βSYN (H). I. Superimposed digital picture. Scale bar: D–I, 10 μm.
Figure 4. Expression of [A30P]aSYN in transgenic mouse brain. A, Schematic drawing of the transgenic construct (not drawn to scale). Hatched box, nThy-1.2 promoter region. Open boxes, Thy-1 exonic sequences; (truncated) exon IV contains the polyadenylation signals. Solid line, Thy-1 intron A. Start and stop codons of the open reading frame for human [A30P]aSYN (filled box; *, A30P mutagenesis site) are directly flanked by XhoI restriction sites (A). Dashed line, 3' -region of the Thy-1 gene. A, NorI restriction sites used to linearize construct and remove vector sequences before microinjection. B, A mixture of two probes specific for the human aSYN transgene and a probe for the mouse β-actin gene was hybridized to a Northern blot of poly(A)+ RNA from [A30P]aSYN mice, as indicated. The sizes of the transcripts were ~1.8 and 2.1 kb, respectively. C-E, Lyophilized heat-stable supernatants of whole brain cytosol (200 µg) from 6- to 10-week-old individuals of the indicated [A30P]aSYN mouse lines were subjected to 15% SDS-PAGE. Equal loading was demonstrated by Brilliant blue staining of the gels after transfer. Western blots were sequentially probed with the mouse-specific antiseraum 7544 (C), Mc42 (D), and the human-specific antibody 3400 (E). Bands were quantified by densitometric scanning (bottom panels). The data are representative for at least three animals per line screened for [A30P]aSYN protein expression. aSYN-immunoreactive double bands (asterisks) comigrating with the 29 kDa standard, a position consistent with the molecular mass of a dimer, were observed at variable intensity. These putative dimeric species could be well resolved in large gels (P10DS; Owl Separation Systems, Portsmouth, NH) (F). Whole brain cytosol (600 µg) from a 10-month-old line 18 mouse was directly subjected to 15% SDS-PAGE, without any concentration step. Western blot was probed with antibody 3400.

Figure 5. Synaptosomal localization of [A30P]aSYN in transgenic mouse brain. Postnuclear supernatant from a 4-month-old male line 18 mouse was processed as described in Figure 2A. The Western blot was sequentially probed with antiserum 3400 (top panel), Mc42 (middle panel), and anti-synaptophysin (bottom panel).

mice. Thus, the transport of synaptic vesicle proteins is not generally perturbed in the mice expressing human aSYN. Rather, the pathological accumulation in neuronal cell bodies and neurites is restricted to the transgenic human aSYN.

DISCUSSION

Using antibodies specific for selected members of the synuclein family, we demonstrated synaptic colocalization of aSYN and βSYN in mouse and human brain. Biochemically, both aSYN and βSYN were present in the same subcellular compartments, namely the cytosol and synaptosomes. Both synucleins were found throughout the brain, most prominently in the synapse-rich molecular layers of cerebellum (Fig. 6), neocortex (Fig. 7), hippocampus, and retina (data not shown). Double-labeled confocal microscopy revealed extensive colocalization of aSYN and βSYN. Most, if not all, synaptophysin-positive presynaptic terminals therefore contain both aSYN and βSYN, at least in human cerebellar cortex. Because the same presynaptic staining was observed in (Thy-1)-h[wt]aSYN and (Thy-1)-h[A30P]aSYN mice, the PD-associated A30P mutation did not abolish anterograde transport to the synaptic compartments in transgenic mouse brain. However, some perturbance of its axonal transport may be indicated by the accumulation of transgenic aSYN in neuronal cell bodies and neurites.

aSYN immunoreactivity in the lower density fractions of the recently described sucrose gradient floatation assay has been interpreted as interaction of aSYN with synaptic vesicles (Jensen et al., 1998). However, centrifugation at relatively low force (16,000 g) depleted synucleins from the floating fractions (Fig. 2C). Centrifugation at this force is sufficient to pellet synaptosomes (Huttner et al., 1983). aSYN and βSYN were indeed present in the synaptosomal pellet P2 upon subcellular fractionation of mouse brain. Lysis of the synaptosomal pellet was followed by recovery of synucleins from the 100,000 g supernatant LS2. In contrast, synaptophysin was quantitatively recovered from the 100,000 g pellet LP2, which contains synaptic vesicles (Huttner et al., 1983). Moreover, some synaptophysin was detected in the 100,000 g pellet P3 (Fig. 2D), as well as in the synuclein-depleted floating fractions from a 16,000 g supernatant (Fig. 2C). Thus, if synucleins are bound to synaptic vesicles, as visualized by the punctate immunostaining of primary neuron cultures (Shibayama-Imazu et al., 1993; Withers et al., 1997) and demonstrated by the in vitro interaction of aSYN with synthetic membranes and a crude vesicle preparation (Davidson et al., 1998; Jensen et al., 1998), the interaction appears to be reversible. Dilution in the process of the floatation assay and the hypotonic lysis of synaptosomes may dissociate the putative synuclein–vesicle complex. Both methods involved at least 10-fold dilution in the sucrose gradient and water, respectively. In contrast, insertion of the membrane-spanning protein synaptophysin (Wiedemann and Franke, 1985)
into synaptic vesicles is expected to be resistant to these procedures. The \( \alpha \)SYN and \( \beta \)SYN present in floating fractions from the brain postnuclear fraction may arise from synaptic vesicles trapped in synaptosomes. Consistent with this interpretation is the identification of the synaptosomal-associated protein SNAP-25, a presynaptic membrane marker (Söllner et al., 1993), in the \( \alpha \)SYN-positive floating fractions (Jensen et al., 1998). Synaptosomal synucleins were also recovered in LS2 in previous studies of rat brain (Maroteaux and Scheller, 1991; Shibayama-Imazu et al., 1993; George et al., 1995). Irizarry et al. (1996), however, reported an equal distribution of \( \alpha \)SYN in the synaptosomal supernatants (LS2) and pellets (LP2) prepared from postmortem human temporal cortex. Methodological (fresh vs postmortem brain) differences may account for this finding.

Immunoreactivity in the cerebellar molecular layer, circumventing Purkinje cells, and particularly intense staining of cerebellar glomeruli in the granule cell layer of rat and canary has been detected previously with antibodies against \( \alpha \)SYN and \( \beta \)SYN (Shibayama-Imazu et al., 1993; Jakes et al., 1994; George et al., 1995; Iwai et al., 1995). The distribution of these proteins was similar in mouse brain. Double-stained confocal microscopy demonstrated virtually complete overlap of \( \alpha \)SYN, \( \beta \)SYN, and synaptofysin. We were not able to identify \( \alpha \)SYN-specific or \( \beta \)SYN-specific synapses. The extensive colocalization of \( \alpha \)SYN and \( \beta \)SYN suggests that they are spatially close and thus in a position to be functionally redundant.

It has been proposed that [A30P]\( \alpha \)SYN is less efficiently transported along the axon (Jensen et al., 1998, 1999). However, we observed a normal synaptosomal localization and presynaptic distribution of wild-type and [A30P]\( \alpha \)SYN in transgenic mouse brain. Thus, anterograde transport of \( \alpha \)SYN in vivo was not severely abolished by the A30P mutation. Likewise, no difference in subcellular targeting was found in primary neurons transfected with wild-type and mutant \( \alpha \)SYN (McLean et al., 2000).

Nevertheless, a perturbation of axonal transport was indicated by the accumulation of \( \alpha \)SYN in neuronal cell bodies and neurites, which was observed in transgenic mice expressing wild-type and [A30P]\( \alpha \)SYN. Endogenous mouse \( \alpha \)SYN was not retained in the pathological cell bodies and neurites. Moreover, neither \( \beta \)SYN nor synaptofysin were found within these [A30P]\( \alpha \)SYN-positive profiles. Thus, somal and neuritic accumulation is a specific feature of transgenic human \( \alpha \)SYN and not simply attributable to an overload of the machinery transporting synaptic vesicle proteins.

Somal accumulation of \( \alpha \)SYN was found in mice subjected to a chronic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) regimen causing apoptotic death of dopaminergic neurons, whereas acute MPTP toxicity had no effect on \( \alpha \)SYN expression (Vila et al., 2000). Similar findings were made in rat and baboon models of dopaminergic neuron apoptosis (Kholodilov et al., 1999; Kowall et al., 2000). We have not detected any apoptotic profiles in the [A30P]\( \alpha \)SYN mice. Thus, the observed somal (and neuritic) accumulation is not a consequence of apoptosis.

While this work was under consideration, human \( \alpha \)SYN (both wild-type and PD mutant) expressed in transgenic Drosophila melanogaster was demonstrated to form the 7–10 nm fibrils that are characteristic for human LBs (Feany and Bender, 2000). The only difference found in this study was a more rapid decline in climbing behavior in the mutant [A30P]\( \alpha \)SYN transgenic animals compared with [wt]\( \alpha \)SYN flies. Recently, a transgenic mouse line greatly overexpressing human wild-type \( \alpha \)SYN under the control of the human PDGF-\( \beta \) promoter was presented (Masliah et al., 2000). These animals showed amorphous precipitates of \( \alpha \)SYN in the cytoplasm but also in the rough endoplasmic reticulum and the nucleus. Decreases in dopaminergic markers and locomotor performance were reported. Our (Tyr-1)-h[A30P]\( \alpha \)SYN mice showed no movement disability, up to 1 year of age. Apparently the neurons were able to cope with the load of transgenic [A30P]\( \alpha \)SYN, despite its tendency to form fibrils in vitro (Giasson et al., 2000).
Figure 7. Accumulation of human αSYN in neuronal cell bodies and neurites of transgenic mouse brain. Abnormal accumulation of human [A30P]αSYN was detected in cell bodies and bulbous neurites in most brain regions, including frontal cortex (A [line 31] and B [line 18]), 15G7). Some [A30P]αSYN-filled neurites (arrows) emanated from neuronal cell bodies with accumulated [A30P]αSYN (A, B). In contrast, expression of βSYN (C, antisemur 6485) and endogenous αSYN (D, antisemur 7544) was restricted to the neuropil and was not found in neuronal cell bodies and neurites accumulating [A30P]αSYN. Pathological bulbous αSYN-positive neurites (arrows) could also be observed in the dentate nucleus of wt (E) and [A30P] [line 31 (F) and line 18 (G); arrows] mice (15G7 staining). H, Lewy neurites (arrows) stained with 15G7 in the hippocampal CA2/3 region of a patient with LB dementia.

The pathological accumulations of overexpressed h[A30P]αSYN might represent early stages of pathological abnormalities, which could finally lead to PD-like phenotype. It remains to be shown whether additional cofactors are required to induce fibril formation and generation of LB-like deposits in vertebrate brain.

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