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Contrasting Effects of α-Synuclein and γ-Synuclein on the Phenotype of Cysteine String Protein α (CSPα) Null Mutant Mice Suggest Distinct Function of these Proteins in Neuronal Synapses*

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Background: α-Synuclein rescues synaptic dysfunction in CSPα-deficient mice, but the effect of γ-synuclein is unknown.
Results: γ-Synuclein binds synaptic vesicles but is unable to interact with synaptobrevin-2/VAMP2 and rescue the phenotype of CSPα-deficient mice.
Conclusion: Functional diversity of the two synucleins in synapses is determined by structural differences within their C-terminal domains.
Significance: Delineating functional similarities and differences within the synuclein family is important for understanding synaptic transmission and pathogenesis of synucleinopathies.

In neuronal synapses, neurotransmitter-loaded vesicles fuse with presynaptic plasma membrane in a complex sequence of tightly regulated events. The assembly of specialized SNARE complexes plays a pivotal role in this process. The function of the chaperone cysteine string protein α (CSPα) is important for synaptic SNARE complex formation, and mice lacking this protein develop severe synaptic dysfunction and neurodegeneration that lead to their death within 3 months after birth. Another presynaptic protein, α-synuclein, also potentiates SNARE complex formation, and its overexpression rescues the phenotype of CSPα null mutant mice, although these two proteins use different mechanisms to achieve this effect. α-Synuclein is a member of a family of three related proteins whose structural similarity suggests functional redundancy. Here, we assessed whether γ-synuclein shares the ability of α-synuclein to bind synaptic vesicles and ameliorate neurodegeneration caused by CSPα deficiency *in vivo*. Although the N-terminal lipid-binding domains of the two synucleins showed similar affinity for purified synaptic vesicles, the C-terminal domain of γ-synuclein was not able to interact with synaptobrevin-2/VAMP2. Consequently, overexpression of γ-synuclein did not have any noticeable effect on the phenotype of CSPα null mutant mice. Our data suggest that the functions of α- and γ-synucleins in presynaptic terminals are not fully redundant.

Neuronal signaling depends primarily on the Ca^{2+}-triggered release of neurotransmitters from presynaptic vesicles into the synaptic cleft with the consequent activation of specific postsynaptic receptors. As with many other types of membrane fusion, a crucial molecular event in the process of vesicular neurotransmitter release is the formation of a complex containing vesicle- and terminal-bound SNARE (soluble NSF attachment protein receptor) proteins. In the case of neurotransmitter release, SNAP-25 (synaptosome-associated protein of 25 kDa) and syntaxin-1 play the role of terminal-bound proteins (t-SNARE), and vesicle-bound synaptobrevin-2/VAMP2 (vesicle-associated membrane protein 2) functions as a v-SNARE (reviewed in Ref. 1). SNARE complex assembly/disassembly occurs in high frequency cycles throughout the lifetime of the neuron. A consequence of this activity is the sustained production of highly reactive, unfolded intermediate forms of SNARE proteins, which are toxic to neurons and therefore should be efficiently neutralized through either their refolding or degradation. The importance of such protection has been clearly demonstrated in mice lacking the presynaptic SNARE complex-associated chaperone cysteine string protein α (CSPα),[3] in which catastrophic synaptic degeneration was observed (2). The neurodegeneration seen in postnatal CSPα null mutant mice correlates with significant reductions in SNARE complex assembly and substantial decreases in the levels of SNAP-25 (3–5), which have recently been robustly shown to be the primary cause of neurodegeneration in this model (6). Although CSPα is involved in various events during the synaptic vesicle recycling process (4, 7), it has been proposed that protection of synapses against degeneration depends on the ability of this protein to maintain the correct conformation of SNAP-25 during synaptic activity. This is facilitated through the formation of an active chaperone complex with Hsc70 (heat shock cognate 70) and SGT (small glutamine-rich tetratricopeptide repeat protein) (8), which deters SNAP-25 degradation, stimulates

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[3] The abbreviation used is: CSPα, cysteine string protein α.

SNARE complex assembly, and consequently prevents accumulation of toxic forms of SNARE proteins (5, 6).

Overexpression of α-synuclein, a small presynaptic protein robustly linked to Parkinson disease and certain other neurodegenerative diseases collectively known as synucleinopathies, ameliorates the phenotype of CSPα null mutant mice (3). Conversely, simultaneous inactivation of α-synuclein and CSPα genes causes even more severe synaptic dysfunction and earlier death of double null mutant mice; moreover, the ablation of both α- and β-synucleins results in further exacerbated CSPα null phenotype (3). Expression of A30P mutant α-synuclein lacking an ability to bind biological membranes efficiently does not facilitate the rescue of this phenotype. These findings imply that, in neuronal synapses, α-synuclein and CSPα act within the same pathway, although significant differences in their structures and binding abilities make it unlikely that there is any direct functional redundancy. This hypothesis is consistent with the finding of elevated CSPα levels in the brains of mice lacking all three members of the synuclein family, α-, β-, and γ-synucleins (9).

γ-Synuclein is structurally similar to α- and β-synucleins (reviewed in Ref. 10). A high degree of functional redundancy has been suggested between the three members of the family, consistent with the observation that mice lacking all three synucleins develop phenotypic changes not seen in mice lacking one or two members of the family (3, 9, 11–14). However, in contrast to α- and β-synucleins, γ-synuclein has a distinct pattern of expression in selected populations of peripheral and central neurons and is abundant not only in the synaptic cytoplasm but also in the axonal and perikaryal cytoplasm (15–18).

To assess how structural differences between α- and γ-synucleins affect their synaptic function, we investigated whether overexpressed γ-synuclein shares the ability of overexpressed α-synuclein to compensate for the CSPα deficiency. CSPα mutant mice (2) were crossed with γ-synuclein transgenic mice (19) to produce CSPα null mutant animals expressing significantly increased levels of mouse γ-synuclein in their neurons. We have demonstrated that despite the ability to bind lipid membranes of presynaptic vesicles, γ-synuclein is unable to interact with synaptobrevin-2/VAMP2 and is incapable of rescuing the phenotype caused by the ablation of the CSPα gene.

EXPERIMENTAL PROCEDURES

Experimental Animals—CSP$^{+/-}$ mice on a mixed Ola129× C57BL/6J background were the kind gift of Dr. Thomas Südhof (Stanford University). The CSPα$^{+/-}$ mouse line was transferred to a pure genetic background through six rounds of backcrossing with C57BL/6J mice obtained from Charles River Laboratories. The production of Thy1mγSN mice has been described in our previous publication (19). Mice were genotyped by PCR analysis of DNA extracted from ear biopsies. For genotyping CSPα mutant mice, primers D (5′-AAAGTCCTATCGGTAAGCAGC-3′), E (5′-CTGCTGGCATACTAATTGCAG-3′), and C (5′-GAGCGCGCGCGGCGGAGTTGTTGAC-3′) were used in a single PCR. Amplification of a 0.6-kb fragment with primers D and E indicated the presence of the wild-type allele, and amplification of a 0.4-kb fragment with primers E and C indicated the presence of the targeted allele. Thy1mγSN transgenic mice were identified by the presence of a 1-kb fragment in the amplification reaction with primers HP45ThyIf2 (5′-ACACCCCTAAAGCATACAGTCAGACC-3′) and HP84mγSN (5′-GGCCTTCTAGTCTTCTCCACTCTTG-3′). For production of experimental cohorts, CSPα$^{+/-}$ mice were intercrossed to produce CSP$^{-/-}$ mice or mated with homozygous Thy1mγSN mice for two generations to produce CSPα$^{+/-}$/Thy1mγSN$^{TG/TG}$ mice. The latter were crossed with CSPα$^{+/-}$ mice to produce experimental cohorts of CSPα$^{-/-}$/Thy1mγSN$^{WT/TG}$ and CSPα$^{+/+}$/Thy1mγSN$^{WT/TG}$ mice. The production and maintenance of triple synuclein null mutant mice have been described previously (11). Mice were caged in groups of five or fewer, with a light cycle of 12 h of light/12 h of dark and *ad libitum* access to food and water. All work on animals was carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act (1986).

Preparation of Synaptic Vesicle Fraction—Vesicle isolation was carried out according to a previously described method (20), with some modifications. Spinal cord or brain tissue was homogenized in 10 volumes of ice-cold buffer containing 0.32 M sucrose, 5 mM HEPES (pH 7.4), and Complete mini EDTA-free protease inhibitors (Roche Applied Science). Nuclei and cell debris were removed by centrifugation at 1000 × *g* for 10 min at 2 °C, and the supernatant was further spun at 20,000 × *g* for 20 min at 2 °C. The pellet was resuspended in 0.32 M sucrose (half-volume of the homogenization buffer used originally) by intense vortexing, transferred to a glass-Teflon homogenizer with 4 volumes of ice-cold distilled H_2O, homogenized, and left on ice for 5 min. 0.25 M HEPES (pH 7.4) and 1 M potassium tartrate were added up to final concentrations of 25 and 100 mM, respectively. Synaptosomal lysate was cleared by centrifugation at 20,000 × *g* for 20 min, and the supernatant (cleared synaptosomal lysate) was further centrifuged at 120,000 × *g* for 40 min. The pellet containing synaptic vesicles was resuspended in SDS gel loading buffer for Western blotting.

Expression of Recombinant Proteins in Bacteria—Human γ-synuclein/α-synuclein cDNA chimeras (PeS, encoding 95 N-terminal amino acids of γ-synuclein followed by 45 C-terminal amino acids of α-synuclein; SyP, encoding 95 N-terminal amino acids of α-synuclein followed by 32 C-terminal amino acids of γ-synuclein; and PSy, encoding 60 N-terminal amino acids of γ-synuclein followed by 80 C-terminal amino acids of α-synuclein) were produced from α- and γ-synuclein cDNAs by conventional PCR and subcloning techniques. Coding regions of human α-synuclein, mutant (A30P) α-synuclein, γ-synuclein, and γ-synuclein/α-synuclein chimeras were subcloned in the pCS19 (21) or pGEX4T-1 (GE Healthcare) expression vector, and the resulting plasmids were used for transformation of *Escherichia coli* KU98 or BL21(DE) cells, respectively. Eukaryotic inserts of all expression plasmids were verified by sequencing. Recombinant protein expression in logarithmically growing bacterial cells was induced by isopropyl β-D-thiogalactopyranoside, and after 6 h of growth at 22 °C, untagged synucleins were purified as described previously (22). GST-fused synucleins were captured from lysates of isopropyl β-D-thiogalactopyranoside-induced bacterial cells using glutathione-Sepharose 4B (GE Healthcare), and beads were thoroughly washed and used in pulldown experiments. Alternatively, GST

fusion proteins were eluted from beads in 5 mM reduced glutathione, dialyzed against 25 mM HEPES (pH 7.4) and 100 mM potassium tartrate, and used for interaction with synaptic vesicles as described below.

Interaction of Synucleins with Synaptic Vesicles in Vitro—Cleared synaptosomal lysate was prepared from the brains of triple synuclein null mutant mice as described above (final volume of 3 ml for two brains). 0.5 ml of the lysate was incubated with 5 μg of recombinant synuclein protein at 30 °C for 30 min, followed by sedimentation of synaptic vesicles by centrifugation at 120,000 × *g* for 40 min. The pellets were washed three times with 25 mM HEPES (pH 7.4) and 100 mM potassium tartrate and resuspended in 60 μl of water. Samples were prepared for SDS-PAGE by the addition of 20 μl of 4× SDS-PAGE loading buffer and incubation at 100 °C for 10 min.

GST Pulldown—To study the interaction of synucleins with endogenous synaptobrevin-2/VAMP2, 0.5 ml of the cleared synaptosomal lysate was incubated with 5 μg of purified GST-fused synucleins, followed by the addition of an equal volume of PBS and 2% Triton X-100 to lyse vesicle membranes. Glutathione-Sepharose beads (20-μl bed volume) were added to this lysate and incubated for 2 h at 4 °C with gentle mixing to pull down GST fusion proteins. After four washes with PBS and 1% Triton X-100, bound proteins were eluted by incubation at 100 °C for 10 min in SDS-PAGE loading buffer. Eluates were analyzed by Western blotting with anti-synaptobrevin-2/VAMP2 antibody.

Western Blotting and Antibodies—Protein separation by SDS-PAGE, transfer to a PVDF membrane by semidry transfer, blocking of membranes in 4% milk in TBS containing 0.1% Tween 20, incubation with primary antibodies and HRP-conjugated secondary antibodies (GE Healthcare), and protein band visualization using enhanced chemiluminescence (ECL$^+$, GE Healthcare) were carried out as described previously (16, 19). For simultaneous detection of two proteins, membranes were incubated in a mixture of rabbit polyclonal and mouse monoclonal primary antibodies, and protein bands were detected using Cy3- or Cy5-conjugated secondary antibodies (Invitrogen) and the FluorChem Q MultiImage III system (Cell Biosciences). Primary antibodies against γ-synuclein (affinity-purified rabbit polyclonal, SK23 (15) or SK109 (23), both diluted 1:500), α-synuclein (mouse monoclonal, clone Syn211, diluted 1:500, Santa Cruz Biotechnology), synaptophysin (mouse monoclonal, clone 2, diluted 1:5000, BD Transduction Laboratories), synaptobrevin-2/VAMP2 (mouse monoclonal, clone 69.1, diluted 1:3000, Synaptic Systems), SNAP-25 (mouse monoclonal, clone 20, diluted 1:1000, BD Transduction Laboratories), syntaxin-1 (mouse monoclonal, clone 78.2, diluted 1:2000, Synaptic Systems), CSPα (rabbit polyclonal, diluted 1:1000, Santa Cruz Biotechnology), dynamin-1/2/3 (rabbit polyclonal, diluted 1:1000, Synaptic Systems), synaptotagmin (mouse monoclonal, clone ASV48, diluted 1:5000, QED), synapsin IIa (mouse monoclonal, clone 1, diluted 1:10,000, BD Transduction Laboratories), and VMAT-2 (rabbit polyclonal, diluted 1:500, Santa Cruz Biotechnology) were used for detection.

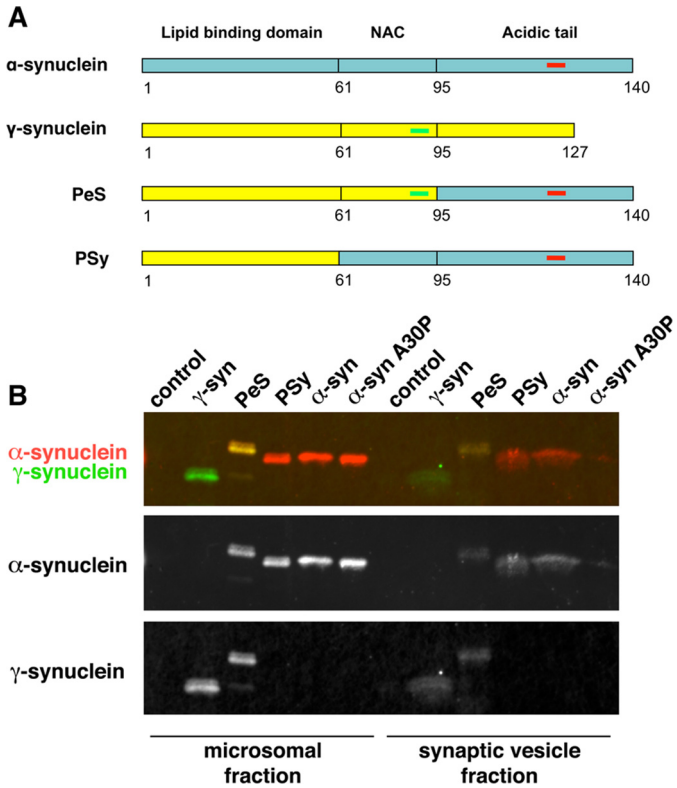

FIGURE 1. **Interaction of synucleins with synaptic vesicles *in vitro*.** *A*, recombinant proteins used for synaptic vesicle binding. NAC designates a non-amyloid component domain. The *small bars* show the positions of epitopes recognized by anti-α-synuclein antibody (*red*) and anti-γ-synuclein antibody (*green*). Amino acid sequences of these epitopes are shown in Fig. 6. *B*, Western blot analysis of synaptic vesicle-bound recombinant synucleins. Cleared synaptosomal lysate prepared from the brains of triple synuclein null mutant mice was incubated with recombinant synuclein (*syn*) proteins, followed by purification of synaptic vesicles as described under "Experimental Procedures." Proteins associated with synaptic vesicles were separated by 16% SDS-PAGE and analyzed by Western blotting using a mixture of mouse monoclonal anti-α-synuclein antibody and rabbit polyclonal anti-γ-synuclein antibody. Protein bands were visualized using Cy5-conjugated anti-mouse and Cy3-conjugated anti-rabbit secondary antibodies. The *middle* and *lower panels* show images from separate detection channels. On the merged image (*upper panel*), protein bands detected by anti-α-synuclein antibody are *red*, those detected by anti-γ-synuclein antibody are *green*, and those detected by both antibodies are *yellow*.

RESULTS

γ-Synuclein Is Abundant on Thy1mγSN Synaptic Vesicles—The ability of α-synuclein to prevent neurodegeneration caused by CSPα ablation was previously suggested to be dependent on its ability to bind lipid membranes at the presynaptic terminal (3). Although γ-synuclein is also known to interact with synthetic membranes (24), its association with synaptic vesicles has never been demonstrated. We used a bacterial expression system (21) to produce recombinant α- and γ-synucleins as well as two chimeric proteins (Fig. 1*A*). Purified proteins were incubated with the cleared synaptosomal lysate isolated from the brains of triple synuclein null mutant mice, followed by sedimentation of synaptic vesicles and thorough washing of the resulting pellets. The presence of synucleins in the synaptic vesicle fraction was assessed by Western blotting using mouse monoclonal Syn211 and rabbit polyclonal SK109 antibodies, which specifically recognize a C-terminal epitope of α-synuclein and an internal epitope of γ-synuclein, respectively

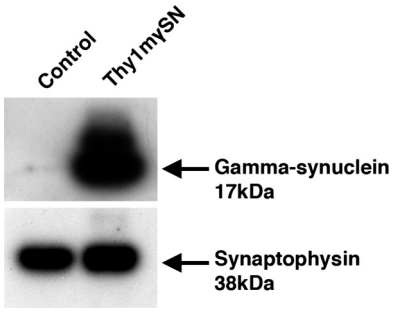

FIGURE 2. **γ-Synuclein is present in the fraction of purified synaptic vesicles from Thy1mγSN mice.** A Western blot of total proteins from the synaptic vesicle fraction isolated from the spinal cords of 9-month-old wild-type control and Thy1mγSN mice was consecutively probed with antibodies specific to γ-synuclein (*upper panel*) and synaptophysin (*lower panel*).

(Fig. 1*A*). The presence of both of these epitopes in the chimeric PeS protein allowed us to accurately compare the amounts of both proteins in different samples on the same Western blot by using a two-color fluorescence detection system. Fig. 1*B* illustrates the results of a typical binding experiment, demonstrating that α- and γ-synucleins had a similar ability to bind synaptic vesicles. Consistent with previous reports, A30P α-synuclein showed very low binding.

Prior to assessing a possible effect of γ-synuclein overexpression on synaptic dysfunction caused by ablation of CSP$\alpha^{-/-}$, it was important to demonstrate that endogenous and, more importantly, overexpressed γ-synucleins are associated with synaptic vesicles *in vivo*. Therefore, we assessed the level of γ-synuclein in a synaptic vesicle fraction isolated from the spinal cord tissue of 9-month-old wild-type and Thy1mγSN mice. Synaptophysin, a resident synaptic vesicle protein, was used for normalization of the amounts of total synaptic proteins in the samples studied. Western blotting revealed low but clearly detectable levels of endogenous γ-synuclein in the wild-type synaptic vesicle fraction (Fig. 2), suggesting that the protein is able to interact with neuronal vesicles under normal physiological conditions. Substantially higher levels of γ-synuclein were found in the synaptic vesicle fraction isolated from the Thy1mγSN transgenic mice, which correlated with the significantly higher levels of γ-synuclein expression in the spinal cords of these mice (19).

Overexpression of γ-Synuclein Does Not Rescue the Pathological Phenotype of CSPα Null Mutant Mice—To exclude background effects when assessing the ability of γ-synuclein to protect against the neurodegeneration induced by CSPα ablation, we first generated a line of mutant animals on a pure genetic background by backcrossing mice of a pre-existing CSP$\alpha^{+/-}$ line on a mixed Ola129×C57BL/6J background (2, 3) with C57BL/6J mice for six generations. Intercrossing of CSP$\alpha^{+/-}$ mice produced litters with a normal Mendelian distribution of wild-type (CSP$\alpha^{+/+}$), heterozygous (CSP$\alpha^{+/-}$), and null mutant (CSP$\alpha^{-/-}$) newborn pups, which was consistent with previously reported observations for mice on a mixed background (2, 3). The null mutant mice, which were at first indistinguishable from their wild-type littermates, stopped gaining weight between postnatal days 10 and 20 (Fig. 3, *A* and *B*). From this point, the health of the pups began to deteriorate progressively, and at the age of 3 weeks, they started to die. 50% of the

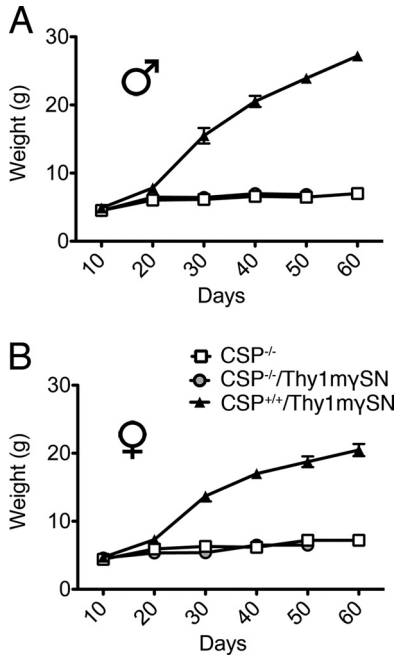

FIGURE 3. **Overexpression of γ-synuclein does not rescue weight loss in CSPα null mutant mice.** The line graphs show the dynamics of the weight gain for male (*A*) and female (*B*) animals from experimental cohorts of CSP$\alpha^{-/-}$, CSP$\alpha^{-/-}$/Thy1mγSN, and CSP$\alpha^{+/+}$/Thy1mγSN mice.

CSP$\alpha^{-/-}$ mice did not survive beyond postnatal day 34, and all of them were dead by postnatal day 52 (Fig. 4). It was noted that the phenotype developed by these mice appeared to be similar to the phenotype of some sublines (2, 3) but slightly more severe than the phenotype of other sublines of CSP$\alpha^{-/-}$ mice (3, 6), suggesting that its severity depends of the mouse genetic background.

To test whether overexpression of γ-synuclein would rescue the lethal phenotype of CSP$\alpha^{-/-}$ mice, we generated cohorts of CSP null mutant and wild-type littermates expressing high levels of γ-synuclein in their neurons due to the presence of an allele of the Thy1mγSN transgene in their genomes, *i.e.* CSP$\alpha^{-/-}$/Thy1mγSN and CSP$\alpha^{+/+}$/Thy1mγSN mice, respectively (for details, see "Experimental Procedures"). Consistent with our previous observations (19), CSP$\alpha^{+/+}$/Thy1mγSN mice showed linear weight increases and no signs of ill health during first 2 months of postnatal development (Fig. 3, *A* and *B*). Any loss of these animals was on par with the normal incidence of death in the colony of wild-type C57BL/6J mice housed in the same animal holding room. In contrast, CSP$\alpha^{-/-}$/Thy1mγSN mice displayed the same restricted growth after postnatal day 10 as CSP$\alpha^{-/-}$ mice. At this stage, mice in both of these groups became progressively lethargic, although they were able to move when prompted. Neither the age of the onset of death nor the survival of CSP$\alpha^{-/-}$/Thy1mγSN and CSP$\alpha^{-/-}$ mice was significantly different (Fig. 4). Similar to the cohort of CSP$\alpha^{-/-}$ mice, 50% of the mice in the CSP$\alpha^{-/-}$/Thy1mγSN cohort died at postnatal day 34, with the majority of mice dying by postnatal day 50, although a small percentage (<10%) of mice in the CSP$\alpha^{-/-}$/Thy1mγSN cohort survived to postnatal day 80 (Fig. 4).

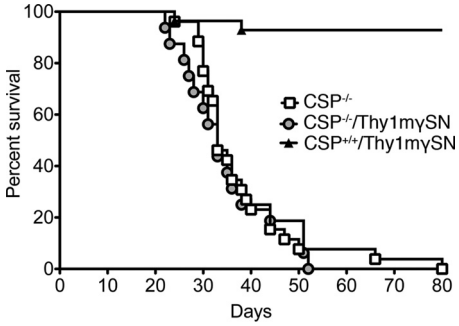

FIGURE 4. **Overexpression of γ-synuclein does not rescue the lethal CSPα null phenotype.** The Kaplan-Meier plot shows survival in cohorts of CSP$\alpha^{-/-}$ (n = 16), CSP$\alpha^{-/-}$/Thy1mγSN (n = 26), and CSP$\alpha^{+/+}$/Thy1mγSN (n = 28) mice. No significant difference was detected in the survival of CSP$^{-/-}$ and CSP$\alpha^{-/-}$/Thy1mγSN mice by log rank (p = 0.5284) and generalized Wilcoxon (p = 0.4109) tests for equality of survival.

γ-Synuclein Does Not Interact with Synaptobrevin-2/VAMP2 and Other Synaptic Vesicle Proteins—The rescue effect of α-synuclein on the phenotype of CSP$\alpha^{-/-}$ mice depends on interaction of its C-terminal domain with the cytoplasmic N-terminal domain of the vesicle membrane-associated protein synaptobrevin-2/VAMP2 (9). Therefore, it was feasible to test whether or not γ-synuclein is able to interact with this v-SNARE protein. We used GST-fused synucleins (Fig. 5*A*) to pull down endogenous synaptobrevin-2/VAMP2 on the surface of synaptic vesicles isolated from the brains of mice lacking all three synucleins. Interaction of synaptobrevin-2/VAMP2 with α-synuclein was observed, but no interaction with γ-synuclein was detected (Fig. 5*B*). Moreover, a chimeric molecule (PeS) bearing the N-terminal domain of γ-synuclein and the C-terminal fragment of α-synuclein also interacted with synaptobrevin-2/VAMP2, whereas the reciprocal chimeric molecule (SyP) did not (Fig. 5*B*). We also assessed if other proteins associated with synaptic vesicles or involved in their function could be pulled down by GST fusion proteins from the cleared synaptosomal lysates. No interaction of γ-synuclein with CSPα, SNAP-25, syntaxin-1, dynamins, synaptotagmin, synaptophysin, synapsin IIa, and VMAT-2 was observed.

DISCUSSION

In this study, we have demonstrated that γ-synuclein is unable to recapitulate the ability of α-synuclein to rescue mice from the neurodegeneration induced by ablation of CSPα. The precise mechanism by which α-synuclein achieves this protection is unclear, although results of recent studies have strongly suggested a link with the promotion of SNARE complex assembly under conditions of increased synaptic activity (2, 3). Two structural domains of the protein appear to be crucial for executing this function: the N-terminal lipid-binding domain (24), which accomplishes docking of α-synuclein to the outer surface of the phospholipid membrane of synaptic vesicles, and the acidic C-terminal region, which is responsible for interaction with another protein associated with the membrane of synaptic vesicles, the v-SNARE synaptobrevin-2/VAMP2 (9). The interaction stimulates this v-SNARE to form a complex with the t-SNARE proteins synapsin-1 and SNAP-25 and thus potentiates the docking of vesicles to the synaptic membrane and the release of a neurotransmitter.

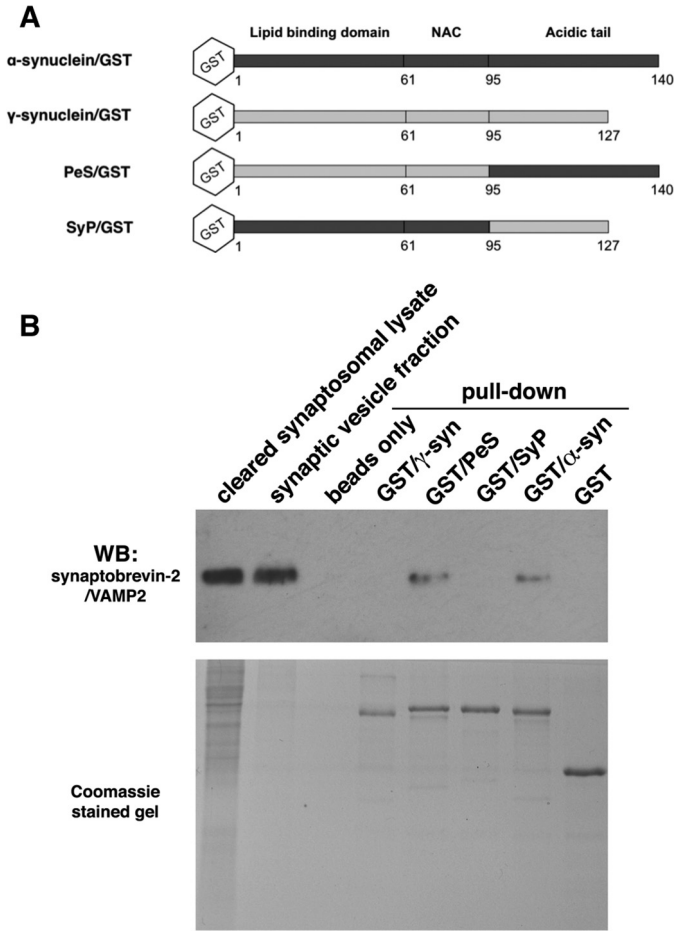

FIGURE 5. ***In vitro*** **interaction of GST-fused synucleins with endogenous synaptobrevin-2/VAMP2.** *A*, GST fusion proteins used in interaction studies. NAC designates a non-amyloid component domain. *B*, Western blot (*WB*) analysis of synaptobrevin-2/VAMP2 (*upper panel*) pulled down by GST fusion proteins (visualized by Coomassie Blue staining; *lower panel*) from synaptosomal lysate isolated from the nervous system of mice lacking all three synucleins (*syn*).

The ability of α-synuclein to interact with biological or synthetic phospholipid membranes is inhibited by amino acid substitutions disrupting the α-helical conformation acquired by the N-terminal domain upon this interaction (24–29). One such substitution, A30P, is caused by the α-synuclein gene mutation associated with a familial form of Parkinson disease (30, 31). Strikingly, A30P α-synuclein was found to be totally unable to prevent the neurodegeneration induced by CSPα ablation (3). In contrast, another Parkinson disease-associated variant of α-synuclein with the A53T substitution, which does not compromise the phospholipid-binding ability of the protein (31), was able to rescue the phenotype of CSPα null mutant mice as efficiently as the wild-type protein (3). These data suggest that the ability of α-synuclein to interact with phospholipids of the synaptic vesicle membrane is essential for its capacity to prevent neurodegeneration induced by CSPα ablation.

Although the N-terminal lipid-binding domain of γ-synuclein has several amino acid substitutions compared with the corresponding domain of α-synuclein (Fig. 6), these substitutions are mainly conservative; the free state residual structures of the two proteins are similar (32); and when bound to detergent micelles the N-terminal domains of these proteins also

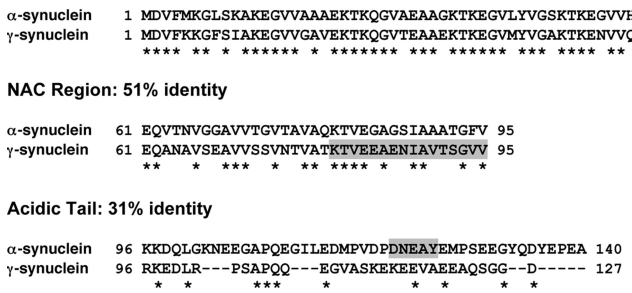

FIGURE 6. **Alignment of amino acid sequences of three regions of human α- and γ-synucleins.** Identical amino acids are shown by *asterisks* below the sequences. NAC designates a non-amyloid component domain. Epitopes recognized by antibody SK109 in γ-synuclein and by antibody Syn211 in α-synuclein are *shaded*.

share very similar structural properties (33). We have shown that α- and γ-synucleins have similar abilities to bind synaptic vesicles isolated from the brains of triple synuclein null mutant mice, *i.e.* native vesicles devoid of prebound synucleins. Moreover, we have demonstrated for the first time the presence of γ-synuclein in a fraction of synaptic vesicles purified from the neural tissue of wild-type and transgenic mice, suggesting that like the other two members of the family, γ-synuclein interacts with these vesicles *in vivo*. This suggests that the two synucleins might have similar functions in the process of synaptic vesicle turnover and neurotransmitter release. Indeed, evidence of functional redundancy has been observed in previous studies of mice lacking each or both α-synuclein and γ-synuclein (13, 14).

Nevertheless, unlike α-synuclein, overexpressed γ-synuclein is unable to rescue the phenotype of CSPα null mutant mice. It is feasible to suggest that this is due to significant structural and functional differences between the C-terminal domains of α- and γ-synucleins. In contrast to the highly conserved N-terminal membrane-binding domains, the C-terminal domains of these two proteins share very limited amino acid similarities (Fig. 6), and in γ-synuclein, the C-terminal domain is much less ordered (32). The functional importance of this domain was illustrated by the finding that a C-terminal truncation of α-synuclein eliminates its ability to interact with synaptobrevin-2/VAMP2 and potentiate SNARE complex assembly (9), which is crucial for rescuing the phenotype of CSPα null mutant mice. However, this result does not prove that the interaction involving full-length α-synuclein is sequence-specific but might simply indicate that it could be instigated by any sequence with multiple negatively charged amino acids located at the C terminus of a synuclein molecule. Therefore, we tested whether the C-terminal domain of γ-synuclein, which is also highly acidic, is able to interact with synaptobrevin-2/VAMP2. To facilitate interactions that normally take place on the surface of synaptic vesicles, we first incubated purified GST-fused synuclein proteins with intact synaptic vesicles from the brains of triple synuclein null mutant mice, followed by the lysis of membranes with a non-ionic detergent and the pulldown of protein complexes using a glutathione affinity matrix. Using this approach, we confirmed the previously observed interaction of α-synuclein with synaptobrevin-2/VAMP2. Using chimeric synuclein proteins, we also demonstrated that the N-terminal lipid-binding domain of either α- or γ-synuclein is able to facilitate this interaction, but the C-terminal domain of α-synuclein, but not γ-synuclein, can bind synaptobrevin-2/VAMP2. These results clearly demonstrate that the specific structure of the C-terminal domain of α-synuclein and not merely its negative charge is crucial for formation of a functional complex with synaptobrevin-2/VAMP2 at the surface of synaptic vesicles. They also explain why γ-synuclein is unable to rescue the phenotype of CSPα null mutant mice and imply that this protein is not directly involved in potentiation of synaptic vesicle fusion with the plasma membrane.

The normal biological function of γ-synuclein and the role of this protein dysfunction in pathological processes remain even less understood than those of the other two synucleins. Recent studies of mouse models have linked increased expression and aggregation of γ-synuclein with neurodegeneration in glaucoma and motor neuron diseases (19, 34, 35). Outside the nervous system, this protein is abundant in white fat adipocytes, and the level of its expression correlates with the degree of obesity (36). Moreover, our latest study demonstrated the role of γ-synuclein in regulation of lipolysis and lipid droplet formation in adipocytes (37). In these cells, γ-synuclein is not associated with lipid droplets but potentiates translocation of cytosolic SNAP-23 to their surface, enhances triacylglyceride incorporation into lipid droplets, and increases their size at times of nutrient excess. These results link γ-synuclein with modulation of another type of SNARE complex by a mechanism different from that of neuronal SNARE complex assembly potentiation by α-synuclein. We also did not find evidence for γ-synuclein interaction with several other proteins involved in synaptic vesicle structure or turnover. Further studies are required to reveal the exact function of γ-synuclein and the functional interplay between the three members of the family in presynaptic terminals.

In conclusion, the results of our study emphasize the importance of sequence-specific interactions of the α-synuclein C-terminal domain with other macromolecules, particularly with synaptobrevin-2/VAMP2, for α-synuclein function in the regulation of presynaptic neurotransmitter release. Our experimental data also strongly suggest that despite many structural similarities between α- and γ-synucleins, their functions in presynaptic terminals are not redundant.

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