Contrasting Effects of α-synuclein and γ-synuclein on the Phenotype of Cysteine String Protein alpha (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 phenotype of CSPα-deficient mice.

Conclusion: Functional diversity of two synucleins in synapses are determined by structural differences within their C-terminal domains.

Significance: Delineating functional similarities and differences within synuclein family is important for understanding synaptic transmission and pathogenesis of synucleinopathies.

SUMMARY

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. Function of a chaperone protein CSPα is important for synaptic SNARE complex formation and mice lacking this protein develop severe synaptic dysfunction and neurodegeneration that led to their death within three 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 the family of three related proteins which 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 to 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 functions of α-synuclein and γ-synuclein in presynaptic terminals are not fully redundant.

Neuronal signaling primarily depends on the Ca2+-triggered release of neurotransmitters from presynaptic vesicles into the synaptic cleft with 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 (synaptosomal-associated protein of 25K) and syntaxin-1 play the role of terminal bound proteins (t-SNARE) and the vesicle bound synaptobrevin-2/VAMP2 (vesicle-associated membrane protein 2) functions as a v-SNARE (reviewed in (27)). 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 alpha (CSPα), in which catastrophic synaptic degeneration was observed (7). The neurodegeneration seen in postnatal CSPα null mutant mice correlates with significant reductions in SNARE-complex assembly and substantial decrease in levels of SNAP-25 (5, 21, 24), that has recently been robustly shown to be the primary cause of neurodegeneration in this model (23). Although CSPα is involved in various events during the synaptic vesicle recycling process (21, 33), 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 heat shock cognate 70 (Hsc70) and small glutamine-rich tetratricopeptide repeat protein (SGT) (31), which deters SNAP-25 degradation, stimulates SNARE-complex assembly, and consequently prevents accumulation of toxic forms of SNARE proteins (23, 24).

Overexpression of α-synuclein, a small presynaptic protein robustly linked to Parkinson’s (PD) and certain other neurodegenerative diseases collectively known as synucleinopathies, ameliorates the phenotype of CSPα null mutant mice (5). 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-synuclein results in further exacerbated CSPα null phenotype (5). Expression of A30P mutant α-synuclein lacking an ability to bind efficiently biological membranes does not facilitate the rescue of this phenotype. These findings imply that in neuronal synapses α-synuclein and CSPα act within the same pathway, though 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 brain of mice lacking all three members of the synuclein family, α-, β- and γ-synucleins (3).

γ-synuclein is structurally similar to α- and β-synucleins (reviewed in (14)). A high degree of functional redundancy has been suggested between the three members of the family, consistent with an observation that mice lacking all three synucleins develop phenotypical changes not seen in mice lacking one or two members of the family (1, 3, 5, 9, 20, 22). 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 synaptic but also in axonal and perikaryal cytoplasm (2, 16, 25, 30).

To assess how structural differences between α-synuclein and γ-synuclein 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 (7) were crossed with γ-synuclein transgenic mice (17) 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 incapable of rescuing the phenotype caused by the ablation of the CSPα gene.

**EXPERIMENTAL PROCEDURES**

*Experimental animals – CSPα* mice on a mixed Ola129/C57Bl6 background were kindly gifted by Thomas Südhof (Stanford University, USA). The CSPα+/- mouse line was transferred to a
pure genetic background through six rounds of backcrossing with C57Bl/6J mice obtained from Charles River. The production of Thy1mySN mice has been described in our previous publication (17). Mice were genotyped by PCR analysis of DNA extracted from ears biopsies. For genotyping CSPα mutant mice primers D (5′ - AAAGTCTATCGGTAAAGCAGC - 3′), E (5′ - CTGCTGGCATACTAAATTGCAG - 3′) and C (5′ – GAGCGCGCGCGGAGGTTGAG - 3′) were used in a single PCR reaction. 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 – the presence of the targeted allele. Thy1mySN transgenic mice were identified by the presence of a 1 kb fragment in the amplification reaction with primers HP45ThyIf2 (5′ - ACACCCCTAAAGCTACGTGTCGAC - 3′) and HP84mySN (5′ - GGCCTTCTAGCTCTCCACTCTTG - 3′). For production of experimental cohorts CSPα+/− mice were intercrossed to produce CSPα−/− mice or mated with homozygous Thy1mySN mice for two generations to produce CSPα−/−/Thy1mySN+/+ mice. The latter were crossed with CSPα+/− mice to produce experimental cohorts of CSPα−/−/Thy1mySNWT/TG and CSPα+/−/Thy1mySNWT/TG mice. The production and maintenance of triple synuclein null mutant mice has been described previously (1). Mice were caged in groups of five or fewer, with a light cycle of 12 hours light/12 hours 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 – Vesicles isolation was carried out according to a previously described method (32), with some modifications. Spinal cord or brain tissue was homogenized in 10 volumes of ice-cold buffer, containing 0.32M sucrose, 5mM HEPES pH 7.4 and Complete Mini-EDTA-free protease inhibitors (Roche). Nuclei and cell debris were removed by centrifugation at 1000 x g for 10 minutes at 2°C and the supernatant was further spun at 20,000 x g for 20 min at 2°C. The pellet was resuspended in 0.32M sucrose (half volume of originally used homogenization buffer) by intense vortexing, transferred to a glass-Teflon homogenizer with 4 volumes of ice-cold dH2O, homogenized and left on ice for 5 min. 0.25M HEPES pH 7.4 and 1M potassium tartrate were added up to a final concentration of 25mM and 100mM, respectively. Synaptosomal lysate was cleared by centrifugation at 20,000 x g for 20 min, and the supernatant (cleared synaptosomal lysate) was further centrifuged at 120,000 x g for 40 min. The pellet containing synaptic vesicles was resuspended in a 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) or A30P α-synuclein, mutant (A30P) α-synuclein, γ-synuclein and γ-synuclein chimeras were cloned in pCS19 (26) or pGEX4T-1 expression vector (GE Healthcare) and resulting plasmids were used for transformation of E.coli KU98 or BL21(DE) cells, respectively. Eukaryotic inserts of all expression plasmids were verified by sequencing. Recombinant protein expression in logarithmically growing bacteria cells was induced by IPTG and after 6 h of growth at 22°C untagged synucleins were purified as described previously (10). GST-fusion synucleins were captured from lysates of IPTG-induced bacterial cells using glutathione-Sepharose 4B (GE Healthcare), beads were thoroughly washed and used in pull-down experiments. Alternatively, GST-fusion proteins were eluted from beads in 5mM reduced glutathione, dialyzed against 25mM HEPES pH 7.4; 100mM 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 brain of triple synuclein null mutant mice as described above (final volume 3 ml for 2 brains). 0.5 ml of the lysate was incubated with 5 µg of a recombinant synuclein protein at 30°C for 30 min followed by sedimentation of synaptic vesicles by centrifugation at 120,000 x g for 40 min. The
pellets were washed three times with 25mM HEPES pH 7.4; 100mM potassium tartrate and resuspended in 60 µl of water. Samples were prepared for SDS-PAGE by adding 20 µl of 4xSDS-PAGE loading buffer and incubation at 100°C for 10 min.

**GST-pull down** – For studying interaction of synucleins with endogenous synaptobrevin-2/VAMP2, 0.5 ml of the cleared synaptosomal lysate was incubated with 5 µg of purified GST-fusion synucleins followed by adding equal volume of PBS; 2% Triton X-100 to lyse vesicular 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; 1% Triton X-100 bound proteins were eluted by incubating 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** – Proteins separation by SDS-PAGE, transfer to a PVDF membrane by semi-dry transfer, blocking of membranes in 4% milk in TBS containing 0.1% Tween 20, incubation in 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, 17). 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 FluorChem Q MultImage III system (Cell Biosciences). Primary antibodies against γ-synuclein (rabbit polyclonal affinity purified SK23 (2) or SK109 (18), both diluted 1:500), α-synuclein (mouse monoclonal, clone 211, Santa Cruz Biotechnology, diluted 1:500), synaptophysin (mouse monoclonal, clone 2, BD Transduction Laboratory, diluted 1:5000), synaptobrevin-2/VAMP2 (mouse monoclonal, clone 69.1, Synaptic Systems, diluted 1:3000), SNAP-25 (mouse monoclonal, clone 20, BD Transduction Laboratory, diluted 1:1000), syntaxin 1 (mouse monoclonal, clone 78.2, Synaptic Systems, diluted 1:2000), CSPα (rabbit polyclonal, Santa Cruz Biotechnology, diluted 1:1000), dynamin 1/2/3 (rabbit polyclonal, Synaptic Systems, diluted 1:1000), synaptotagmin (mouse monoclonal, clone ASV48, QED, diluted 1:5000), synapsin IIa (mouse monoclonal, clone 1, BD Transduction Laboratory, diluted 1:10000) and VMAT-2 (rabbit polyclonal, Santa Cruz Biotechnology, diluted 1:500) were used for detection.

**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 (5). Although γ-synuclein is also known to interact with synthetic membranes (6), its association with synaptic vesicles has never been demonstrated. We used a bacterial expression system (26) to produce recombinant α-synuclein and γ-synuclein as well as two chimeric proteins (Fig. 1A). Purified proteins were incubated with the cleared synaptosomal lysate isolated from the brain of triple synuclein null mutant mice followed by sedimentation of synaptic vesicles and thorough washing of 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 that specifically recognize a C-terminal epitope of α-synuclein and an internal epitope of γ-synuclein, correspondingly (Fig. 1A). The presence of both these epitopes in the chimeric PeS protein allowed us to accurately compare amount of both proteins in different samples on the same Western blot by using two-color fluorescence detection system. Figure 1B illustrates results of a typical binding experiment demonstrating that α-synuclein and γ-synuclein had similar ability to bind synaptic vesicles. Consistent with the 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 CSPα23 it was important to demonstrate that endogenous and more importantly, overexpressed γ-synuclein 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 amounts of total synaptic proteins in studied samples. Western blotting revealed low, but clearly detectable, level of endogenous γ-synuclein in the wild type synaptic vesicle fraction, suggesting that the protein is able to interact with neuronal vesicles under normal physiological conditions (Fig. 2). Substantially higher level of γ-synuclein was found in the synaptic vesicle fraction isolated from the Thy1mγSN transgenic mice, which correlated with significantly higher levels of γ-synuclein expression in the spinal cord of these mice (17).

Overexpression of γ-synuclein does not rescue 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 preexisting CSPα+/- line on a mixed Ola129xC57Bl/6J background (5, 7) to C57Bl/6J mice for 6 generations. Intercrossing of CSPα+/- mice produced litters with normal Mendelian distribution of wild type (CSPα+/-), heterozygous (CSPα+/-) and null mutant (CSPα-/-) newborn pups, which was consistent with previously reported observations for mice on a mixed background (5, 7). The null mutant mice that were at first indistinguishable from their wild type littersmates, stopped gaining weight between postnatal days 10 and 20 (Fig. 3A, B). From this point the health of pups began to deteriorate progressively and from the age of 3 weeks they started to die. 50% of CSPα+/- mice died progressively and from the age of 34 days, with the majority of mice dying by P50, although a small percent (<10%) of mice in the CSPα+/+/Thy1mγSN cohort survived to P80 (Fig. 4).

γ-synuclein does not interact with synaptobrevin-2/VAMP2 and other synaptic vesicle proteins – The rescue effect of α-synuclein on the phenotype of CSPα-/- mice depends on interaction of its C-terminal domain with cytoplasmic N-terminal domain of the vesicular membrane-associated protein synaptobrevin-2/VAMP2 (3). Therefore it was feasible to test whether or not γ-synuclein is able to interact with this v-SNARE protein. We used GST-fusion synucleins (Fig. 5A) to pull-down endogenous synaptobrevin-2/VAMP2 on the surface of synaptic vesicles isolated from the brain of mice lacking all three synucleins. Interaction of synaptobrevin-2/VAMP2 was observed with α-synuclein, but no interaction with γ-synuclein was detected (Fig. 5B). Moreover, a chimeric molecule PeS bearing N-terminal domain of γ-synuclein and C-terminal fragment of α-synuclein also interacted with synaptobrevin-2/VAMP2, whereas a reciprocal chimeric molecule SyP did not (Fig. 5B). 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, dynamin 1, synaptotagmin, synaptophysin, synapsin IIA and VMAT-2 was observed.
DISCUSSION

In the present 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, though results of recent studies have strongly suggested a link with the promotion of SNARE complex assembly in conditions of increased synaptic activity (3, 5). Two structural domains of the protein appear to be crucial for executing this function, the N-terminal lipid-binding domain (6) that accomplishes docking of α-synuclein to the outer surface of the phospholipid membrane of synaptic vesicles and the acidic C-terminal region responsible for interaction with another protein associated with the membrane of synaptic vesicles, the v-SNARE synaptobrevin-2/VAMP2 (3). 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 neurotransmitter.

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 (4, 6, 8, 11, 13, 15). One of such substitutions, A30P, is caused by the α-synuclein gene mutation associated with a familial form of PD (12, 19). Strikingly, A30P α-synuclein was found to be totally unable to prevent the neurodegeneration induced by CSPα ablation (5). In contrast, another PD-associated variant of α-synuclein with the A53T substitution that does not compromise phospholipid binding ability of the protein (19), was able to rescue the phenotype of CSPα null mutant mice as efficiently as the wild type protein (5). 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 to the corresponding domain of α-synuclein (Fig. 6), these substitutions are mainly conservative, the free-state residual structures of two proteins are similar (28), and when bound to detergent micelles the N-terminal domains of these proteins also share very similar structural properties (29). We have shown that α-synuclein and γ-synuclein have similar abilities to bind synaptic vesicles isolated from the brain of triple synuclein null mutant mice, i.e. native vesicles devoid of pre-bound 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 two synucleins might have similar functions in the process of synaptic vesicle turnover and neurotransmitter release. Indeed, evidences of functional redundancy have been observed in previous studies of mice lacking each or both α-synuclein and γ-synuclein (20, 22).

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 C-terminal domains of α- and γ-synuclein. In contrast to 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 (28). A functional importance of this domain was illustrated by the finding that the C-terminal truncation of α-synuclein eliminates its ability to interact with synaptobrevin-2/VAMP2 and potentiate SNARE complex assembly (3), which is crucial for rescuing 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-fusion synuclein proteins with intact synaptic vesicles from the brain of the triple synuclein null mutant mice followed by the lysis of membranes with a non-ionic.
detergent and the pull-down of protein complexes using the Glutathione affinity matrix. Using this approach we have confirmed previously observed interaction of \(\alpha\)-synuclein with synaptobrevin-2/VAMP2. Using chimeric synuclein proteins we have also demonstrated that the N-terminal lipid-binding domain of either \(\alpha\)-synuclein or \(\gamma\)-synuclein is able to facilitate this interaction but the C-terminal domain of \(\alpha\)-synuclein and not of \(\gamma\)-synuclein can bind synaptobrevin-2/VAMP2. These results clearly demonstrate that specific structure of the C-terminal domain of \(\alpha\)-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 \(\gamma\)-synuclein is unable to rescue phenotype of CSP\(\alpha\) null mutant mice and imply that this protein is not directly involved in potentiation of synaptic vesicle fusion with the plasma membrane.

Normal biological function of \(\gamma\)-synuclein and the role of this protein dysfunction in pathological processes remain even less understood than those of other two synucleins. Recent studies of mouse models have linked increased expression and aggregation of \(\gamma\)-synuclein with neurodegeneration in glaucoma and motor neuron diseases (25, 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 \(\gamma\)-synuclein in regulation of lipolysis and lipid droplet formation in adipocytes. In these cells \(\gamma\)-synuclein is not associated with lipid droplets but potentiates translocation of cytosolic SNAP-23 to their surface, enhances TAG incorporation into lipid droplets and increases their size at times of nutrient excess (revised version of the manuscript under review in PNAS). These results tie \(\gamma\)-synuclein with modulation of another type of SNARE complex by a mechanism different from the mechanism of neuronal SNARE complex assembly potentiation by \(\alpha\)-synuclein. We also did not find evidence for \(\gamma\)-synuclein interaction with several other proteins involved in synaptic vesicle structure or turnover. Further studies are required to reveal exact function of \(\gamma\)-synuclein and functional interplay between three members of the family in presynaptic terminals.

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

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FOOTNOTES

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The abbreviations used are: CSPα, cysteine string protein alpha; SNARE, soluble NSF attachment protein receptor proteins; SNAP-25, synaptosomal-associated protein of 25K; VAMP2, vesicle-associated membrane protein 2; Hsc70, heat shock cognate 70; SGT, small glutamine-rich tetratricopeptide repeat protein; PD, Parkinson’s disease; TAG, triacylglyceride

FIGURE LEGENDS

Figure 1. Interaction of synucleins with synaptic vesicles in vitro. (A) Recombinant proteins used for synaptic vesicles binding. Small bars show position of epitopes recognized by anti-α-synuclein antibody (red) and anti-γ-synuclein antibody (green). Amino acid sequences of these epitopes are shown in Figure 6. (B) Western blot analysis of synaptic vesicle-bound recombinant synucleins. Cleared synaptosomal lysate prepared from the brain of triple synuclein null mutant mice was incubated with recombinant synuclein proteins followed by purification of synaptic vesicles as described in Materials and Methods. Proteins associated with synaptic vesicles were separated in 16% SDS-PAGE and analysed 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 antibody. Black and white panels show images from separate detection channels. On the merged image (colour panel) protein bands detected by anti-α-synuclein antibody are red, detected by anti-γ-synuclein antibody are green and detected by both antibodies are yellow.

Figure 2. γ-synuclein is present in the fraction of purified synaptic vesicles of Thy1mγSN mice. Western blot of total proteins from the synaptic vesicle fraction isolated from the spinal cord of 9-month old wild type control and Thy1mγSN mice was consecutively probed with antibodies specific to γ-synuclein (top panel) and synaptophysin (bottom panel).

Figure 3. Overexpression of γ-synuclein does not rescue weight loss in CSPα-null mutant mice. Line graphs show dynamics of the weight gain for male (a) and female (b) animals from experimental cohorts of CSPα−/−, CSPα−/−/Thy1mγSN and CSPα+/+/Thy1mγSN mice.

Figure 4. Overexpression of γ-synuclein does not rescue the lethal CSPα-null phenotype. Kaplan-Meier plot shows survival in cohorts of CSPα−/− (n=16), CSPα−/−/Thy1mγSN (n=26) and Thy1mγSN (n=28) mice. No significant difference was detected in the survival of CSPα−/− and CSPα−/−/Thy1mγSN by log rank (p=0.5284) and generalized Wilcoxon (p=0.4109) tests for equality of survival.

Figure 5. In vitro interaction of GST-fusion synucleins with endogenous synaptobrevin-2/VAMP2. (A) GST-fusion proteins used in interaction studies. (B) Western blot analysis of synaptobrevin-2/VAMP2 (upper panel) pulled-down by GST-fusion proteins (visualized by Coomassie staining, bottom panel) from synaptosomal lysate isolated from the nervous system of mice lacking all three synucleins.

Figure 6. Alignment of amino acid sequences of three regions of human α-synuclein and γ-synuclein. Identical amino acids are shown by stars below the sequences. Epitopes recognized by SK109 antibody in γ-synuclein and by Syn211 antibody in α-synuclein are highlighted.
Figure 1.
Figure 2.

Figure 3.
Figure 4.

Figure 5.
Lipid Binding Domain: 76% identity

\[
\begin{align*}
\alpha\text{-synuclein} & \quad 1 \text{ MDVF\_KGLSKA\_KEG\_V\_VA\_AE\_K\_T\_KEG\_VL\_Y\_GSK\_T\_KEG\_VV\_HV\_AV\_TEK\_K} \quad 60 \\
\gamma\text{-synuclein} & \quad 1 \text{ MDVF\_K\_G\_FS\_IA\_KEG\_V\_G\_A\_VE\_K\_T\_Q\_G\_V\_T\_E\_A\_E\_K\_T\_K\_E\_V\_Y\_G\_A\_K\_T\_K\_E\_N\_V\_Q\_S\_T\_V\_E\_K\_K} \quad 60 \\
\end{align*}
\]

NAC Region: 51% identity

\[
\begin{align*}
\alpha\text{-synuclein} & \quad 61 \text{ EQVT\_N\_G\_G\_A\_V\_T\_V\_A\_Q\_T\_V\_E\_G\_A\_G\_S\_I\_A\_A\_T\_G\_F\_V} \quad 95 \\
\gamma\text{-synuclein} & \quad 61 \text{ EQAN\_A\_V\_E\_A\_V\_S\_S\_V\_N\_V\_T\_A\_K\_T\_V\_E\_E\_A\_N\_I\_A\_V\_T\_S\_G\_V\_V} \quad 95 \\
\end{align*}
\]

Acidic Tail: 31% identity

\[
\begin{align*}
\alpha\text{-synuclein} & \quad 96 \text{ KKDQL\_G\_N\_E\_G\_A\_P\_Q\_E\_G\_I\_L\_E\_D\_M\_P\_V\_D\_P\_N\_E\_A\_Y\_E\_M\_P\_S\_E\_E\_G\_Y\_Q\_D\_E\_P\_E\_A} \quad 140 \\
\gamma\text{-synuclein} & \quad 96 \text{ RKEDL\_R\_P\_A\_P\_Q\_E\_G\_V\_A\_K\_E\_E\_V\_A\_E\_A\_Q\_S\_G\_G\_D\_E\_D\_D\_D} \quad 127 \\
\end{align*}
\]

Figure 6.
Contrasting Effects of α-synuclein and γ-synuclein on the Phenotype of Cysteine String Protein alpha (CSP α) Null Mutant Mice Suggest Distinct Function of these Proteins in Neuronal Synapses

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