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α-Synuclein is localized in a subpopulation of rat brain synaptic vesicles

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α-Synuclein is a neuronal protein implicated both in synaptic transmission and in neurodegenerative diseases. Although it is evident that this protein is enriched in the presynaptic terminals of neurons, localization in synaptic vesicles has not been conclusively determined. Here, we show that α-synuclein is present, but not enriched, in synaptic vesicles using highly purified synaptic vesicle preparations from rat brain homogenate. Immunoisolation of vesicles using antibodies against synaptophysin or synaptobrevin confirmed the presence of α-synuclein in synaptic vesicles. Additional separation of synaptic vesicles by sucrose velocity centrifugation showed that there are different subpopulations of synaptic vesicles and that α-synuclein is present only in a specific subpopulation, whereas synaptophysin and synaptobrevin were found in all the synaptic vesicles. Presence of α-synuclein only in a subset of synaptic vesicles suggests that this protein may have a specific function in synaptic vesicle cycling, hence in synaptic transmission.

Key words: Parkinson's disease, neurotransmission, synaptophysin, synaptobrevin

α-Synuclein (α-syn) is a 140-amino acid protein that is highly expressed in brain and spontaneously forms amyloid-like fibrils in solution (Goldberg and Lansbury 2000). The fibrillar form of this protein is considered a major constituent of proteinacious inclusions found in many neurodegenerative diseases, including Parkinson's disease (PD), dementia with Lewy bodies, and multiple system atrophy (Goedert 2001). Furthermore, three missense mutations and several gene multiplication mutations in the α-syn locus were linked to familial PD (Singleton et al. 2004). Animal models overexpressing α-syn developed pathological and behavioral phenotypes that resembled human PD, further supporting the etiological role of α-syn in neurodegenerative processes (Maries et al. 2003).

α-Syn has been implicated in synaptic plasticity and synaptic transmission. Synelfin, the Zebra finch homolog of α-syn, was induced in the lateral magnocellular nucleus, a brain region that participates in seasonal song learning (George et al. 1995). Targeted null mutation or overexpression of α-syn gene resulted in subtle abnormalities in synaptic activities as well as synaptic vesicle (SV) biogenesis (Abeliovich et al. 2000, Cabin et al. 2002, Liu et al. 2004, Yavich et al. 2004). Identification of α-syn-binding proteins has implied that α-syn functions through the molecular interactions with a variety of other proteins (Engelender et al. 1999, Ostrerova et al. 1999, Jensen et al. 1999, 2000, Ghee et al. 2000, Iwata et al. 2001). However, the precise physiological activity of this protein is still unknown.

Information on the precise localization of a protein often proves essential for the elucidation of the protein's function. Previous immunohistochemical studies showed the enrichment of α-syn in presynaptic terminals of neurons (Iwai et al. 1995). Biochemical fractionations of brain homogenate also consistently showed the presence of α-syn in synaptosomal fractions (George et al. 1995, Lee et al. 2002). However, given the crude nature of the preparations used in these studies, precise localization of α-syn in the

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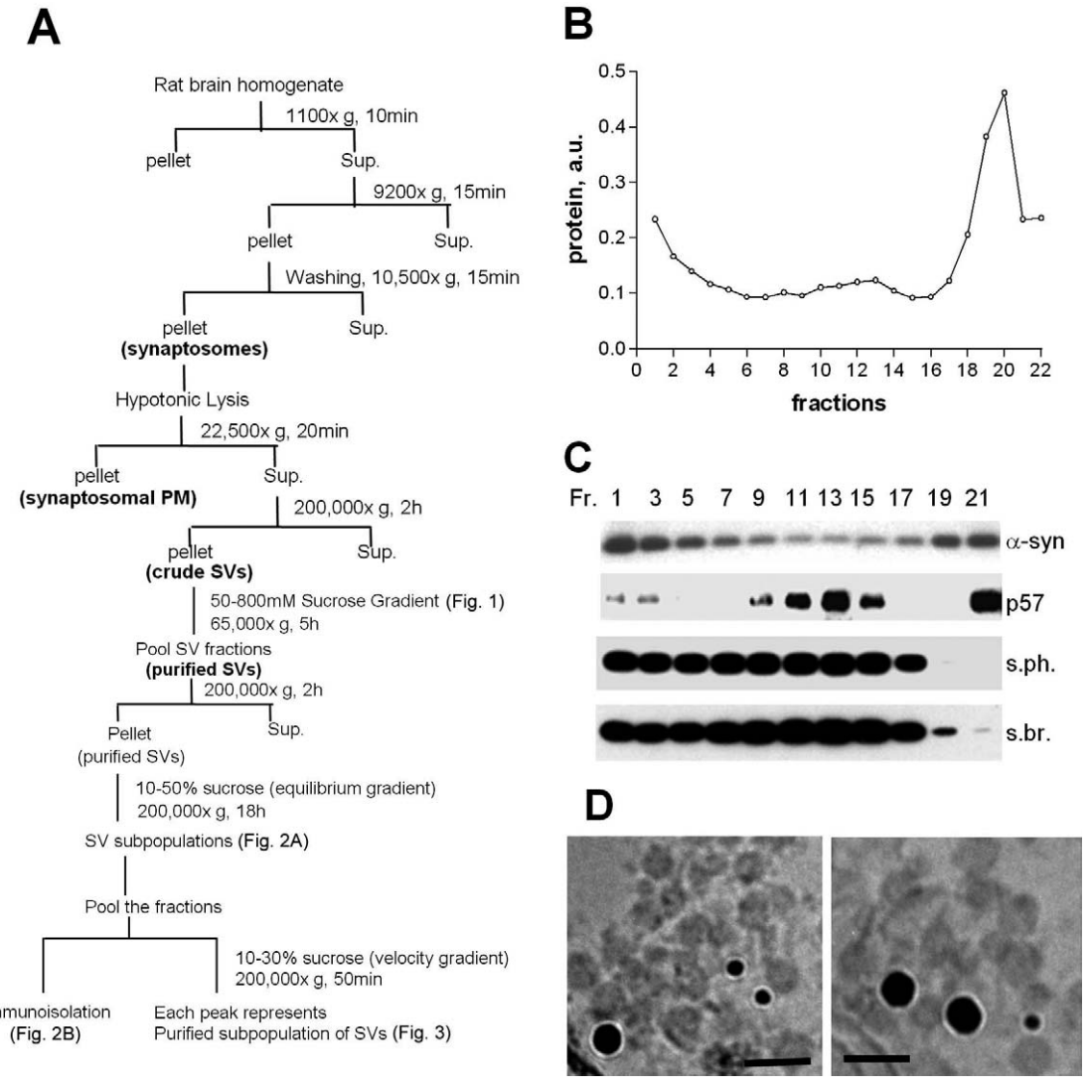

Fig. 1. Isolation of SVs in a 50–800 mM sucrose velocity gradient. (A) Schematic flow-chart of the fractionation procedure. For details, see the text. (B–D) Crude SV preparation was loaded onto a linear sucrose density gradient (50–800 mM) and centrifuged at 65 000× g for 5 h. Fractions were collected from the bottom of the gradient, and equal volume aliquot of each fraction was subjected to protein assay (B) and Western blot analyses (C). The graph on the top shows the protein profile. (α-syn) α-synuclein; (p57) V-type H^+-ATPase; (s.ph.) synaptophysin; (s.br.) synaptobrevin. (D) EM images with immunoglod labeling. Fractions from 9 to 15 were pooled and subjected to EM. For the detection of synaptophysin and α-syn, gold particles with the sizes of 10 nm and 20 nm were used, respectively. Scale bar is 50 nm.

presynaptic terminals remains to be determined. Here, we provide evidence that α-syn is indeed a component of SVs by preparing highly purified SVs using a series of density gradient centrifugations and immunoabsorption of SVs. Moreover, α-syn was found in a specific subpopulation of SVs, suggesting that this protein might play a specific role in the SV cycling and/or neural transmission.

SVs were isolated from rat brains according to Huttner and colleagues (1983) and further fractionated as depicted in Fig. 1. Female Sprague-Dawley rats with the age of 8–10 weeks were purchased from Charles River and euthanized on the day of arrival. The rats were euthanized using carbon dioxide in a closed chamber. Animal euthanization was performed under a protocol approved by the Institutional Animal Care and Use Committee at Konkuk University. Six rat cerebrums were washed in homogenization buffer (HB; 4 mM Hepes, pH 7.4, 320 mM sucrose) and homogenized in the same buffer in a motor-driven

Teflon-glass homogenizer. The homogenate was centrifuged for 10 min at 1 100× g. The resulting supernatant was centrifuged for 15 min at 9 200× g and the pellet was resuspended in HB, followed by a centrifugation for 15 min at 10 500× g. The resulting pellet (synaptosomes) was resuspended in HB and diluted with 9 volume of H_2O to release SVs by hypotonic lysis of synaptosomes. After 1 M Hepes, pH 7.4, was added to a final concentration of 7.5 mM, this suspension was incubated on ice for 30 min and centrifuged for 20 min at 22 500× g. The supernatant was centrifuged again for 2 h at 200 000× g, and the pellet (crude SVs) was resuspended in 1 ml of HB. To obtain pure SVs, the suspension was loaded on a continuous gradient of 50–800 mM sucrose in 4 mM Hepes, pH 7.4, and centrifuged for 5 h at 65 000× g. Fractions from the middle portion of the gradient that contain SV marker proteins were pooled and centrifuged for 2 h at 200 000× g. Further fractionation of the SVs into subpopulations was performed according to Thoidis and others (1998). The pellet of SVs was resuspended in buffer A (10 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM EGTA, 0.1 mM $MgCl_2$), loaded on an 10–50% sucrose gradient in buffer A, and centrifuged for 18 h at 200 000× g. Each half of the major protein peak was pooled separately (Fig. 3A), loaded on a 10–30% sucrose gradient, and centrifuged for 50 min at 200 000× g. All the fractions were collected from the bottom of the gradient, and protein content was determined with the BCA kit (Pierce Cheminal Co., Rockford, IL). Fractions were analyzed by Western blotting as described (Lee et al. 1998).

Mouse monoclonal antibodies against synaptophysin, synaptobrevin, and α-syn were purchased from Chemicon International Inc. (Temecula, CA; MAB5258; 1:2 000), Synaptic Systems GmbH (Gottingen, Germany; Cat. 104 001; 1:10 000), and BD Transduction Laboratories (San Diego, CA; Cat. 610787; 1:1 500), respectively. Anti-V-type H^+-ATPase antibody was a gift from G. Bowman, University of California, Santa Cruz.

For immuno-isolation of SVs, fractions from the 10–50% gradient were pooled and centrifuged for 1 h at 260 000× g. The pellet was resuspended in buffer A containing 0.5% bovine serum albumin (BSA). The vesicle suspension was incubated at 4°C for 1 h with mouse monoclonal antibody (1.25 μg) against synaptophysin, synaptobrevin, or α-syn, or with mouse IgG. For each reaction, 1/6 of total vesicle preparation (approximately 80 μg protein) was used. The vesicle-

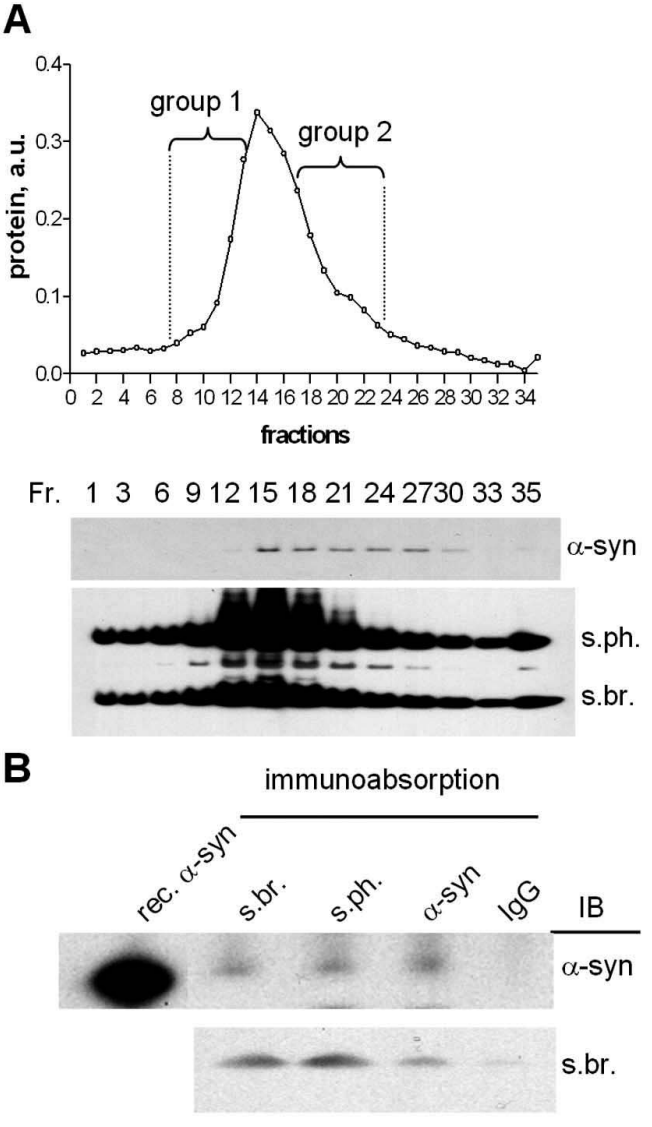

Fig. 2. Fractionation of SVs into subpopulations. (A) SVs (fr. 8–15 of 50–800 mM sucrose gradient) were applied onto a 10–50% linear sucrose density gradient and centrifuged until the vesicles reach their equilibrium densities (200 000× g, 18 h). Fractions were collected from the bottom of the gradient and subjected to protein assay and Western blotting. Note that all the fractions contain SV markers, synaptophysin and synaptobrevin, whereas α-syn is detected only in certain area of the gradient. (B) Fractions 15–27 were pooled and immunoabsorbed by mouse IgG or by antibodies against synaptobrevin, syanptophysin, or α-syn. Immunoisolated vesicles were analyzed using antibodies for α-syn or synaptobrevin.

antibody complexes were then incubated with paramegnetic beads (4×10^7 beads) conjugated with goat anti-mouse IgG (Dynabead M-450; Dynal Inc., Lake Success, NY) at 4°C for 1 h. Prior to use, the beads were saturated with 5% BSA in buffer A. The bead-

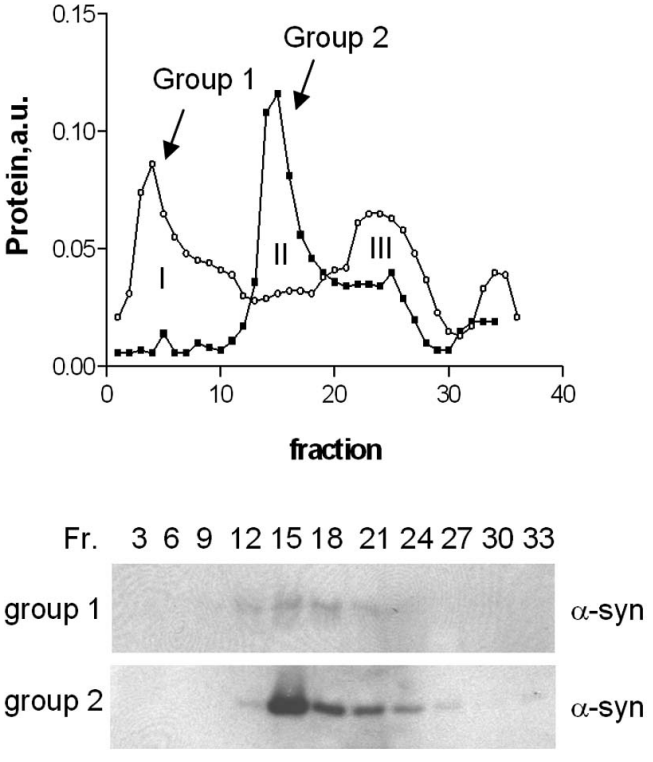

Fig. 3. Isolation of a subpopulation of SVs that contains α-syn. Fractions from 10–50% sucrose equilibrium gradient were pooled into two groups (see Fig. 2A; group 1, fr. 8–13; group 2, fr. 17–23) and each group of vesicles was subsequently applied onto a 10–30% sucrose velocity gradient. After the centrifugation (200 000× g, 50 min), fractions collected from the bottom of the gradient and analyzed for protein content (open circle, group 1 vesicles; closed square, group 2 vesicles) and α-syn distribution. Note that only the peak II vesicles contain α-syn.

vesicle complexes were washed with buffer A/0.5% BSA three times, before the proteins were eluted from the bead-bound vesicles with 1× Laemmli sample buffer.

For immunogold cryo-electron microscopy, the vesicle pellet was rehydrated with 35 μl distilled water and then loaded onto holey-carbon film-supported grids. The labeling was done by anti-synaptophysin antibody and 10 nm immunogold, followed by anti α-synuclein antibody and 20 nm immunogold. Grids were incubated with each antibody for 20 min at room temperature, and washed with 0.1% BSA in PBS buffer after each step. The labeled vesicles on grids were plunged into liquid ethane before the thin samples began to evaporate. Vitrobot (FEI) was used to make a thin aqueous film blotting with filter paper and the immediate plunging. The frozen grids were stored in liquid nitrogen and transferred to a cryotransfer holder (Gatan) under liquid nitrogen at approximately −180°C. Cryo-TEM (Tecnai-F20, FEI) images were then acquired with a CCD camera.

To obtain highly pure SVs, crude SVs were isolated from rat brain homogenate and further separated by velocity centrifugation in a 50–800 mM sucrose gradient (Fig. 1A). Fractions from the middle portion of the gradient were enriched in SVs as indicated by an enrichment of marker proteins such as synaptophysin, synaptobrevin, and V-type H^+-ATPase (p57). Most α-syn was found in the bottom and top fractions while a relatively small amount co-fractionated with the SV marker proteins in the middle part of the gradient (Fig. 1B,C), suggesting that α-syn is present but not enriched in SVs. The presence of α-syn in SVs was confirmed by electron microscopy (EM) with immunogold labeling. EM showed that our procedure yielded highly homogeneous SV preparations, and these vesicles were labeled with the antibodies against synaptophysin and α-syn (Fig. 1D)

Recent studies have demonstrated the presence of different populations of SVs (Thoidis et al. 1998, Provoda et al. 2000). To determine the precise localization of α-syn within the subpopulations of SVs, the fractions that contain SV marker proteins (fr. 8–15) were pooled, and the SVs were further fractionated by equilibrium density gradient centrifugation on a 10–50% sucrose gradient. Fractions were collected and the localization of α-syn and SV marker proteins was determined (Fig. 2A). Since the material loaded on the density gradient was a purified preparation of SVs, all the fractions contained the SV marker proteins, synaptophysin and synaptobrevin, and the distrubution of these proteins correlated well with the protein profile. On the other hand, α-syn was found only in a subregion of the gradient (Fig. 2A), suggesting that unlike synaptophysin and synaptobrevin, α-syn is present in a subset of SVs. In order to confirm the co-localization of α-syn and SV marker proteins in individual vesicles, α-syn-containing vesicles (fr. 15–27) were pooled, immunoabsorbed with antibodies for synaptobrevin or synaptophysin, and isolated using paramagnetic beads. The immunoabsorbed vesicles were eluted with SDS-containing Laemmli sample buffer, and protein components were analyzed by Western blotting. Both synaptophysin- and synaptobrevin-immunoabsorbed SVs contained α-syn as well as synaptobrevin (Fig. 2B).

Immunoabsorption of vesicles with α-syn antibody also resulted in the isolation of synaptobrevin-positive SVs (Fig. 2B). These results clearly show that α-syn is indeed present in SVs.

Although the profile of α-syn in the 10–50% equilibrium density gradient was indicative of the presence of subsets of SVs, we were not able to clearly separate the SV subpopulations in this density-based fractionation. To improve the separation among the subpopulations of SVs, the vesicles from the 10–50% equilibrium centrifugation were pooled into two groups (Fig. 2A); the vesicles that did not contain α-syn (group 1; fr. 8–13) and the vesicles that did contain α-syn (group 2; fr. 17–23). Each group of vesicles was subsequently subjected to 10–30% sucrose velocity centrifugation, by which SVs were separated according to their sedimentation velocity. This step produced three major peaks; peak I and peak II were derived from group 1 and 2 vesicles, respectively, whereas both group 1 and 2 contained peak III (Fig. 3). All the peaks contained synaptophysin, suggesting that these vesicles are SVs (data not shown). However, α-syn was localized only in peak II, which was a major component of group 2 vesicles (Fig. 3). α-Syn-containing-peak II was also weakly detected in the fractions from group 1 vesicles, representing a minor contamination of group 2 vesicles and verifying specific localization of α-syn in peak II vesicles (Fig. 3). These results confirmed the existence of different subpopulations of SVs and demonstrated that α-syn is specifically localized in a certain subset of SVs.

Although it has been known for more than a decade that α-syn is enriched in presynaptic terminals of neurons, its localization in SVs was not clearly addressed. In the present study, we have shown that a portion of presynaptic α-syn is present in SVs, and further fractionation of SVs revealed that only a specific subpopulation of SVs contains α-syn. These results are in good agreement with previous studies in an animal model and a cell culture system. Targeted deletion of the α-syn gene in mice resulted in increased neurotransmitter release in response to paired stimuli in the nigrostriatal dopamine system, whereas the mice showed no abnormality in dopamine discharge and reuptake after single electrical stimulation (Abeliovich et al. 2000). Another study with α-syn knock-out mice suggested that this protein is required for the biosynthesis and/or maintenance of the reserve pool of SVs (Cabin et al. 2002). These results suggest that α-syn is involved in a specific process or pathway of neurotransmission and regulates a specific pool of SVs. Isolation of a specific subset of SVs that contain α-syn in our study not only provides biochemical evidence supporting this idea but also offers opportunities to determine the identity of this specific pool of SVs.

Aggregation of α-syn has been found in a number of neurodegenerative diseases. In a recent study, we have shown that vesicular form of α-syn has a higher tendency for aggregation than the cytosolic form, and that the aggregates can grow rapidly in the membrane by recruiting cytosolic α-syn (Lee et al. 2002, 2005). Aggregation of vesicular α-syn was also found in the crude SV fraction from rat brain (Lee et al. 2002, 2005). According to the recent finding by Volles and coauthors (2001), certain forms of oligomeric α-syn have high affinity to lipid membranes and form pores that permit passive transport of ions and small molecules across the membrane. Therefore, by permeabilizing vesicular membranes, α-syn aggregates might cause deacidification of SVs and leakage of vesicular contents, preventing enrichment of neurotransmitters in SVs. Especially, permeabilization of dopaminergic SVs could be more detrimental to the cells than that of other SV types, because of dopamine's ability to generate toxic free radicals (Olanow and Tatton 1999). Consistent with this idea, transgenic mice that overexpress α-syn and form non-fibrillar aggregates showed a degeneration of tyrosine hydroxylase-positive nerve terminals in striatum (Masliah et al. 2000). Analysis of vesicle permeabilization using our SV preparations should aid in understanding the role of α-syn in synaptic degeneration.

In conclusion, we provide evidence that at least in SV pools, α-syn is localized in a specific subset of vesicles, characterization of which might lead to better understanding of the normal function of α-syn in synaptic transmission. Furthermore, studies on the effects of α-syn aggregation on integrity of these particular SVs could provide insights into the pathogenic mechanisms of α-synucleinopathies.

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