Synphilin-1 is developmentally localized to synaptic terminals and its association with synaptic vesicles is modulated by \( \alpha \)-synuclein

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Running Title: Presynaptic localization of synphilin-1

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Abbreviations: CNS, central nervous system; GST, glutathione S-transferase; Parkinson’s disease.
Abstract

α-Synuclein is the major component of Lewy bodies in patients with Parkinson’s disease and mutations in α-synuclein gene are responsible for some familial forms of the disease. α-Synuclein is enriched in the presynapse but its synaptic targets are unknown. Synphilin-1 associates in vivo with α-synuclein promoting the formation of intracellular inclusions. Additionally, synphilin-1 was found to be an intrinsic component of Lewy bodies in patients with PD. To understand the role of synphilin-1 in PD, we sought to define its localization and function in the brain. We now report that, like α-synuclein, synphilin-1 is enriched in neurons. In young rats, synphilin-1 is prominent in neuronal cell bodies but gradually migrates to neuropil during development. Immunoelectron microscopy of adult rat cerebral cortex demonstrates that synphilin-1 is highly enriched in presynaptic nerve terminals. Synphilin-1 co-immunoprecipitates with synaptic vesicles, indicating a strong association with these structures. In vitro binding experiments demonstrate that the N-terminus of synphilin-1 robustly associates with synaptic vesicles, and that this association is resistant to high salt washing but is abolished by inclusion of α-synuclein in the incubation medium. Our data indicates that synphilin-1 is a synaptic partner of α-synuclein and it may mediate synaptic roles attributed to α-synuclein.
Introduction

Parkinson’s disease (PD) is one of the most common neurodegenerative disorders. At post-mortem examination, brains from PD patients show loss of dopaminergic neurons in the substantia nigra and surviving neurons develop a characteristic inclusion called Lewy body (1,2). While most cases of Parkinson’s disease are sporadic, a few families have PD caused by mutations in the α-synuclein gene (3,4). α-Synuclein is also the major component of Lewy bodies in sporadic PD, suggesting that it is implicated in the pathogenesis of the disease (5-7).

α-Synuclein is highly enriched in the brain and it progressively accumulates into nerve terminals during development (8,9). Although the physiologic role of α-synuclein is still unclear, there is evidence that it regulates the size of the synaptic vesicular pool (10). In addition, targeted disruption of the α-synuclein gene causes accelerated recovery of dopamine release when the neuron is presented with multiple stimuli, suggesting that α-synuclein is a negative regulator of dopamine neurotransmission (11). Thus, even though α-synuclein has a synaptic role, its protein partners in the synapase and how it contributes to the death of dopaminergic neurons in PD are not known.

We have previously characterized synphilin-1 as a protein that associates with α-synuclein and leads to the formation of inclusion bodies when co-transfected with the NAC portion of α-synuclein in cultured cells (12). The interaction of synphilin-1 and α-synuclein was recently confirmed by biochemical and FRET techniques (13,14). Synphilin-1 co-localizes with carboxy-terminally truncated α-synuclein constructs in cytoplasmic inclusions of H4 transfected cells (15), supporting the idea that α-synuclein-synphilin-1 interaction may be relevant for inclusion formation. In accordance, synphilin-
1 was found to be an intrinsic component of Lewy bodies in PD indicating that it may be involved in the pathology of PD (16).

Parkin, a protein implicated in juvenile PD, was also shown to interact with and ubiquitinate synphilin-1. Co-transfection of synphilin-1, α-synuclein and parkin elicits the formation of ubiquitin-positive Lewy-body-like cytosolic inclusions, suggesting that synphilin-1 may link α-synuclein and parkin into a common pathogenic mechanism (17). Disease mutations in parkin failed to ubiquitinate synphilin-1 raising the possibility that accumulation of synphilin-1 could also be implicated in the pathogenesis of PD (17).

Although there are compelling data supporting the association of synphilin-1 with α-synuclein and PD, the distribution and role of synphilin-1 in the brain are not known. Because α-synuclein is a regulator of the synaptic vesicle pool, we now raise the possibility that synphilin-1 is a synaptic target for α-synuclein. Here, we show that synphilin-1 is predominantly expressed in neurons and gradually migrates to presynaptic terminals during development. Synphilin-1 specifically associates with synaptic vesicles and this interaction is negatively modulated by α-synuclein. Thus, synphilin-1 seems to be a synaptic partner of α-synuclein, implying that this interaction mediates the synaptic effects of α-synuclein.
Materials and methods

Synaptosomal preparation and subcellular fractionation- Purified synaptosomes were prepared by Percoll gradient from Wistar rat brains by pooling interfaces of 10-15% and 15-23% Percoll as described (18). Subcellular fractionations were carried out as described (19). Briefly, brains of 5 adult rats were homogenized in buffer containing 0.32 M sucrose, 4 mM Hepes, pH 7.4, and protease inhibitors (Complete, Roche). The crude homogenate (H) was centrifuged for 10 min at 800 g, producing the P1 pellet. The supernatant (S1) was centrifuged for 15 min at 9,200 g, producing the pellet P2 and supernatant S2. P2 was washed once with the original volume of homogenization buffer and centrifuged for 15 min at 10,000 g to produce the final P2 pellet. S2 supernatant was centrifuged for 2 h at 165,000 g to give the supernatant S3 and pellet P3. Washed P2 was resuspended in a small volume of homogenization buffer and lysed hypotonically with 9 vol. ice-cold water containing a cocktail of protease inhibitors. The pH was quickly adjusted by the addition of concentrated Hepes, pH 7.4. The lysed P2 was centrifuged for 20 min at 25,000 g, giving rise to the supernatant LS1 and pellet LP1. LS1 was centrifuged for 2 h at 165,000 g, producing the supernatant LS2 and the crude synaptic vesicle fraction LP2. Protein samples (50 µg) were denatured for 5 min at 100 °C in SDS sample buffer and analyzed by Western blot.

Cell transfection. N2A cells were grown in DMEM containing 10% FBS in a 5% CO₂ atmosphere. Cells were transiently transfected with 10 µg full-length synphilin-1 in pRK5-HA plasmid (12) utilizing Lipofectamine 2000 (Life Technologies). Cells were harvested 48 hours after transfection and synphilin-1 was detected by Western blot with a polyclonal antibody produced against synphilin-1 (12).
**Western blot analysis.** Samples were homogenized in 50 mM Tris-HCl, pH 7.4, 140 mM KCl, 2 mM EDTA, 0.5% triton X-100 and protease inhibitors cocktail (Complete, Roche), and clarified by centrifugation at 5,000 x g for 10 min. Protein samples (50 µg) were fractionated on 10% SDS-page and transferred to PVDF membranes (Schleicher & Schuell) at 200mA for 16 h. The blots were probed with antibodies against synphilin-1 (1µg/ml), α-synuclein (1:2,000; Transduction Laboratories) and synaptophysin (1:10,000; Sigma). Western blots were developed with ECL detection reagents and the yielded luminescence was captured on a hyperfilm ECL autoradiography film (Amersham).

**Immunohistochemistry.** Rats were anesthetized and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were dissected, fixed for additional 4 hours in 4% paraformaldehyde and cryoprotected in 20% sucrose in phosphate buffer, pH 7.4. Ten micrometer-thick sections were cut in a cryostate and immunoreacted with antibodies against synphilin-1 (2.5µg/ml) and tyrosine hydroxylase (1:300, Chemicon). Immunostaining was developed using the avidin-biotin-peroxidase complex method with diaminobenzidine as a chromogen (ABC Elite kit, Vector Laboratories). The specificity of the immunostaining was checked by incubating adjacent sections with preabsorbed anti-synphilin-1 antibody. For this, antibody against synphilin-1 was incubated with excess synphilin-1 antigen as described (12).

**Electron microscopy.** Adult Wistar rats were perfused with 4% paraformaldehyde and 0.08% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were dissected and immersed into 4% paraformaldehyde overnight at 4 ºC. Fifty micrometers-thick sections were cut using a vibratome and collected into phosphate buffer (pH 7.4). Sections were
cryoprotected into 30% sucrose for 6 hours and kept at –70ºC. After thawing, sections were immunostained with anti-synphilin-1 antibody (2.5µg/ml) and were detected using the avidin-biotin-peroxidase complex method with diaminobenzidine as a chromogen (ABC Elite kit, Vector Laboratories). Stained sections were postfixed for 1 hour in 2.5% glutaraldehyde in 0.1 M cacodilate buffer, pH 7.4, followed by 15 min incubation in 1% OsO4 and dehydrated with increasing concentrations of acetone. Sections were immersed for 6 hours in Epon diluted with acetone 1:1 and infiltrated for 24 h in 100% Epon. Polymerization of Epon was done by incubating the sections between two slides for 24 hr at 60 ºC. Pieces from selected areas were mounted on Epon capsules and ultrathin sections were collected over copper grids and counterstained with lead citrate. Images were obtained using the Zeiss 900 electron microscope.

For immunogold labeling, vibratome sections were embedded and polymerized in Unicryl. Ultra-thin sections were mounted over niquel grids, blocked with 50 mM ammonium chloride, 3% BSA and 0.02% tween 20 and further incubated with anti-synphilin-1 antibody overnight at 4 ºC. Ten nanometer gold anti-rabbit antibody was used to develop the reaction. Immunogold-stained sections were counterstained with 2% glutaraldehyde, 1% OsO4 and lead citrate. Blanks were carried out by omitting primary antibody. Images were obtained using the Zeiss 900 electron microscope.

**Immunoprecipitation**- For synaptic vesicle immunoprecipitation, Dynal beads (M-450) conjugated to secondary anti-mouse antibody were first coated with a monoclonal antibody to synaptophysin (Sigma) or mouse control IgG by overnight incubation in PBS containing 1% BSA followed by four washes in the same buffer. Then, LP2 brain subcellular fraction was incubated overnight with beads coupled to either anti-
synaptophysin or mouse IgG in buffer containing PBS, 1% BSA and protease inhibitor cocktail. The beads were washed five times with the same buffer and subjected to Western blot analysis with antibodies against synaptophysin, synphilin-1 and α-synuclein.

**Yeast two-hybrid constructs and experiments.** The yeast two-hybrid experiments were performed as previously described (12,20). Different synphilin-1 truncations were fused in frame into yeast two-hybrid vector containing the GAL4 DNA-binding domain (pPC97) and N-terminal α-synuclein (1-65 aa) was fused to the GAL4 DNA-activation domain in pPC86 vector. The yeast strain Y190 (MATa, ura3-52, his3-A200, ade 2-101, trp 1-901, leu 2-3, 112, gal4Δgal80Δ, URA::GAL-lacZ, cyh2, LYS::GAL-HIS3) was used for all transformations. Synphilin-1/pPC97 constructs were co-transformed with alpha-synuclein/pPC86 into Y190. The transformants were grown for 4 days in Trp⁻, Leu⁻, His⁻. The β-galactosidase filter lift assay was carried out for all grown colonies and monitored within one hour at 37°C. c-Jun (246-335 aa) in pPC86 and c-Fos (117-197 aa) in pPC97 were used as positive controls for the yeast two-hybrid assays.

**In vitro binding assays.** N-terminal synphilin-1(1-349)-GST or FKBP12-GST-fusion proteins were produced and purified on glutathione-sepharose 4B beads according to the manufacturer instructions (Pharmacia Biotech). For binding experiments, LP2 brain fraction was incubated with 20 μg/ml of GST-fusion proteins for 1 h at 4°C in buffer containing 50 mM Tris-HCl, pH 7.4, 500 mM NaCl, 1% BSA and protease inhibitor cocktail (Complete, Roche). Beads were washed 5 times with the same buffer and bound proteins were eluted from the beads with SDS sample buffer and synaptic vesicles pulled down by synphilin-1-GST were detected with anti-synaptophysin antibody.
Results

To ascertain synphilin-1 localization, we carried out immunohistochemistry and found that synphilin-1 is widely distributed in the brain and highly enriched in neurons. In post-natal day 7 rat, highest expression is observed in the cerebral cortex, hippocampus and cerebellum (Fig. 1A to 1C). Synphilin-1 densities are higher in neuronal cell bodies of layers II, III and VI of the cerebral cortex (Fig. 1A). In the hippocampus, pyramidal neurons (CA1 to CA3 regions) are strongly labeled as well as the granule cells of the dentate gyrus (Fig. 1B). In the cerebellum, Purkinje cells (P) are strongly stained with substantial staining also observed in the granule cell layer but negligible staining in the molecular layer (Fig. 1C). High densities of synphilin-1 are also observed in the caudate-putamen and associated structures, such as nucleus accumbens, as well as in the olfactory bulb and anterior commissure (data not shown). Staining is prominent in most nuclei of the brainstem (data not shown). Synphilin-1 immunostaining was specific since no labeling was observed when brain tissues were reacted with anti-synphilin-1 antibody preabsorbed with antigen (Fig. 1D and data not shown). Expression of synphilin-1 is developmentally regulated. At higher magnification, the immunoreactivity of synphilin-1 in P3 brain is prominent in the soma and processes of neurons of the cortex, hippocampus and cerebellum (Figs. 1E, 1H and 1K). However, when we analyzed the labeling of synphilin-1 in P14 brain, we observed a decrease of synphilin-1 densities in the cell bodies and a concomitant increase in the neuropil (Fig. 1F, 1I and 1L). This is better observed in adult brain (3 months) in which synphilin-1
immunoreactivity exhibits a diffuse punctuated pattern with no labeling of cell bodies, suggestive of nerve terminal staining (Figs. 1G, 1J and 1M). Thus, in the adult cerebellum, staining of Purkinje and granule cells is little or absent and immunoreactivity is prominent in the molecular layer, which contains abundant synapses of the parallel fibers originating from granule cell layer. Neither cell bodies nor neuropil were stained when brain sections were incubated with anti-synphilin-1 antibody preabsorbed with synphilin-1 antigen (Figs. 1N to 1P and data not shown). Different from other regions in the brain, immunostaining of synphilin-1 in substantia nigra pars compacta persisted in cell bodies and process of neurons even in the adult brain with a pattern similar to that observed for tyrosine hydroxylase immunoreactivity (Fig. 3). However, by analyzing adjacent sections, we found that more neuronal cell bodies were stained for tyrosine hydroxylase than for synphilin-1. This may be due to differences in the quality of the antibodies employed or to partial migration of synphilin-1 to neuropil. Nevertheless, synphilin-1 is expressed in human dopaminergic neurons and it is present in the majority of Lewy bodies in PD, indicating that synphilin-1 co-localizes with the neurons affected by the disease (16, 21). Synphilin-1 immunostaining of substantia nigra neurons was specific since no labeling was observed when sections were incubated with preabsorbed anti-synphilin-1 antibody (Fig. 2, inset).

We have previously shown that anti-synphilin-1 antibody recognizes a protein of approximately 90 kDa in adult brain, which is smaller than the predicted size based on its cDNA sequence (12). The decrease in cell body staining in adult brain is not caused by a decrease in synphilin-1 expression, since synphilin-1 is expressed at similar levels in all animal ages. Nevertheless, we observed a significant change in synphilin-1 apparent
molecular weight during development (Fig. 3A). Synphilin-1 mobility is identical to the recombinant full-length protein until P14 (Fig. 3). At P30 and older ages, synphilin-1 gel mobility changes to approximately 90 kDa (Fig. 3). Thus, redistribution of synphilin-1 toward the neuropil seems to parallel the decrease of its apparent size during development, implying a post-translational modification.

Similar to that previously reported for α-synuclein (9,22), synphilin-1 staining of neuropil in the adult brain indicate that it progressively accumulates in nerve terminals during development. In accordance, we found that synphilin-1 is enriched in biochemically purified nerve terminals prepared from adult but not young animals (Fig. 4A). A similar pattern is observed for α-synuclein expression (Fig. 4B). By contrast, synaptophysin is enriched in synaptosomes since early ages (Fig. 4C).

To further investigate the intracellular distribution of synphilin-1, we prepared subcellular fractions from rat brain tissue. We found that synphilin-1 is highly concentrated in LP2, a fraction enriched in synaptic vesicles (Fig. 4D). The integral synaptic vesicle synaptophysin was used as an internal control for the preparation (Fig. 4E).

To directly demonstrate synphilin-1 localization in nerve terminals, we carried out electron microscopy studies. When revealed with DAB, synphilin-1 immunoreactivity is observed at presynaptic nerve terminals of adult rat frontal cortex (Figs. 5A and B). Synphilin-1 immunoreactivity is absent from cell bodies or postsynaptic densities of mature cortical neurons (data not shown). Immunogold electron microscopy shows that synphilin-1 immunoreactivity is located in the vicinity or associated to synaptic vesicles (arrows) (Fig. 5C). Gold particles are not found at
postsynaptic densities. No specific DAB or immunogold staining was observed when sections were incubated with preabsorbed anti-synphilin-1 antibody (Figs. 5D-5H), suggesting that synphilin-1 decorates synaptic vesicles in a specific manner.

In order to determine the degree of association between synaptic vesicles and synphilin-1, synaptic vesicles were immunoprecipitated with anti-synaptophysin antibody and checked for the presence of synphilin-1. Synphilin-1 specifically co-immunoprecipitates with synaptic vesicles, suggesting that it associates \textit{in vivo} with these structures (Fig. 6A). The interaction of synphilin-1 with synaptic vesicles is specific since no immunoprecipitation was observed with beads coupled to mouse IgG as a control.

In previous report, the region of interaction between synphilin-1 and $\alpha$-synuclein was not thoroughly defined (12). Utilizing Yeast Two-Hybrid analysis, we now mapped the first 349 amino acid residues of synphilin-1 as the minimal region for interaction with the first 65 amino acid residues of $\alpha$-synuclein (Fig. 6C). Further deletions of the N-terminal region of synphilin-1 abolished the interaction. To verify if the region that interacts with $\alpha$-synuclein suffices for binding synaptic vesicles, we expressed the N-terminal synphilin-1 region and checked for its ability to pull down synaptic vesicles from LP2 fraction. We found that synphilin-1 (1-349)-GST robustly associates with synaptophysin-containing synaptic vesicles and this association is resistant to high salt washing (Fig. 7). No binding to synaptic vesicles was observed with FKBP12-GST control.

Because the region of synphilin-1 that binds to $\alpha$-synuclein contains the region that associates to synaptic vesicles, it is possible that $\alpha$-synuclein anchors synphilin-1 to
the vesicles. However, this possibility is unlikely since biochemical and density gradient centrifugation experiments failed to detect the presence of α-synuclein in synaptic vesicle fraction (23). Thus, it has been suggested that the interaction of α-synuclein with synaptic vesicles is reversible and the biochemical fractionation possibly dissociates the synuclein-synaptic vesicle complex. In accordance, we found that most of α-synuclein is cytosolic and only a minor fraction is present in LP2 (data not shown). In addition, levels of α-synuclein in immunopurified synaptic vesicles were undetectable under our experimental conditions (data not shown). Thus, we now raised the possibility that α-synuclein modulates the binding of synphilin-1, rather than anchoring it to vesicles. We explored this possibility by adding purified α-synuclein to the incubation mixture and measuring the degree of association of synphilin-1 with synaptic vesicles. We found that both wild type and A53T mutant α-synuclein abolish the binding of synphilin-1 to synaptic vesicles, suggesting that α-synuclein negatively regulates the association of synphilin-1 with synaptic vesicles (Fig. 7). We did not detect binding of synaptic vesicles to α-synuclein-GST under our experimental conditions utilizing high salt concentration (data not shown). Thus, it is unlikely that α-synuclein competes with synphilin-1 for the same site in the surface of the vesicles.
Discussion

We previously demonstrated that synphilin-1 specifically co-immunoprecipitates with α-synuclein from brain homogenates (12). We now provide evidence supporting the notion that synphilin-1 is a synaptic partner of α-synuclein. We found that synphilin-1 is widely distributed in the brain and that it is localized to neurons. Like α-synuclein, synphilin-1 is found in the cell bodies of immature neurons and is redistributed toward the presynaptic nerve terminal during development. Similar to α-synuclein, segregation of synphilin-1 into synaptic terminals appears late in the development of central nervous system suggesting that synphilin-1 would be important in the maturation or perhaps modulation of synapses. α-Synuclein was shown to undergo fast axonal transport (24). The characterization of synphilin-1 transport toward the synaptic terminal may give some clues about the process that lead to abnormal accumulation of synphilin-1 and α-synuclein in the cell body of neurons in PD.

The decrease on the apparent molecular weight of synphilin-1 in older animals raise the possibility that synphilin-1 undergo post-translational modification during development, such as proteolysis. Supporting this idea is the fact that we previously found only one size of synphilin-1 mRNA (12). Furthermore, the parallel accumulation of synphilin-1 at nerve terminals and the decrease of its apparent molecular weight suggest that synphilin-1 may be more vulnerable to post-translational modification at the presynaptic nerve terminal. Further studies will be necessary to clarify the mechanism of synphilin-1 post-translational modification.

α-Synuclein was shown to regulate the size of the presynaptic vesicular pool in primary hippocampal neurons and transgenic mice overexpressing α-synuclein have loss
of dopaminergic terminals (10,25). α-Synuclein knockout mice display increased dopamine release under stimulated conditions, suggesting that α-synuclein is a negative regulator of dopamine neurotransmission (11). We now showed that α-synuclein also abolished the interaction of synphilin-1 with synaptic vesicles. Thus, it is conceivable that synphilin-1 plays a role on synaptic function in concert with α-synuclein.

In addition, it was recently described that synphilin-1 can be phosphorilated in cultured cells, and that inhibition of synphilin-1 phosphorilation markedly reduces its ability to interact with α-synuclein (26). Therefore, modulation of synphilin-1-α-synuclein interaction by phosphorilation may also be important to determine the amount of synphilin-1 bound to synaptic vesicles.

Hyman and co-workers suggested that the C-termini of synphilin-1 and α-synuclein specifically interact using FRET technique (13). Our data now reveals that the first 349 amino acid residues of synphilin-1 are required for binding the N-terminus of α-synuclein. Thus, both N- and C- termini of synphilin-1 may be required for interaction with α-synuclein. We were not able to further restrict the α-synuclein binding region of synphilin-1 by yeast two-hybrid system, suggesting that synphilin-1 may require a tertiary structure to interact with α-synuclein. In addition, we found that the N-terminal region of synphilin-1 binds strongly to synaptic vesicles.

α-Synuclein interacts in vitro with synthetic membranes (27,28). An association of α-synuclein with synaptic vesicles was proposed based on the finding that it co-localizes with synaptophysin in brain sections and primary neuronal cultures (8,10). However, when more comprehensive brain fractionation experiments were done, α-synuclein was found to be depleted from the synaptic vesicle-enriched fraction (LP2) and
instead it was show to be enriched in a vesicle-depleted supernatant, suggesting that the association of α-synuclein with synaptic vesicles is weak and reversible (23,29). By contrast, synphilin-1 remains strongly bound to immunopurified synaptic vesicles (Fig. 6A). Thus, it is unlikely that α-synuclein anchors synphilin-1 to synaptic vesicles. Instead, our data imply that synphilin-1 may reversibly link α-synuclein to the surface of synaptic vesicles.

Synphilin-1 was shown to accumulate in Lewy bodies, indicating that abnormal distribution of synphilin-1 might also play a role in the pathogenesis of the disease (16,30). Different from other regions of the brain, we detected synphilin-1 in cell bodies of dopaminergic neurons from substantia nigra of adult animals (Fig. 2A), suggesting that synphilin-1 could be available as a core for Lewy body formation. In agreement, synphilin-1 is found in the core of Lewy bodies from patients with PD (16). The persistence of synphilin-1 in the soma of neurons from substantia nigra may facilitate the formation of Lewy bodies that are characteristically found in this region.

Taken together, our data support the idea that synphilin-1 is a partner for α-synuclein in the synapse and that it could mediate the synaptic role of α-synuclein. Understanding the regulation of synphilin-1-α-synuclein interaction will help clarify the possible synaptic contribution for the pathogenesis of PD. The association of synphilin-1 and α-synuclein may be important for the pathogenesis of PD and target disruption of their interaction could have therapeutic implications.

Recently, parkin was shown to weakly associate with synaptic vesicles (31) and it also interacts with synphilin-1 and α-synuclein (17,32). Thus, synphilin-1 may be physiologically important to keep both α-synuclein and parkin in close association with
synaptic vesicles. A better understanding of the dynamics of the interactions among these proteins may help determine the pathogenesis of the disease.
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References


Figure legends

Figure 1. Synphilin-1 is widely expressed in rat brain and accumulates in neuropil during development. Synphilin-1 immunoreactivity in neurons of post-natal day 7 rat cerebral cortex (A), hippocampus (B) and cerebellum (C). In all regions examined, cell bodies and processes of neurons were immunoreactive for synphilin-1. As shown for the hippocampus, immunostaining for synphilin-1 is specific since adjacent sections incubated with anti-synphilin-1 antibody preabsorbed with antigen yielded no signal (D). Synphilin-1 immunoreactivity in rat cerebral cortex (E-G), hippocampus (H-J) and cerebellum (K-M). In P3 animals, cell bodies and processes of neurons were immunoreactive for synphilin-1. In P14 and adult rats (3 months), a progressive decrease of neuronal cell body staining was observed in parallel to an increase of neuropil staining. Inset depicts cell bodies and processes of neurons from P14 cerebral cortex still strongly immunoreactive for synphilin-1. In 3 month rats, synphilin-1 immunostaining was found only in the neuropil. Specificity of synphilin-1 immunostaining was confirmed by incubating adjacent sections with preabsorbed anti-synphilin-1 antibody. For instance, we demonstrate in panels N to P that cerebellum at different ages show no staining when incubated with preabsorbed antibody. Scale bar, 900 µm (A-D) and 100 µm (E-P). Cb, cerebellum; Ctx, cerebral cortex; DG, dentate gyrus; Gr, granule cell layer; Hip, hippocampus; Mol, molecular cell layer; Or, Stratum oriens; P, Purkinje cell layer; Pyr, pyramidal neurons; Rad, stratum radiatum.

Figure 2. Expression of synphilin-1 in rat substantia nigra. Different from other regions, the immunoreactivity of synphilin-1 in substantia nigra pars compacta of adult
rat was still present in cell bodies and processes of neurons (A). Adjacent sections were incubated with tyrosine hydroxylase antibody (B). Inset depicts adjacent midbrain section that was incubated with anti-synphilin-1 antibody preabsorbed with antigen. Scale bar, 100 µm. SNC, substantia nigra pars compacta; SNR, substantia nigra pars reticulata; TH, tyrosine hydroxylase.

Figure 3. Expression of synphilin-1 protein during development. Rat brain protein lysates (50 µg) were obtained from animals with different ages, P0 until adult (3 months), and analyzed by Western blot using our polyclonal antibody against synphilin-1. Protein extract of N2A cells expressing recombinant synphilin-1 by transfecting full-length synphilin-1 in pRK5-HA plasmid was used as control (first lane). Levels of synphilin-1 in the brain are similar during development and adulthood. The apparent molecular weight of synphilin-1 decreased in rats older than P14. The blot is representative of four independent experiments with three different preparations. B, Table shows the migration pattern of synphilin-1 in the brain compared to that of recombinant synphilin-1.

Figure 4. Enrichment of synphilin-1 into synaptic terminals during development. Western blot analysis of rat brain total homogenates and synaptosomes, detected using anti-synphilin-1 antibody (A), anti-α-synuclein (B) and anti-synaptophysin antibodies (C). The total homogenate was analyzed side-by-side with purified synaptosomal fraction to determine the degree of synphilin-1 enrichment within synaptosomes. Synphilin-1 and α-synuclein are enriched in synaptosomes of adult but not P5 rats, suggesting that they accumulate in presynaptic terminals of adults. The figure is representative of three
experiments with three different synaptosomal preparations. To determine the subcellular distribution of synphilin-1, subcellular fractions were obtained by a series of differential centrifugations of adult rat brain homogenate. Equal amounts of protein from different fractions were analyzed by Western blot using anti-synphilin-1 (D) and anti-synaptophysin antibodies (E). Similar to synaptophysin, synphilin-1 was found to be enriched in the high-speed pellet of the lysed synaptosomal fraction (LP2), which is a fraction enriched in synaptic vesicles.

**Figure 5. Presynaptic localization of synphilin-1.** Electron microscopy analysis show a presynaptic localization for synphilin-1. (A and B) Fifty micrometers-thick sections of adult rat frontal cortex immunostained for synphilin-1 were revealed with DAB. Stained frontal cortex slices were embedded in Epon and ultra thin sections were analyzed in a Zeiss 900 electron microscope. Robust DAB staining was found concentrated in presynaptic terminals. No obvious DAB staining was observed in post-synaptic terminals. (C) Immunogold staining, slices of rat adult cerebral cortex slices were embedded into Unycril. Ultra thin sections were incubated with anti-synphilin-1 antibody followed by incubation with 10 nm gold anti-rabbit IgG. Gold-labeled synphilin-1 (arrows) was detected on or close to synaptic vesicles. No specific immunogold labeling was observed in post-synaptic densities. We verified the specificity of the immunostainings by incubating sections with preabsorbed anti-synphilin-1 antibody; DAB (D-F) and gold (G and H). Scale bar, 150 nm (A), 120 nm (B) and 80 nm (C). Pre, presynapse; Post, postsynapse.
Figure 6. Synphilin-1 co-immunoprecipitates with synaptic vesicles. Synaptic vesicles were immunoprecipitated from adult rat brain LP2 fraction using monoclonal affinity-purified antibody to synaptophysin coupled to magnetic beads. Immunoprecipitates were analyzed for synphilin-1 (A) and synaptophysin (B) by Western blot analysis. Synphilin-1 was found to co-immunoprecipitate with intact synaptic vesicles. Co-immunoprecipitation of synphilin-1 with synaptic vesicles was specific since beads coupled to mouse IgG failed to co-precipitate synphilin-1. C, Mapping the α-synuclein-binding region in synphilin-1. The scheme shows the result of β-galactosidase filter assays of Y190 co-transformed with different synphilin-1 truncations subcloned into pPC97 together with the first 65 amino acids of α-synuclein subcloned into pPC86. Positive interactions (+) were observed for fragments containing the first 349 amino acids of the N-terminus.

Figure 7. α-Synuclein negatively modulates the interaction between synphilin-1 and synaptic vesicles. Purified FKBP12-GST or synphilin-1-GST (1-349 aa) bound to glutathione-sepharose were incubated with LP2 fraction in buffer containing 50 mM Tris-HCl, pH 7.4, 500 mM NaCl and 1% BSA. Beads were washed 5 times with the same buffer and analyzed by Western blot using anti-synaptophysin antibody. Synphilin-1-GST was able to pull down intact synaptic vesicles as observed by the presence of synaptophysin in the synphilin-1-GST beads even after several washes with buffer-containing high salt. This synaptic vesicle binding was specific since a control protein, FKBP12-GST, failed to pull down synaptic vesicles. Addition of 20 µg/ml soluble α-synuclein, WT or A53T mutant, to the incubation mixture with synphilin-1-GST and
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Figure 1.
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Figure 4.
Figure 5.
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Figure 7.

![Image of a gel electrophoresis experiment with molecular weight markers and bands labeled as Input, FKBP12-GST, Synphilin-1-GST, Synph-GST WT, and Synph-GST A53T. The band labeled Synaptophysin is indicated.](image-url)
Additions and Corrections


Synphilin-1 is developmentally localized to synaptic terminals, and its association with synaptic vesicles is modulated by α-synuclein.

Cátiia S. Ribeiro, Katia Carneiro, Christopher A. Ross, João R. L. Menezes, and Simone Engelender

Figs. 1–7 for this article are reprinted here.

**Fig. 1.** Synphilin-1 is widely expressed in rat brain and accumulates in neuropil during development. Synphilin-1 immunoreactivity in neurons of postnatal day 7 rat cerebral cortex (A), hippocampus (B), and cerebellum (C). In all regions examined, cell bodies and processes of neurons were immunoreactive for synphilin-1. As shown for the hippocampus, immunostaining for synphilin-1 was specific since adjacent sections incubated with anti-synphilin-1 antibody preabsorbed with antigen yielded no signal (D). Synphilin-1 immunoreactivity is shown in rat cerebral cortex (E–G), hippocampus (H–J), and cerebellum (K–M). In postnatal day 3 (P3) animals, cell bodies and processes of neurons were immunoreactive for synphilin-1. In postnatal day 14 (P14) and adult rats (3 months), a progressive decrease of neuronal cell body staining was observed in parallel to an increase of neuropil staining. The inset depicts cell bodies and processes of neurons from postnatal day 14 cerebral cortex still strongly immunoreactive for synphilin-1. In 3-month-old rats, synphilin-1 immunostaining was found only in the neuropil. Specificity of synphilin-1 immunostaining was confirmed by incubating adjacent sections with preabsorbed anti-synphilin-1 antibody. For instance, N–P demonstrate that cerebellum at different ages showed no staining when incubated with preabsorbed antibody. Scale bar, 900 μm (A–D) and 100 μm (E–P). Cb, cerebellum; Ctx, cerebral cortex; DG, dentate gyrus; Gr, granule cell layer; Hip, hippocampus; Mol, molecular cell layer; Or, stratum oriens; P, Purkinje cell layer; Pyr, pyramidal neurons; Rad, stratum radiatum.

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.
Fig. 2. Expression of synphilin-1 in rat substantia nigra. Different from other regions, the immunoreactivity of synphilin-1 in substantia nigra pars compacta of adult rat was still present in cell bodies and processes of neurons (A). Adjacent sections were incubated with tyrosine hydroxylase antibody (B). The inset depicts an adjacent midbrain section that was incubated with anti-synphilin-1 antibody preabsorbed with antigen. Scale bar, 100 μm. SNC, substantia nigra pars compacta; SNR, substantia nigra pars reticulata; TH, tyrosine hydroxylase.

Fig. 3. Expression of synphilin-1 protein during development. A, rat brain protein lysates (50 μg) were obtained from animals with different ages, postnatal day 0 (P0) until adult (3 months) and analyzed by Western blot using a polyclonal antibody against synphilin-1. Protein extract of N2A cells expressing recombinant synphilin-1 by transfecting full-length synphilin-1 in pRK5-HA plasmid was used as control (first lane, Sph1-HA). Levels of synphilin-1 in the brain are similar during development and adulthood. The apparent molecular weight of synphilin-1 decreased in rats older than postnatal day 14 (P14). The blot is representative of four independent experiments with three different preparations. B, the table shows the migration pattern of synphilin-1 in the brain compared with that of recombinant synphilin-1. P, postnatal day; Sph1, synphilin-1.
FIG. 4. Enrichment of synphilin-1 into synaptic terminals during development. Western blot analysis of rat brain total homogenates and synaptosomes detected using anti-synphilin-1 antibody (A), anti-α-synuclein (B), and anti-synaptophysin antibodies (C) is shown. The total homogenate was analyzed side-by-side with purified synaptosomal fraction to determine the degree of synphilin-1 enrichment within synaptosomes. Synphilin-1 and α-synuclein are enriched in synaptosomes of adult but not postnatal day 5 (P5) rats, suggesting that they accumulate in presynaptic terminals of adults. The figure is representative of three experiments with three different synaptosomal preparations. To determine the subcellular distribution of synphilin-1, subcellular fractions were obtained by a series of differential centrifugations of adult rat brain homogenate. Equal amounts of protein from different fractions were analyzed by Western blot using anti-synphilin-1 (D) and anti-synaptophysin antibodies (E). Similar to synaptophysin, synphilin-1 was found to be enriched in the high speed pellet of the lysed synaptosomal fraction (LP2), which is a fraction enriched in synaptic vesicles. H, homogenate.

FIG. 5. Presynaptic localization of synphilin-1. Electron microscopy analysis shows a presynaptic localization for synphilin-1. A and B, fifty-micrometer-thick sections of adult rat frontal cortex immunostained for synphilin-1 were revealed with DAB. Stained frontal cortex slices were embedded in Epon, and ultrathin sections were analyzed by a Zeiss 900 electron microscope. Robust DAB staining was found concentrated in presynaptic terminals. No obvious DAB staining was observed in postsynaptic terminals. C, immunogold staining; slices of rat adult cerebral cortex slices were embedded into Unicryl. Ultrathin sections were incubated with anti-synphilin-1 antibody followed by incubation with 10-nm gold anti-rabbit IgG. Gold-labeled synphilin-1 (arrows) was detected on or close to synaptic vesicles. No specific immunogold labeling was observed in postsynaptic densities. We verified the specificity of the immunostainings by incubating sections with preabsorbed anti-synphilin-1 antibody: DAB (D–F) and gold (G and H). Scale bar, 150 nm (A), 120 nm (B), and 80 nm (C). Pre, presynapse; Post, postsynapse.
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Synphilin-1 is developmentally localized to synaptic terminals and its association with synaptic vesicles is modulated by \( \alpha \)-synuclein

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