Supplementary Materials and Methods

Identification of the IR puncta

Glutamate in Mg^{2+}-free bath solution, or normal bath solution (control) was added to 10-20 day old culture dishes and washed out after 1 minute by exchanging the solution with PBS (Antonova et al., 2001). In some experiments 200µM D-APV (RBI), was present in the bath solution for 1 hour before and during glutamate application. In other experiments 20nM NO paired with 200µM D-APV or 100µM NO-synthase inhibitor, N^W^-methyl-L-arginine (L-NMA), paired with glutamate in Mg^{2+}-free bath solution were prepared and applied as previously described (Arancio et al., 1996). The cultures were fixed starting approximately 5 or 30 minutes after glutamate, NO, L-NMA or control solution. Primary antibodies were either mouse monoclonal anti-α-Syn (Transduction Lab.; 10µg/ml in 10% goat serum in PBS) or affinity-purified rabbit anti-Sys I (Molecular Probes) diluted 1:200. Secondary antibodies were either goat anti-mouse conjugated with Cy5 (Jackson) diluted 1:200 in 10% goat serum in PBS or goat anti-rabbit conjugated with Cy3 (Jackson) diluted 1:200. Cells were excited using the 568 nm and 647 nm lines of a krypton-argon laser to image Cy3 and Cy5, respectively. Kalman averages of 4 scans were collected for each image and puncta in a representative field were identified. To minimize possible sources of bias, ten neurons in each culture dish were selected at random and analyzed by an observer who was blind to the experimental treatment. α-Syn and Sys I puncta in a representative field (94 x 142µm) around the neuron were quantified using a computer program written in IDL (Research Systems). The fields were chosen to
have roughly equal number and length of neuronal processes. Individual puncta were identified based on having a fluorescence intensity that exceeded a threshold set above background and a diameter between 0.5 and 5 µm. Puncta of different types were said to be colocalized if their boundaries overlapped or came within 1 µm of each other. For all measures, the mean result from the 10 neurons in the dish was normalized to the mean from control dishes in the same culture batch because variability between different culture batches was greater that between dishes in the same batch. These values were used for statistical comparison of dishes that received different experimental treatments using t-tests.

**Western Blot**

Primary antibodies consisted of guinea pig polyclonal anti-α-Syn 1:1000 (Calbiochem), rabbit anti-Sys I 1:200 (Molecular Probes), and mouse monoclonal anti-Syp (Chemicon International, 2 µg/ml). As a control, blots were also stained for NSE 1:2000 (Polysciences) to normalize the amount of neuronal protein. Cell cultures from α-Syn KO mice were normalized to the signal obtained in the same lane with an anti-actin antibody. Secondary antibodies consisted of anti-guinea pig IgG conjugated with Alkaline Phosphatase 1:5000 (Sigma), anti-rabbit IgG conjugated with Alkaline Phosphatase 1:12500 (Sigma), and anti-mouse IgG conjugated with Alkaline Phosphatase 1:12500 (Sigma).

**Antisense Treatments**

Cells were placed in serum-free (Sf) medium on the day before and during AS treatments to provide better delivery of oligonucleotides to the cells. Cultures
were treated with 5µM AS, S, Scr, or Sf only solution starting at nine days in vitro. The treatment was repeated every other day and lasted for 4 days.

**Electrophysiology**

mEPSCs were recorded with 1µM tetrodotoxin in the bath to block action potentials, and were counted using Mini Analysis Program (Synaptosoft). EPSCs between pairs of pyramidal neurons were recorded with the dual whole cell ruptured patch technique (Arancio et al, 1995). Glutamate (200µM) in Mg²⁺-free bath solution was introduced through the perfusion system and washed out in 1 minute. In some experiments D-APV 50µM (RBI) was added to the bath solution.

**Electron microscopy**

Cultured cells from α-Syn null animals and their wild-type littermates were grown on a glass coverslip and fixed with 1% paraformaldehyde and 4% glutaraldehyde in 0.1M sodium cacodylate buffer at pH=7.2. Individual coverslips were rinsed with 0.1M cacodylate, pH=7.4 to remove the fixative, and post-fixed with 1% osmium tetroxide in 0.1M cacodylate, pH=7.4 on ice for 1 hour. Coverslips were then treated with 0.5% aqueous uranyl acetate for 30 minutes at room temperature, and dehydrated in graded series of ethanol that was replaced by Embed 812 (Electron Microscopy Sciences) and allowed to infiltrate for 3 hours at room temperature. The last step was to remove most of the resin in the dish and place several resin filled BEEM capsule upside down over the coverslip. The whole dish was then placed in a 60°C degree oven to polymerize for 48 hours. The polymerized block was removed from the coverslip, trimmed and sections were made parallel to the block face with a diamond knife. Ultrathin sections
were collected on 200 mesh copper grids, stained with saturated uranyl acetate and lead citrate. The stained sections were examined with a JEOL 100X electron microscope operated at 80kV at a final magnification of 50,000X. Photographic negatives from knock-out and wild-type cultures were scanned at 300dpi to make digital images and then were coded and randomized for blind analysis with NIH image software. No more than 2 synapses per negative were selected as previously described (Murphy et al., 2000). Briefly, only synapses with a well-defined postsynaptic density were included in the counting assay. Cross-sectional area of each synapse was measured, and the number of vesicles counted as previously described (Murphy et al., 2000). Vesicles located up to one-vesicle-diameter distance from the membrane were counted as docked. The others were counted as part of the distal vesicular pool. Counts were divided by the cross-sectional area of the synapse, and data were expressed as mean ± SEM. Statistical analysis was performed with unpaired t-test.

**FM 1-43 staining**

Loading of FM 1-43 was induced by changing the perfusion medium from normal saline bath solution (119mM NaCl, 2.5mM KCl, 2mM CaCl₂, 2mM MgCl₂, 25mM HEPES and 30mM glucose) to hyperkalemic bath solution (31.5mM NaCl, 90mM KCl, 2mM CaCl₂, 2mM MgCl₂, 25mM HEPES and 30mM glucose) with 5µM FM 1-43 for 45 seconds. The hyperkalemic solution was then changed back to normal bath solution for 10 minutes to wash out the dye from the external medium. ADVASEP-7 (1mM, CyDex), an anionic cyclodextrin-complexing agent was introduced for 60 seconds in the washing bath solution at 1 and 6 minutes of
washing for enhanced removal of the dye from the external medium. After 10 minutes (this time was sufficient for the complete recycling and repriming of the dye-stained population of synaptic vesicles), an image was taken to record the loading of FM 1-43 in the synaptic boutons. The culture was then exposed to multiple 15 seconds application of hyperkalemic bath solution (without FM 1-43) to evoke repeated cycles of exocytosis, which facilitated release of the dye from the vesicles. An image was taken after 30 minutes of repeated cycles of exocytosis and washing with normal bath solution. The difference between the images before and after multiple exposures to hyperkalemic solution provided the measure of FM 1-43 stained vesicles. To study glutamate-induced presynaptic plasticity changes, the culture was exposed to 200µM glutamate in 0 Mg²⁺ saline for approximately 1 minute and then washed out in approximately 1 minute. Thirty minutes after glutamate application, the staining and destaining procedure was repeated. NMDA receptor antagonist, D-AP5 (40µM) and non-NMDA receptor antagonist, CNQX (20µM) were included in the hyperkalemic solution to block possible recurrent excitation and induction of activity-dependent plasticity. All images were acquired using Nikon D-Eclipse C1 confocal microscope. Cultures were viewed with 20×/0.50NA water immersion objective. An investigator blinded to experimental conditions obtained quantitative data using NIH Image (v. 1.61). The total number of boutons were assessed from randomly selected fields (30.8 X 30.8 µm).
**Protein preparation**

For the preparation of unmodified, murine α-Syn, a glutathione (GST)–α-Syn fusion construct was generated by PCR amplification of the α-Syn gene with specific primer sets described below. The protein coding regions of the full length α-Syn (residues 1-140) was amplified by PCR with the 5’–oligonucleotide primer GAT ACA TCT TTA **GGA TCC** GCC ATG GTG TTC ATG AAA GGA containing the underlined BamHI restriction site and 3’–oligonucleotide primer AGA ACT TGT ACG **CAG CTG** CCA TGG AAG AAC ACC TGG containing underlined Sall restriction site, respectively. The amplified DNAs was gel purified, digested with appropriate enzymes, ligated into the pGEX vector 4T-3 (Amersham Pharmacia) that had been digested with the appropriate restriction enzymes, and gel purified. The GST–α-Syn fusion constructs were then transformed into E-coli strain BL21 (DE3) for protein expression. The transformed bacteria was grown in 2x YTA medium with 0.1 mg/ml ampicillin at 37°C to an A$_{600}$ of 0.6 and then cultured for 3 hours after being induced with 1mM isopropyl beta-D-thio-galactopyranoside (IPTG) (Roche Diagnostics). The cells were harvested by centrifugation at 8000 rpm for 10 minutes. Pellets were washed in ice cold PBS (pH=7.5), and then disrupted by ultrasonication 10 seconds per cycle for 1 minute on ice. After removal of cell debris, the supernatant was loaded onto a GST-Sepharose 4B column (Amersham Pharmacia) equilibrated with PBS. After being washed with 5mM glutathione in PBS, the corresponding fusion protein was cleaved by thrombin on column and the purified untagged protein fraction was eluted with PBS.
For the preparation of α-Syn–GFP fusion protein, His-tagged α-Syn was subcloned from a plasmid into a pEGFP plasmid (Clontech). 1ng of resulting DNA was used for transformation of BL21(DE3)pLysS bacteria (Novagen). Large-scale preparation of protein extract was subsequently performed according to the "pET System Manual (Novagen)". Syn fusion protein that was His tagged was affinity purified from bacterial lysates using His Tag system (Novagen). All constructs were verified by DNA sequencing.

**Protein injection**

A perfluoroethylene-propylene (FEP) tube was pulled to a very fine diameter, filled with the electrode solution containing unmodified α-Syn or α-Syn-GFP, and inserted to within 300µm of the tip of the electrode. At the time of injection, a motorized micrometer spindle begun pushing a Hamilton syringe connected to the FEP tubing.

**References**


S1: The average baseline values were 36 ± 12 min⁻¹, n=7 (Baseline), 39 ± 14 min⁻¹, n=9 (Glu), and 43 ± 10 min⁻¹, n=8 (Glu + D-APV), not significantly different by a one-way ANOVA.
The average baseline values were 48 ± 13 min\(^{-1}\), \(n=10\) (AS), 44 ± 8 min\(^{-1}\), \(n=9\) (S), 41 ± 12 min\(^{-1}\), \(n=8\) (Scr), and 45 ± 12 min\(^{-1}\), \(n=8\) (Sf), not significantly different by a one-way ANOVA.
S3: The average baseline values were 46 ± 11 min⁻¹, n=8 (-/- plus glutamate), 42 ± 9 min⁻¹, n=10 (+/+ plus glutamate), 47 ± 14 min⁻¹, n=7 (-/- alone baseline), and 39 ± 13 min⁻¹, n=7 (+/+ alone baseline), not significantly different by a one-way ANOVA.
S4: The average baseline values were 60 ± 16pA, n=8 (+/+ mice), 48 ± 24pA, n=4 (-/- mice), and 53 ± 14pA, n=8 (+/+ mice in APV) not significantly different.
S5: The average baseline values were 42 ± 11 min⁻¹, n=7 (α-Syn-pre), 38 ±10 min⁻¹, n=6 (α-Syn-post), and 44 ± 14 min⁻¹, n=6 (vehicle), not significantly different by a one-way ANOVA.
**S6:** The average baseline values were 63 ± 21pA, $n=10$ (presynaptic $\alpha$-Syn paired with weak tetanus), 89 ± 25pA, $n=6$ (presynaptic $\alpha$-Syn alone), 54 ± 26pA, $n=7$ (postsynaptic $\alpha$-Syn paired with weak tetanus), 73 ± 19pA, $n=6$ (postsynaptic $\alpha$-Syn alone), not significantly different.
S7: The average baseline values were 44 ± 12 min⁻¹, n=6 (α-Syn-pre), 39 ± 11 min⁻¹, n=6 (α-Syn-post), not significantly different by a one-way ANOVA.
The average baseline values were 50 ± 3.8pA, \( n=7 \) (presynaptic \( \alpha \)-Syn paired with weak tetanus), 66 ± 3.5pA, \( n=5 \) (postsynaptic \( \alpha \)-Syn paired with weak tetanus), not significantly different.
A

-/+  -/-

synapsin I
synaptophysin
actin
α-synuclein

B

Docked Vesicle cluster

C

Docked Vesicle cluster

Number of vesicles (normalized)
Supplementary Fig. 1:  

A) Example of Western blot demonstrating that hippocampal cell cultures from α-Syn null mice do not exhibit changes in the expression of presynaptic proteins Sys I, and Syp. Actin levels remained constant. 

B) Examples of α-Syn null mice and wt synapses with a well defined postsynaptic density. Scale bar=100 nm. 

C) Average number of vesicles in the docked pool and in the vesicle cluster (number of wt docked vesicles=4.60 ± 0.6 vs α-Syn null mice docked vesicles=4.97 ± 0.6, P=0.73, and wt vesicle cluster=22.98 ± 2.8 vs α-Syn null mice vesicle cluster=20.87 ± 3.6, P=0.63). 

Vesicles located up to one-vesicle-diameter distance from the membrane were counted as docked. The others were counted as part of the distal vesicular pool. Counts were divided by the cross-sectional area of the synapse.
A

Presynaptic Postsynaptic

HP

B

C

D

E

α-synuclein-GFP (pre)
GFP
α-synuclein-GFP (post)

mEPSC frequency (% of baseline)

-10 -5 0 5 10 15 20 25 30 35 40 45

Time (min)
**Supplementary Fig. 2:** α-Syn-green fluorescent protein (GFP) introduction into the presynaptic neuron induces a long-lasting increase in frequency of spontaneous release of neurotransmitter. **A)** Schematic representation of an island containing two neurons with two patch pipettes on the cell body and a fast active perfusion device applied to one of the two cells. **B)** Phase contrast image of two neurons grown on islands of glial cells. Two recording electrodes are placed on the cell body. In all experiments, either the presynaptic or the postsynaptic cells were filled with α-Syn-GFP or GFP alone through a fast active internal perfusion system after recording a 10 minutes baseline. Scale bar=100 µm. **C)** Diffusion of α-Syn-GFP to distal processes of a cultured hippocampal neuron. α-Syn-GFP was included in the pipette solution during ruptured patch whole-cell recording, and the neuron was imaged with a Nikon Digital camera DXM-1200 attached to an epifluorescence microscope 4 minutes after injecting the protein into the presynaptic cell. Scale bar=30 µm. **D)** Same neuron as in C after gentle removal of the pipette. **E)** Presynaptic injection of α-Syn-GFP (filled diamonds) produced an immediate and long-lasting mEPSC frequency increase in microcultured neurons. Presynaptic injection of GFP (filled squares) and postsynaptic injection of α-Syn-GFP (filled triangles) did not change the mEPSC frequency. Data were normalized to the average baseline value during the 10 minutes before glutamate in each experiment. The average baseline values were 40 ± 13 min⁻¹, n=7 (GFP), 37 ± 12 min⁻¹, n=7 (α-Syn-GFP-pre), and 45 ± 11 min⁻¹, n=7 (α-Syn-GFP-post), not significantly different by a
one-way ANOVA. The horizontal bar indicates the period during which the protein was introduced into the neuron in this and the following graph.
Time (min)

mEPSC frequency (% of baseline)

α-synuclein + glutamate
α-synuclein + vehicle
vehicle + glutamate

α-synuclein

glutamate
Supplementary Fig. 3: Presynaptic injection of unmodified α-Syn occludes the occurrence of glutamate-induced mEPSC frequency increase. Cultured hippocampal neurons were either treated with glutamate 30 minutes after presynaptic injection of α-Syn (filled diamonds), or with vehicle 30 minutes after presynaptic injection of α-Syn (open squares), or with glutamate 30 minutes after presynaptic injection of vehicle (filled triangles). The average baseline values were $54 \pm 15 \text{ min}^{-1}$, $n=6$ (α-Syn plus glutamate), $42 \pm 10 \text{ min}^{-1}$, $n=6$ (α-Syn plus vehicle), $56 \pm 16 \text{ min}^{-1}$, $n=6$ (vehicle plus glutamate), not significantly different by a one-way ANOVA.
(A) Weak tetanus

(B) Pre β-synuclein + weak tetanus

Post β-synuclein + weak tetanus

Post γ-synuclein + weak tetanus

EPSC amplitude (% of baseline)

Time (min)
**Supplementary Fig. 4:** β-Syn and γ-Syn introduction into the presynaptic neuron does not induce potentiation. **A)** Injection of β-Syn into pre or postsynaptic (open circle and filled triangle, respectively) neuron paired with weak tetanus does not produce long lasting EPSC amplitude increase in cultured neurons. Data were normalized to the average baseline value during the 10 minutes before weak tetanus or β-Syn paired with weak tetanus. **B)** Injection of γ-Syn into pre or postsynaptic (open circle and filled triangle, respectively) neuron paired with weak tetanus does not produce long lasting EPSC amplitude increase in cultured neurons. Data were normalized to the average baseline value during the 10 minutes before weak tetanus or γ-Syn paired with weak tetanus.
Ca^{2+} / Calmodulin Kinase → NO-Synthase → NO

Ca^{2+} concentration

α-synuclein

synaptophysin

synapsin-I
Supplementary Fig. 5: Schematic representation of the mechanistic link of α-Syn with the glutamate-induced enhancement of neurotransmitter release during synaptic plasticity. Glutamate binding to postsynaptic NMDA receptors produces a series of concomitant changes at both postsynaptic and presynaptic level. Postsynaptic changes include increase of AMPA receptors (Antonova et al, 2001; Carroll et al, 1999; Lissin et al, 1999; Luscher et al, 2000; Shi et al, 1999), and release of the retrograde messenger, NO (Arancio et al, 1996; Micheva et al, 2003). Presynaptic changes of which α-Syn is an integral part, include activation of the cGMP/cGK pathway (Arancio et al, 1995; Arancio et al, 2001) and increase of presynaptic vesicle-associated molecules, Syp and Sys I (Antonova et al, 1999; Antonova et al, 2001). As the action potential reaches the presynaptic terminal, it causes a dissociation of Sys I from vesicles and their dispersion into the cytosol (Sihra et al, 1989; Torri-Tarelli et al, 1992; Hosaka et al, 1999). Sys I, in a few hundred milliseconds, returns to the synaptic vesicle cluster (Chi et al, 2001). α–Syn may regulate the interaction of Sys I with the vesicles by binding with vesicle phospholipids through the formation of alpha helices (Davidson et al, 1998).

References


