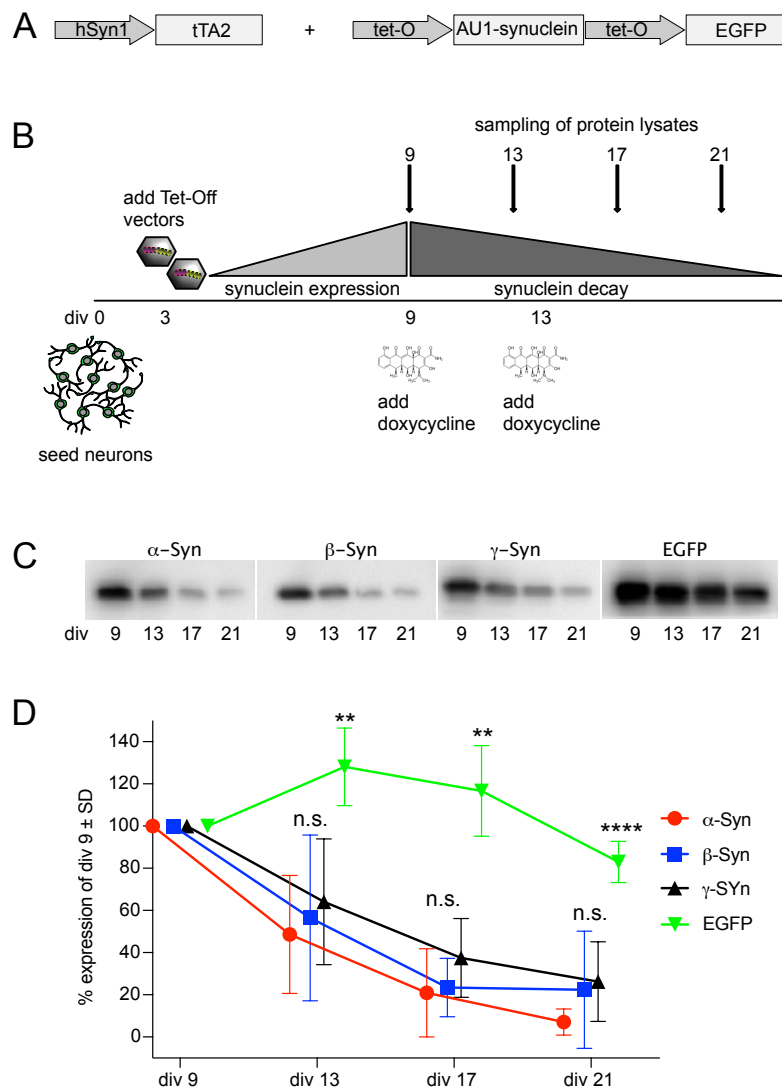


Supplemental Figures S1 – S7 for:

DOPAMINE PROMOTES THE NEURODEGENERATIVE POTENTIAL OF β -SYNUCLEIN

Anupam Raina, Kristian Leite, Sofia Guerin, Sameehan U. Mahajani, Kalyan S. Chakrabarti, Diana Voll, Stefan Becker, Christian Griesinger, Mathias Bähr, Sebastian Kügler

Supplemental Figure S1 adds information to those presented in Figure 1C of the main manuscript, demonstrating equal half-life of all synucleins and thus equal expression levels at late time points of the study.



Supplemental Figure S1: α -, β -, and γ -synuclein possess identical protein half-lives

A) Schematic representation of the Tet-Off vector system used, where the transactivator (tTA2) is expressed from a neuron-specific hSyn1 promoter of one vector genome, while from a second vector genome the respective synucleins (tagged N-terminally with the AU1 epitope tag, DTYRYI) are expressed from tetO elements activated by tTA2 in absence of doxycycline.

B) Schematic representation of the experimental setup used to study half-life of the synucleins. Neuron cultures were transduced at div3, expression of the synucleins and EGFP lasted up to div9, where doxycycline was added to stop the expression. A second dose of doxycycline was added on div 13. On div 9, 13, 17 and 21 protein lysates were obtained and analysed for synuclein and EGFP expression levels.

C) Randomly selected samples of detection of α -, β -, and γ -synuclein by means of the anti-AU1 antibody (Abcam ab3401; RRID:AB_303770) at div 9, 13, 17, and 21 and of EGFP (from a β -synuclein expressing culture; anti-EGFP (Roche 11814460001; RRID:AB_390913)).

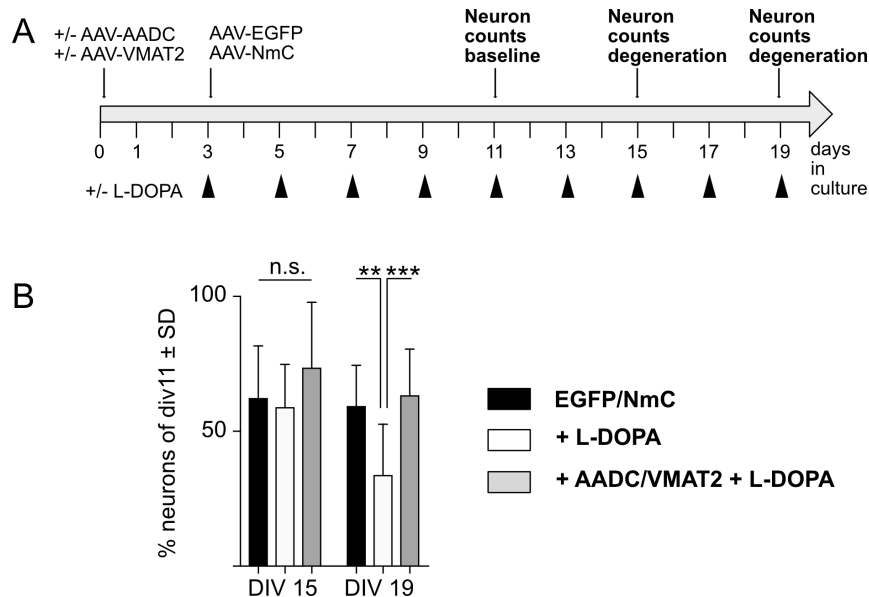
D) Quantitative assessment of the decay kinetics of α -, β -, and γ -synuclein and of EGFP in neuron cultures. Data are given as percentage of expression level of div 9 (at times when expression was stopped by doxycycline addition).

Statistics by 2-way ANOVA with Tukey's post hoc test for multiple comparisons. For EGFP versus the synucleins: ** $p < 0.01$, **** $p < 0.0001$; for α - versus β - versus γ -synuclein: n.s. not significant. Statistical power ($1 - \beta$ error probability) > 0.95 for all conditions. $N = 5$ independently prepared and transduced neuron cultures with 3-4 blots prepared from each culture per time point.

In Figure 1C of the main manuscript we describe equal expression levels for α -, β -, and γ -synuclein at div 9. Given that experiments are conducted up to div 19, it is necessary to demonstrate that expression levels for α -, β -, and γ -synuclein are still identical at later times of the study. This proof is difficult to achieve by probing protein lysates harvested at late time points, due to the differences in neurodegeneration that are ongoing in the respective cultures and the unavailability of an antibody detecting all three human synucleins with the same sensitivity.

Thus, we demonstrate identical half-lives (resulting in constant expression levels) of the three synucleins by an alternative approach. AU1 epitope – tagged α -, β -, and γ -synuclein are expressed in neuron cultures by means of Tet-Off vectors (Suppl. Fig. 1A). Synuclein expression of these vectors can be stopped by adding doxycycline into the cell culture medium (Suppl. Fig. 1B), allowing to study the decay of the three synucleins comparatively in primary neurons (Suppl. Fig. 1C). This analysis demonstrated that i) EGFP decayed much slower as compared to the synucleins, with a decay rate significantly different from the synucleins at all time points, but that ii) α -, β -, and γ -synuclein did not differ in their decay rates, suggesting that identical expression levels demonstrated at div 9 are likely maintained through the whole course of the experiment up to div 19 (Suppl. Fig 1D).

Supplemental Figure S2 adds information to those presented in Figure 2 of the main manuscript. Supplemental Figure S2 shows that in absence of AADC/VMAT2, a significant loss of neurons takes place due to addition of L-DOPA into the cell culture medium. This effect is prevented by expression of AADC/VMAT2, which convert L-DOPA into dopamine and sequester dopamine in synaptic vesicles.



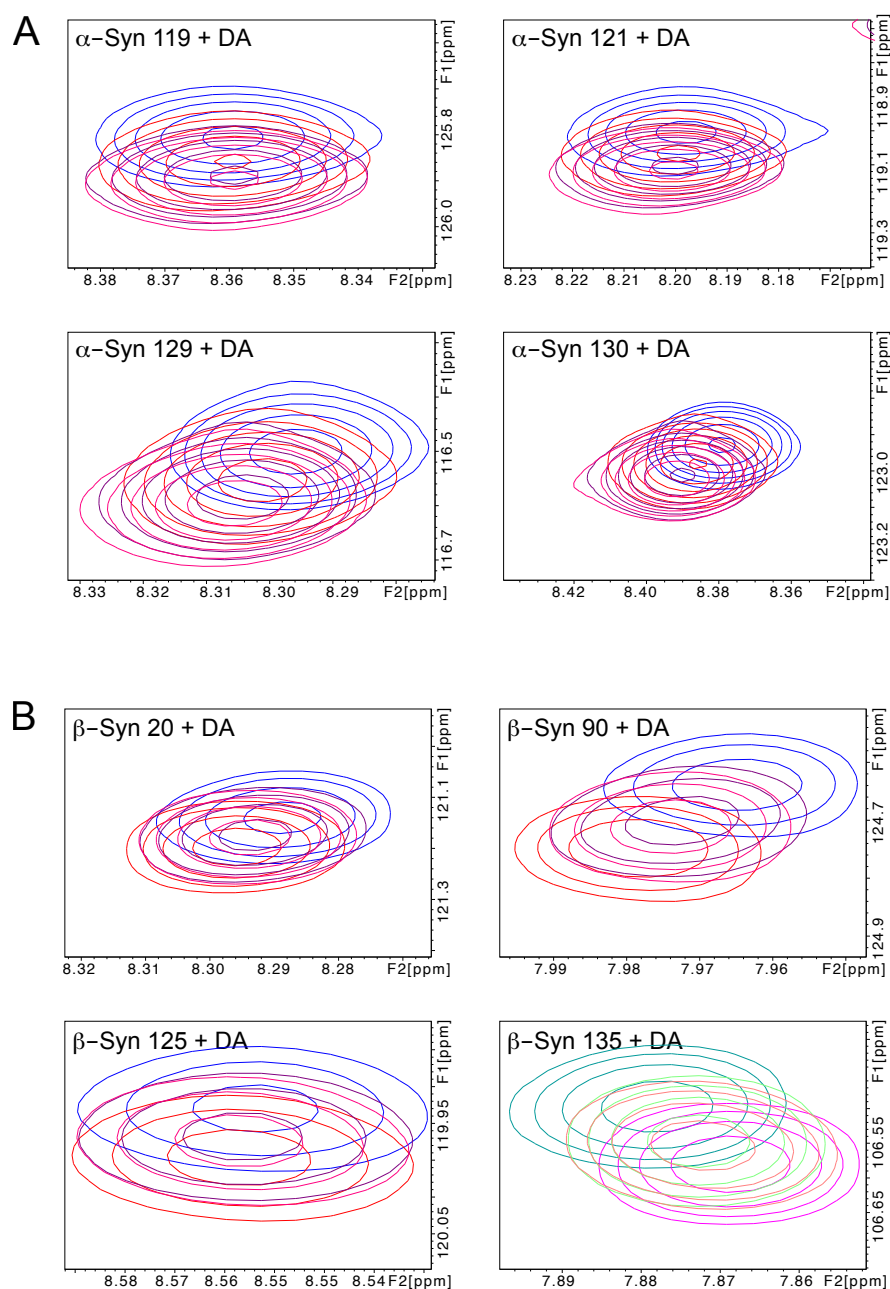
Supplemental Figure S1: Conversion of L-DOPA into dopamine is essential to prevent L-DOPA-associated neurotoxicity

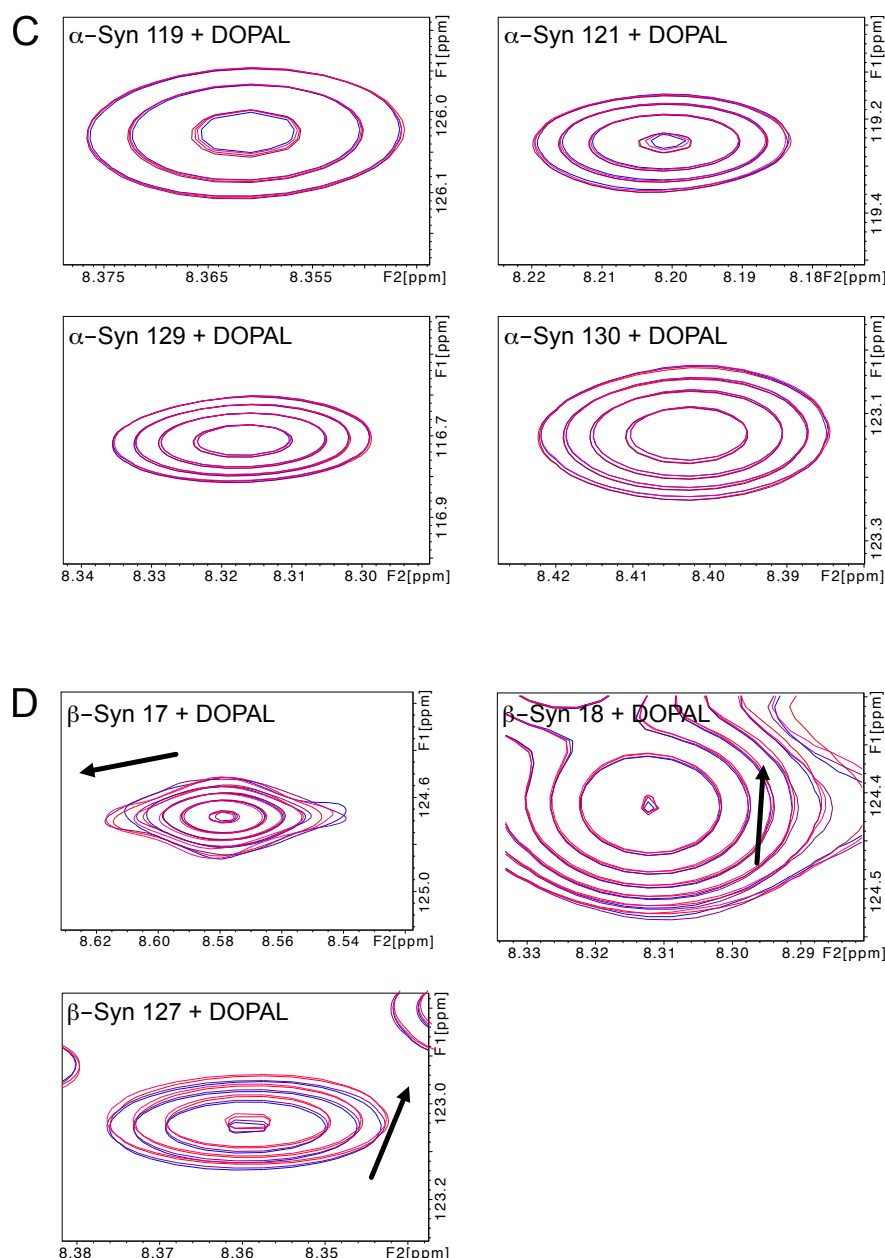
A) Schematic outline of the experimental conditions, which are identical to those described in Figure 2. 10 μ M L-DOPA was added at the times indicated by arrowheads, baseline counts of neuron numbers were performed at DIV 11, and neuron counts to determine L-DOPA's neurotoxicity were performed at DIV 15 and 19. These cultures did not express any synuclein, but only fluorophores exploited for cell counting.

B) Percentage of neurons surviving at DIV 15 and 19 in cultures expressing only fluorophores (black bars), expressing fluorophores and supplied with 10 μ M L-DOPA (white bars), or expressing fluorophores plus AADC/VMAT2 and supplied with 10 μ M L-DOPA (grey bars). Statistics by 1-way ANOVA with Dunnett's post hoc test for multiple comparisons. ** $p < 0.05$, *** $p < 0.01$; Statistical power ($1 - \beta$ error probability) > 0.95 for all conditions. $N = 6$. Bars represent means $\pm$ standard deviation.

Supplemental Figure S3 – S6 add information to those presented in Figure 6 of the main manuscript, providing more details regarding titration of CSPs and modelling of affinity values. Figure S3 shows accuracy of CSP determination even in cases of very small shifts, figures S4 and S5 show individual titration profiles, demonstrate that ^1H and ^{15}N spectra are almost identical, and suggest a 1:1 fitting model, while figure S6 shows error bars incorporated into the panels of Figure 6 E – H of the main manuscript.

Supplemental figure S3:

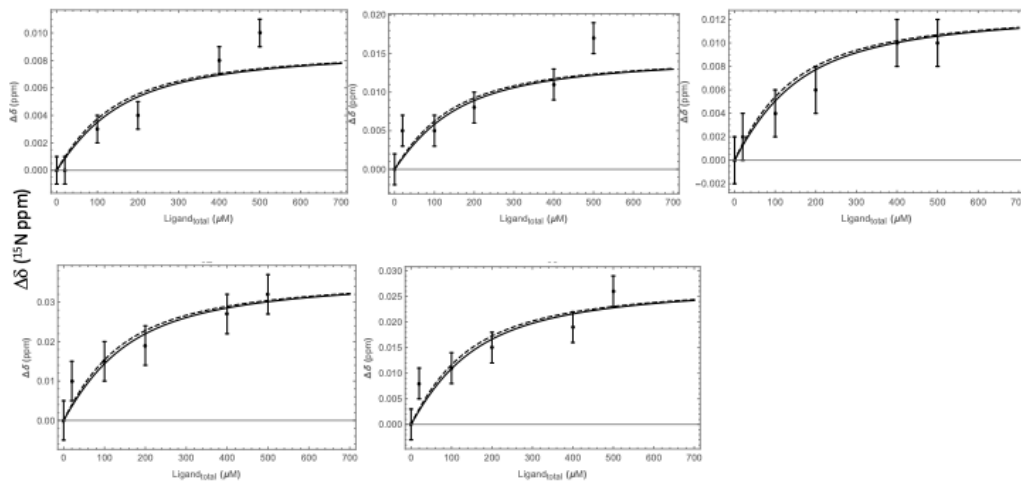




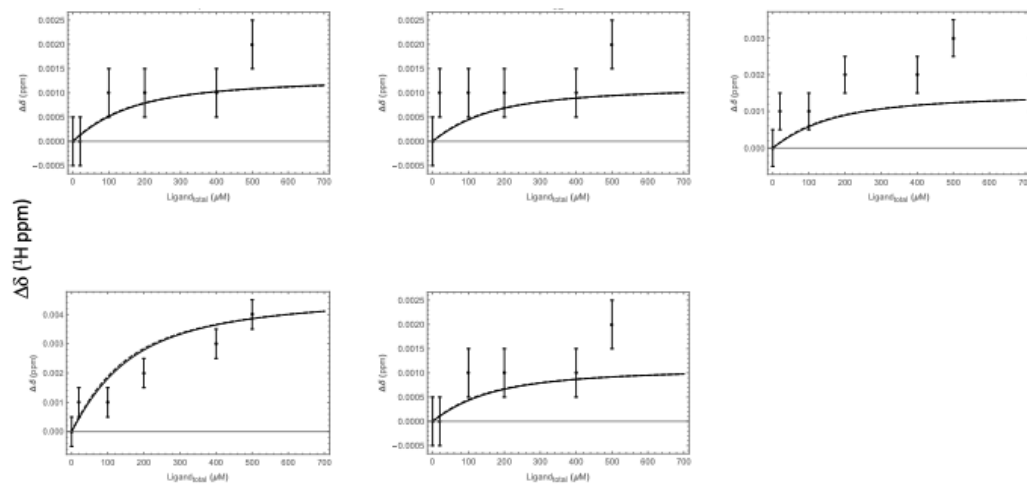
Supplemental Figure S3: The spectral superimposition of chemical shift perturbation upon titration of (A) DA into α -synuclein, (B) DA into β -synuclein, (C) DOPAL into α -synuclein and (D) DOPAL into β -synuclein (with black arrows pointing to the direction of shifts). The superimposed spectra follow the color gradient with the free protein spectra in blue and increasing ligand resulting in higher proportion of red. In (A) & (B) the highest DA concentration is 600 μ M and in (C) & (D) the highest DOPAL concentration is 2 mM. The patterns of chemical shifts show that the exchange regime is fast in NMR timescale and the shift is linear. The linear chemical shift patterns imply that the binding 1:1 or 1:n with equal affinity for all binding sites (Ref: Williamson, M. P., (2013) Using chemical shift perturbation to characterize ligand binding, *Progress in Nuclear Magnetic Resonance*, **73**: 1-16.).

Supplemental Figure S4:

A



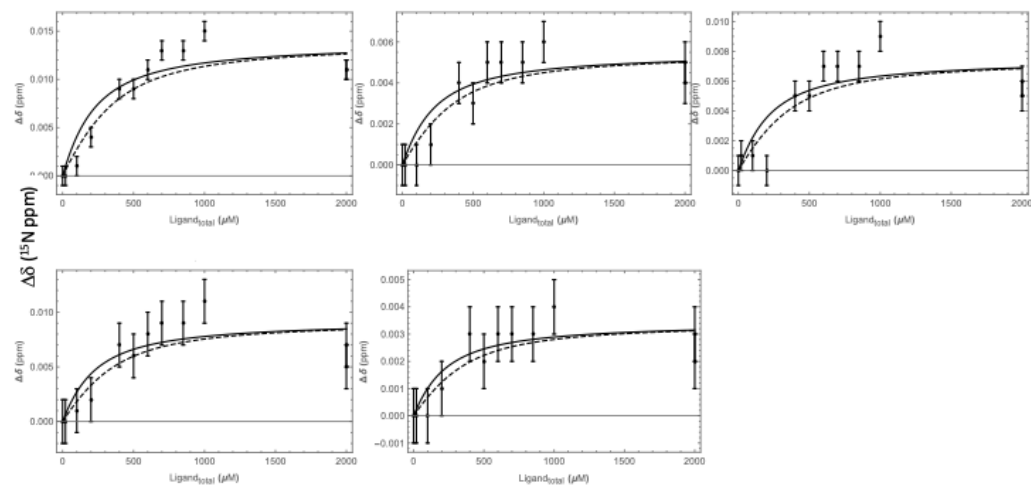
B



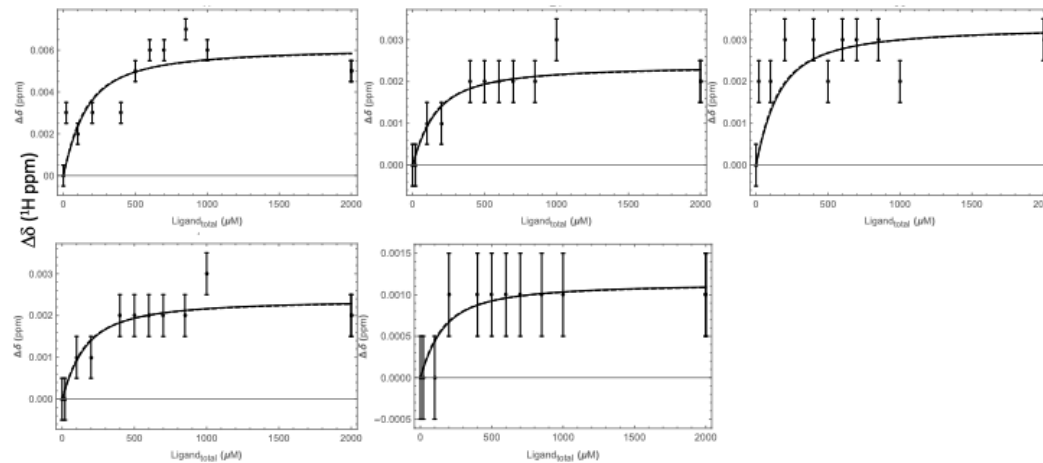
Supplemental Figure S4. Individual titration profiles of representative residues are shown for the interaction of α -synuclein and DA (corresponding to figure 6A in the main manuscript). The data were fit using the equation $\Delta\delta_{obs} = \Delta\delta_{max} \{ (n[P]_t + [L]_t + K_d) - [(n[P]_t + [L]_t + K_d)^2 - 4n[P]_t[L]_t]^{1/2} \} / (2n[P]_t)$, where $\Delta\delta_{obs}$ is the observed difference in chemical shift, $[P]_t$ is the total protein concentration, $[L]_t$ is the total ligand concentration, K_d is the overall dissociation constant and n is the number of sites with equal affinity for binding the ligand (Ref: Williamson, M. P., (2013) Using chemical shift perturbation to characterize ligand binding, *Progress in Nuclear Magnetic Resonance*, **73**: 1-16.). **(A)** The individual titration profiles along the ^{15}N dimension for the residues 20,101, 103, 105 and 139 fit to a 1:1 binding model in black curve ($K_d = 96 \pm 75 \mu\text{M}$). The dashed curve shows the fitting of the data to 1:n equal affinity binding model ($K_d = 94 \pm 264 \mu\text{M}$, $n = 0.8 \pm 3$). **(B)** The individual titration profiles along the ^1H dimension for the same residues fitted to the 1:1 (unbroken curve) ($K_d = 102 \pm 113 \mu\text{M}$) and 1:n equal affinity model (dashed curve) ($K_d = 104 \pm 450 \mu\text{M}$, $n = 0.8 \pm 5$). The n was interpreted to be 1.

Supplemental Figure S5:

A



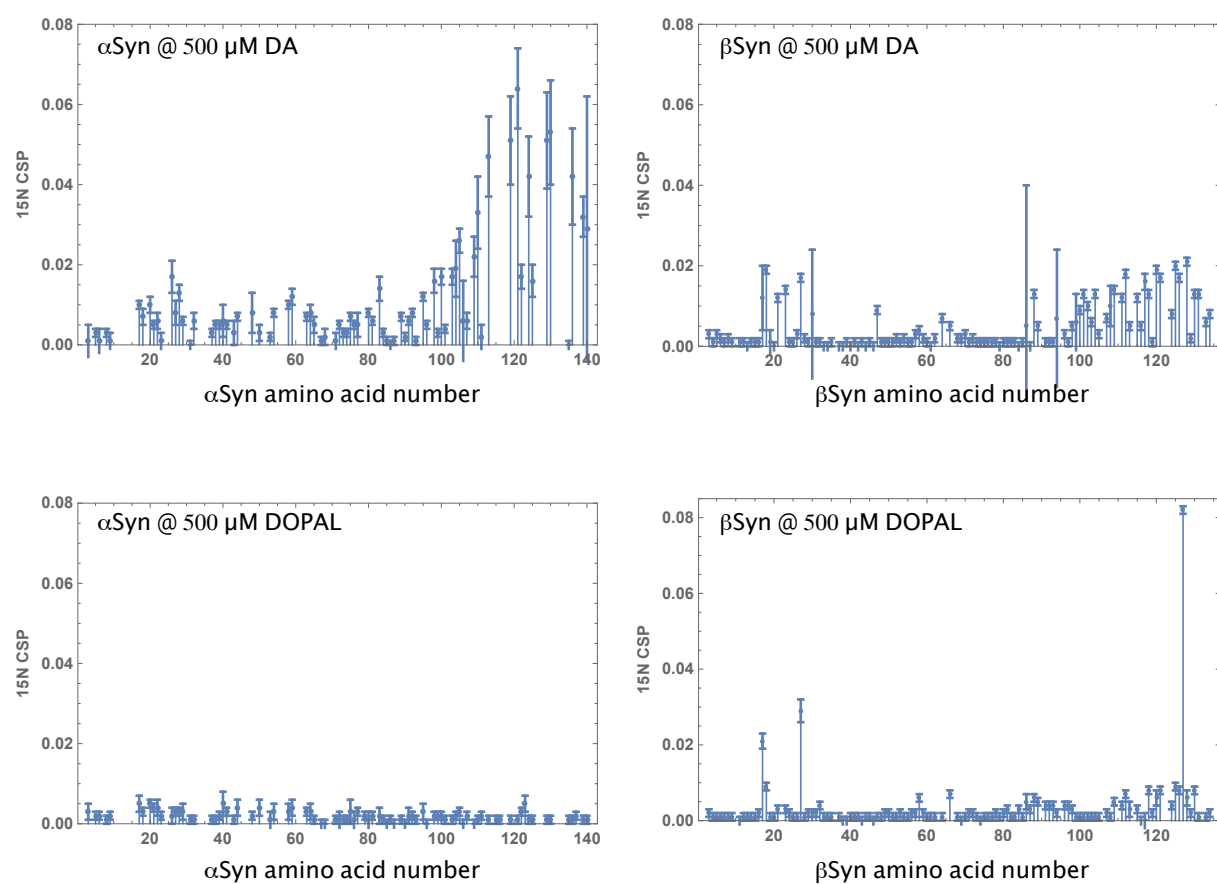
B



Supplemental Figure S5. Individual titration profiles of representative residues are shown for the interaction of β -synuclein and DOPAL (corresponding to figure 6D in the main manuscript). The data were fit using the equation $\Delta\delta_{obs} = \Delta\delta_{max} \{ (n[P]_t + [L]_t + K_d) - [(n[P]_t + [L]_t + K_d)^2 - 4n[P]_t[L]_t]^{1/2} \} / (2n[P]_t)$, where $\Delta\delta_{obs}$ is the observed difference in chemical shift, $[P]_t$ is the total protein concentration, $[L]_t$ is the total ligand concentration, K_d is the overall dissociation constant and n is the number of sites with equal affinity for binding the ligand (Ref: Williamson, M. P., (2013) Using chemical shift perturbation to characterize ligand binding, *Progress in Nuclear Magnetic Resonance*, **73**: 1-16.). **(A)** The individual titration profiles along the ^{15}N dimension for the residues 18, 27, 109, 120 and 134 were fit to a 1:1 binding model in black curve ($K_d = 170 \pm 37 \mu\text{M}$). The dashed curve shows the fitting of the data to 1:n equal affinity binding model ($K_d = 170 \pm 37 \mu\text{M}$, $n = 2.9 \pm \text{ND}$). **(B)** The individual titration profiles along the ^1H dimension for the same residues fitted to the 1:1 (unbroken curve) ($K_d = 102 \pm 22 \mu\text{M}$) and 1:n equal affinity model (dashed curve) ($K_d = 103 \pm 52 \mu\text{M}$, $n = 0.8 \pm 0.9$).

Due to the presence of both models within the error margins of the data, the quality of the fit parameters for a variable no of binding sites (standard error was not determined (ND) and the wide discrepancy between the ^{15}N and ^1H data), the n was interpreted to be 1.

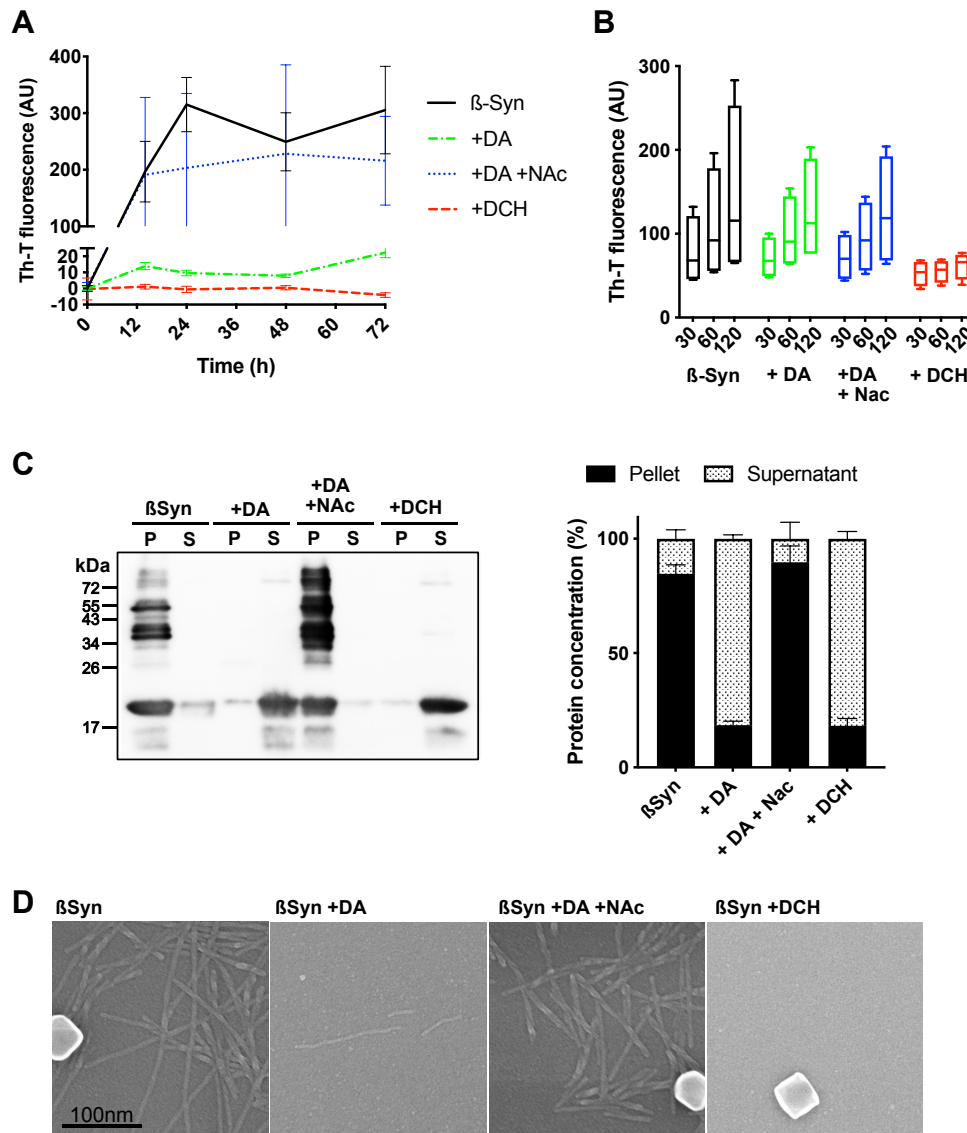
Supplemental Figure S6:



Supplemental Figure S6: The sites of the interaction of DA and DOPAL with α -synuclein and β -synuclein are shown with error bars (from duplicated measurements) corresponding to Figure 6 E – H of the main manuscript.

Supplemental Figure S7:

Supplemental Figure S7 adds information to those presented in Figure 7 of the main manuscript. It shows kinetic data the Th-T assay (Supple. Fig. 7A), the interaction of DA, Nac and DCH with binding of Th-T to β -synuclein fibrils (Suppl. Fig. 7B), an analysis of pelleted and soluble protein fractions obtained after aggregation of β -synuclein in presence of DA, DA + Nac and DCH (Suppl. Fig. 7C), and SEM pictures of fibrillar material obtained after incubation of β -synuclein with DA, DA + Nac and DCH (Suppl. Fig. 7D).



Supplemental Figure S7: Additional data regarding the inhibition of β -synuclein fibrillation by dopaminochrome

A) Kinetic assessment of the early stages of β -synuclein fibrillation. β -synuclein was incubated either alone, or in presence of DA, DA + Nac, or DCH, and Th-T incorporation was recorded in a plate reader over 3 days. The assay was conducted at pH 7.4 with addition of 0.001% (w/w) of β -synuclein pre-formed fibrils as seeds. N = 6 independent replicates for each condition.

B) The potential influence of DA, DA + Nac and DCH on binding of Th-T to fibrillized β -synuclein was evaluated by incorporation of β -synuclein fibrils as generated for Figure 7A (by incubation for 7 days) with Th-T and either DA, DA + Nac or DCH for 30, 60 and 120 minutes at 37°C. While there is a clear trend for DCH to limit Th-T incorporation into β -synuclein fibrils over time, this effect was not statistically significant according to analysis by 1-way ANOVA with Tukey's post hoc test. N = 4 independent replicates per condition.

C) Given the potential influence of DCH on Th-T incorporation into β -synuclein fibrils, the relative amounts of protein present in supernatant (= soluble) and pellet (= fibrillar) were quantified by sedimentation assay. Samples obtained from incubation as shown in Suppl. Fig 7A were centrifuged with 100000 x g for 30 min and analysed by SDS-PAGE and western blot. This assay confirmed that incubation with both DA under conditions were DA can auto-oxidize, and with DCH result in robust inhibition of β -synuclein aggregation. Quantification was performed from N = 4 western blots. P = pellet; S = supernatant.

D) Scanning electron microscopy (SEM) of fibrils obtained after incubation of β -synuclein alone or together with either DA, DA + Nac or DCH for 7 days as shown in Figure 7A. White objects are salt crystals identifying the correct focus level on the surface of the coverslip. Scale bar = 100 nm.

Open Practices Disclosure

Manuscript Title: Dopamine promotes the neurodegenerative potential of β -Synuclein
Corresponding Author: Sebastian Kügler

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