

# $\beta$ -synuclein promotes synaptic vesicle dopamine uptake and rescues dopaminergic neurons from MPTP-induced death

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## Research article

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# Abstract

## Background.

Previous studies demonstrated that dopaminergic neurons in the substantia nigra pars compacta (SNpc) of mice with null mutations for genes encoding  $\alpha$ -synuclein and/or  $\gamma$ -synuclein are resistant to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity. An original straightforward interpretation of these results was that these proteins are directly involved in the mechanism of MPTP-induced degeneration and this view has become commonly accepted. Here we provide evidence that a plausible alternative explanation of this resistance is not the absence of these synucleins per se but their substitution on the membrane of synaptic vesicles by the third member of the family,  $\beta$ -synuclein.

## Methods.

An effect of sub-chronic MPTP regimen on dopaminergic neurons of SNpc was studied in mice lacking members of the synuclein family in all possible combinations. Dopamine uptake was assessed in synaptic vesicles isolated from synuclein null mutant mice. Protein composition of synaptic vesicles was studied by mass spectrometry.

## Results.

Dopaminergic neurons of mice lacking  $\beta$ -synuclein singularly or in combination with the loss of other synucleins, were sensitive to the toxic effect of MPTP. Dopamine uptake by synaptic vesicles isolated from the striatum of triple  $\alpha/\beta/\gamma$ -synuclein deficient mice was significantly reduced, while reintroduction of  $\beta$ -synuclein either *in vivo* or *in vitro* reversed this effect. Proteomic analysis of complexes formed on the surface of synuclein-free synaptic vesicles after addition of recombinant  $\beta$ -synuclein identified multiple integral constituents of these vesicles as well as typically cytosolic proteins, including key enzymes involved in dopamine synthesis, tyrosine hydroxylase (TH) and aromatic L-amino acid decarboxylase (AADC).

## Conclusions.

Of the three members of the synuclein family, only  $\beta$ -synuclein can play a scaffolding role for the assembly of molecular complexes that potentiate the ability of synaptic vesicles to uptake and sequester dopamine and other structurally similar molecules, including 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>), a toxic metabolite of MPTP. The increased presence and activity of  $\beta$ -synuclein at the synaptic vesicles, and not the absence of other synucleins per se, explains the decreased sensitivity to MPTP toxicity of SNpc dopaminergic neurons in mice lacking  $\alpha$ -synuclein and/or  $\gamma$ -synuclein.

# Background

Three members of the synuclein family,  $\alpha$ -,  $\beta$ - and  $\gamma$ -synuclein, are involved in various important molecular processes in presynaptic terminals. Gain-of-toxic-function is believed to be the main mechanism of the involvement of these proteins,  $\alpha$ -synuclein in particular, in pathogenesis of Parkinson's disease and certain other neurodegenerative diseases collectively known as synucleinopathies, but the loss-of-function can also be a factor contributing to synaptic dysfunction associated with these diseases [1–5]. However, null mutations inactivating one or even two synuclein-encoding genes have limited effects on mouse physiology. The most notable consequence of  $\alpha$ -synuclein,  $\gamma$ -synuclein or double  $\alpha/\gamma$ -synuclein null mutations for nigrostriatal system function is a partial [6–9] or even complete [6, 9–12] resistance of dopaminergic neurons of the SNpc to various regimens of the Parkinsonian mimetic toxin MPTP. These observations have led to the hypothesis that these two synucleins are directly involved in, and required for, the MPTP-induced degeneration of dopaminergic neurons in the SNpc.

This straightforward assumption, nevertheless, does not take into consideration a possibility that the third member of the family,  $\beta$ -synuclein, may play a role in the resistance of SNpc neurons to MPTP toxicity.  $\beta$ -synuclein is highly expressed in the same neurons as  $\alpha$ -synuclein, including dopaminergic neurons of the SNpc, and co-localises with  $\alpha$ -synuclein in their presynaptic terminals [13–15]. Together with the high similarity between amino acid sequences of synucleins, this suggests that when a family member is absent, it can be replaced by another one. This can partially compensate for the lost function but also can bring certain “added values”, i.e. functional changes that manifest only when normal synaptic physiology is challenged. Indeed, increased expression of  $\beta$ -synuclein was observed in the midbrain of both  $\alpha$ -synuclein and  $\alpha/\gamma$ -synuclein null mutant mice and restoration of  $\alpha$ -synuclein expression reversed this effect [9, 12]. In several experimental *in vivo* systems, expression of  $\beta$ -synuclein ameliorated neuronal pathology caused by overexpression of  $\alpha$ -synuclein [16–21]. General neuroprotective abilities of  $\beta$ -synuclein have been attributed to inhibition of pro-apoptotic and activation of pro-survival signaling pathways but through unknown mechanisms [9, 16].

Here we demonstrate that  $\beta$ -synuclein plays a pivotal role in developing resistance to MPTP toxicity by SNpc dopaminergic neurons lacking the normal balance of synucleins. This might be due, at least in part, to improved sequestering of MPP<sup>+</sup> into synaptic vesicles, which is concordant with an observed ability of  $\beta$ -synuclein to potentiate dopamine uptake by striatal synaptic vesicles.

# Methods

**Generation of experimental animals.** Generation of  $\alpha$ -synuclein KO (from original mouse line described in [22]),  $\beta$ -synuclein KO (from original mouse line described in [23]),  $\gamma$ -synuclein KO [24], and  $\alpha/\gamma$ -synuclein double KO mice on C57Bl6J (Charles River) background was described previously [12, 25]. Homozygous  $\alpha/\gamma$ -synuclein double KO and  $\beta$ -synuclein KO mice were crossed to generate triple heterozygous mice. Intercrossing of these mice produced founders of TKO, double KO, single KO and wild type colonies used in this study. Therefore, founders of all these colonies were either first-, or second-generation siblings and

all experimental animals were on the same C57Bl6J genetic background. Mouse genotyping was carried out as described previously [12, 23, 24]. Unless otherwise stated, 4–5 months old male mice were used in all experiments. Neuron counts were performed by investigators blinded with respect to the sample genotype and treatment. All animal work was carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act (1986).

**Subchronic MPTP treatment, immunohistochemistry and neuronal cell counts.** Four-month old male mice were injected i.p. once a day for 5 consecutive days with 30 mg/kg of MPTP (Sigma). 21 days after the last MPTP injection animals were euthanized by phenobarbital overdose and brains were dissected. Fixation, processing, embedding, preparing of microtome sections and their immunostaining with antibody against TH (mouse monoclonal, clone TH-2 from Sigma, diluted 1:1000) were performed as in our previous studies [12, 24]. Stereological counting of neurons was carried out as previously described for single and double synuclein KO mice [26, 27].

**Immunoblotting.** SDS gel electrophoresis and Western blotting were performed as described in our previous publications [28, 29]. Primary antibodies against AADC (rabbit polyclonal, Synaptic Systems, 1:1000), TH mouse monoclonal, clone 2, (mouse monoclonal, clone TH-2, Sigma, 1:5000), synaptophysin (mouse monoclonal, clone 2, BD Transduction Laboratories, 1:25000), synapsin IIa (mouse monoclonal, clone 1, BD Transduction Laboratories, 1:5000), VAMP2 (mouse monoclonal, clone 69.1, Synaptic Systems, 1:1000), VMAT-2 (rabbit polyclonal, Sigma, 1:1000),  $\alpha$ -synuclein (mouse monoclonal, clone 42, BD Transduction Laboratories, 1:500),  $\beta$ -synuclein (mouse monoclonal, clone 8, BD Transduction Laboratories, 1:5000) and  $\gamma$ -synuclein (rabbit polyclonal, SK23 [24], 1:1000), secondary HRP-conjugated anti-mouse or anti-rabbit antibodies (GE Healthcare, 1:3000) and ECL + system (GE Healthcare) were used for detection of target proteins.

**Recombinant  $\beta$ -synuclein.** Expression of  $\beta$ -synuclein in *E.coli* and purification of the recombinant protein were performed as described previously [30]. For CLIP experiments a fragment encoding SAWSHPQFEK sequence tag was cloned in frame with the  $\beta$ -synuclein ORF in the pRK172 expression vector and the same expression and purification protocols were used to produce  $\beta$ -synuclein with C-terminal Strep-tag.

**Preparation of synaptosomal and synaptic vesicle fractions.** All procedures were carried out at 4°C. Dorsal striata dissected from 6 mice were homogenised in 1 ml of the 0.32 M sucrose; 5 mM HEPES, pH 7.4 with protease inhibitors (Complete Mini from Roche) using a glass homogenizer (if more striata were used all volumes were scaled up accordingly). Nuclei and cell debris were sedimented by centrifugation at 1,000 x g for 10 min and the supernatant (S1) was further centrifuged at 20,000 x g for 20 min to obtain cytosolic (S2, supernatant) and crude synaptosomes (P2, pellet) fractions. For synaptosomal dopamine uptake assay, the P2 pellet was resuspended by vortexing in 0.5 ml of synaptosomal uptake assay buffer (10 mM HEPES, pH 7.4; 5.6 mM glucose; 120 mM NaCl; 5 mM KCl; 1.2 mM CaCl<sub>2</sub>; 1.2 mM MgCl<sub>2</sub>; 1 mM ascorbic acid; 10  $\mu$ M pargyline). For isolation of synaptic vesicles, the P2 pellet was resuspended by vortexing in 0.5 ml of the 0.32 M sucrose, diluted with 2 ml of deionised H<sub>2</sub>O, homogenised in a glass/teflon homogeniser and left on ice for 10 min before adding 0.3 ml of 250 mM

HEPES, pH 7.4 and 0.3 ml of 1 M potassium tartrate. Synaptic membranes (P3) were separated by centrifugation at  $20,000 \times g$  for 20 min and the supernatant (S3) was centrifuged at  $120,000 \times g$  for 40 min to obtain the synaptic vesicle pellet (P4) that was resuspended in 1 ml of vesicular uptake assay buffer (25 mM HEPES, pH 7.4; 100 mM potassium tartrate; 0.1 mM EDTA; 0.05 mM EGTA; 1.7 mM ascorbic acid; 2 mM ATP) using a syringe fitted with the 25 gauge needle.

## Cross-linking with recombinant $\beta$ -synuclein, immunoprecipitation and mass spectrometry

Synaptic vesicle containing synaptosomal fraction (S3) prepared as above from 80 TKO striata, was divided into two equal aliquots and incubated with or without 50  $\mu$ g recombinant Strep-tagged  $\beta$ -synuclein for 5 min at 30°C. DTSSP (Pierce Biotechnology) was added to both samples to the final concentration of 5 mM. After 25 min of incubation at 30°C, crosslinking was terminated by the addition of Tris-HCl pH 7.5 to 50 mM and synaptic vesicles were spun down by centrifugation at  $120,000 \times g$  for 40 min at 2°C. Pellets were washed twice with 50 mM Tris/HCl pH 7.7; 150 mM NaCl. Synaptic vesicle membranes were lysed in solubilisation buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100 with Roche Complete Mini protease inhibitors) and lysates were incubated with 200  $\mu$ l of a 50% suspension of Tactin beads (Iba GmbH Lifesciences) in solubilisation buffer for 25 min at 4°C. Beads were washed four times with solubilisation buffer, and cross-linked proteins were eluted from  $\beta$ -synuclein (that remained attached to beads via its Strep-tag) by incubation of beads with 50  $\mu$ l of 25 mM Tris/Cl pH 7.5; 50 mM DTT for 40 min at 37°C with occasional mixing. Total protein content in two samples was normalised using a Quick Start Bradford protein assay (Bio-Rad). Proteins were processed for LC-MS analysis by in-solution digestion with reduction and alkylation using 10 mM DTT and 25 mM iodoacetamide respectively, followed by overnight trypsinisation (Promega). Post-digestion, samples were acidified with trifluoroacetic acid, de-salted using reversed-phase spin tips (Glygen Corp.) and dried using a vacuum centrifuge. Dried peptides were solubilised in trifluoroacetic acid and analysed in technical duplicates with separation using an Ultimate 3000 UHPLC system by loading onto a trap column (Acclaim Pepmap 100, 100  $\mu$ m  $\times$  2 cm, C18) at 8  $\mu$ L/min in 2% acetonitrile, 0.1% trifluoroacetic. Peptides were then eluted on-line to an analytical column (Acclaim Pepmap RSLC, 75  $\mu$ m  $\times$  50 cm, C18) and separated using a 120 min gradient. Eluted peptides were analysed by an LTQ Velos Orbitrap mass spectrometer as described previously [31] and Maxquant v1.5.3.8 software was used to align all raw data files. Variable modifications of oxidation (methionine), acetylation (protein N-terminal) and CAM thiopropanoyl (lysine and protein N-terminal) were permitted. The reduced DTSSP crosslinker gives a + 145.01975 Da addition to potential amine-reactive sites. Fixed modification of carbamidomethylation (cysteines) and up to 4 missed cleavages were permitted. Label-free quantitation (LFQ), 're-quantify', 'match between runs' and 'iBAQ' options were all selected. Raw data were searched against the UniprotKB *Mus musculus* database (downloaded November 2015 containing 79,900 protein sequences) and results were filtered to a 1% false discovery rate. Proteins were filtered for those containing at least two unique peptides and with undetectable abundance in control pulldowns.

**Synaptic vesicle dopamine uptake.** Protein concentration in the synaptic vesicle fraction was measured using Quick Start Bradford protein assay (Bio-Rad). For each uptake reaction 100  $\mu$ l of this suspension was mixed in a glass tube with 125  $\mu$ l of vesicular uptake assay buffer and preincubated at 30°C for 15 min. The uptake was initiated by adding 25  $\mu$ l of 0.1  $\mu$ M  $^3$ H-dopamine (34.6 Ci/mmol, Perkin Elmer) in the same buffer and after incubation at 30°C for 5 min terminated with 3 ml of ice-cold wash buffer (vesicular uptake assay buffer without ascorbic acid and ATP). To determine non-specific adsorption, control tubes were incubated on ice. To assess VMAT-2-independent uptake, control reactions were performed at 30°C in the presence of 10  $\mu$ M tetrabenazine. The level of tetrabenazine-resistant uptake was similar to background levels of uptake at 4°C and are therefore not shown. Reaction mixtures were filtered through GF/F filters. After 3 washes with the wash buffer, filters were air dried, placed in vials with SafeScint fluid and radioactivity measured using a Beckman LS6000 Scintillation Counter. For uptake kinetic studies, increasing amounts of cold dopamine (0–10  $\mu$ M) was added to the reactions. Obtained data were used to calculate  $V_{max}$  and  $K_m$  by nonlinear regression method (GraphPad Prism 4.0, GraphPad Software).

**Synaptosomal dopamine uptake.** Protein concentration in the crude synaptosome fraction was measured using Quick Start Bradford protein assay (Bio-Rad) and concentration of samples was adjusted to 2 mg protein per ml. For each reaction, 25  $\mu$ l of this suspension was mixed in a glass tube with 200  $\mu$ l of synaptosomal uptake assay buffer and preincubated at 30°C for 15 min. The uptake was initiated by adding 25  $\mu$ l of 9.8  $\mu$ M dopamine/0.2  $\mu$ M  $^3$ H-dopamine mixture in the same buffer and after 10 min incubation at 30°C terminated with 3 ml of ice-cold wash buffer (synaptosomal uptake assay buffer without ascorbic acid and pargyline). To determine non-specific adsorption, control tubes were incubated on ice. To assess DAT-independent uptake, parallel reactions were performed at 30°C in the presence of 10  $\mu$ M nomifensine. The level of nomifensine-resistant uptake was similar to background levels of uptake at 4°C and are therefore not shown. Reaction mixtures were filtered through GF/F filters. After 3 washes with the wash buffer, filters were air dried, placed in vials with SafeScint fluid and radioactivity measured using a Beckman LS6000 Scintillation Counter.

## **Stereotaxic delivery of lentivirus expressing $\beta$ -synuclein**

A fragment of human  $\beta$ -synuclein cDNA that included the coding region and the upstream Kozak sequence was cloned into the unique BamHI site of the pCCLsin.cPPT.PGK.eGFP.WPRE lentiviral expression vector.  $9 \times 10^5$  virus transducing units were stereotaxically injected at a rate of 1.0  $\mu$ l/min for 90 sec into the substantia nigra (AP = - 3.0, ML = - 1.2, DV = - 4.5, relative to Bregma) of 9-month old male TKO mice. The same amount of control virus particles prepared using “empty” vector DNA were injected into the contralateral substantia nigra. Dorsal striata from the ipsilateral and contralateral hemisphere were dissected 8 weeks after injections and used for comparing vesicular dopamine uptake.

**Statistical analysis.** All data are presented as means  $\pm$  s.e.m. Statistical analysis was performed using GraphPad Prism 8.2.1 (GraphPad Software).

## Results And Discussion

A widely accepted hypothesis suggests that intrinsic toxicity of  $\alpha$ -synuclein and  $\gamma$ -synuclein is a prerequisite for the demise of dopaminergic neurons in the SNpc of MPTP-treated mice. A logical extrapolation of this presumption would be that SNpc neurons of triple  $\alpha/\beta/\gamma$ -synuclein null mutant (TKO) mice should be at least as resistant to the toxic effect of MPTP as neurons lacking  $\alpha$ -synuclein and  $\gamma$ -synuclein. However, morphometric analysis of the number of TH-positive neurons in the SNpc of wild type (WT) and TKO mice treated with a subchronic MPTP regimen [10, 12], revealed the same degree of neuronal loss for both genotypes (Fig. 1). This result clearly demonstrated that none of the three synucleins are required for MPTP-induced death of dopaminergic neurons in the SNpc and suggests a protecting role of  $\beta$ -synuclein may in the single and double  $\alpha/\gamma$ -synuclein knockout scenarios.

The sensitivity of dopaminergic neurons to MPTP depends on the ability of its active toxic metabolite, MPP<sup>+</sup>, to enter the cell via the dopamine transporter (DAT)- and to avoid being sequestered in synaptic vesicles by a vesicular monoamine transporter (VMAT-2)-driven mechanism [32, 33]. Thus, dopaminergic neurons with a higher DAT/VMAT-2 ratio, such as SNpc neurons, are more sensitive to MPTP than neurons with a lower ratio, for instance ventral tegmental area (VTA) neurons. We have previously demonstrated that the function of DAT in synapses of SNpc neurons is not affected by the absence of synucleins [25, 34]. This was further confirmed by studies of *in vitro* dopamine uptake by synaptosomes isolated from the striatum of wild type and TKO mice that revealed no difference in this DAT-dependent uptake between the two animal groups (Additional file 1). These observations suggest that increased sensitivity of midbrain dopaminergic neurons of TKO mice to MPTP may be related to attenuated VMAT-2-dependent MPP<sup>+</sup> uptake by vesicles lacking these proteins. Consistent with the well-documented ability of synucleins to interact with various biological and synthetic membranes, particularly with high curvature vesicles [35–37], we showed that all three family members co-purify with striatal synaptic vesicles despite being predominantly cytosolic proteins in the striatum of wild type mice (Fig. 2a). To check whether vesicle-associated synucleins affect VMAT-2-dependent uptake we compared the ability of synaptic vesicles isolated from the striatum of 5-month old male TKO and wild type mice to take up <sup>3</sup>H-dopamine. It has previously been shown that the basal level of cytosolic dopamine in dopaminergic neurons is below 0.1  $\mu$ M, which is the detection limit of the intracellular patch electrochemistry technique [38]. Therefore, to imitate physiological conditions, uptake was measured with 10 nM dopamine in the reaction mixture. We observed a  $38.5 \pm 4.71\%$  reduction of tetrabenazine-sensitive (i.e. VMAT-2-dependent) dopamine uptake by synaptic vesicles isolated from the striatum of 5-month old male TKO mice when compared to synaptic vesicles from wild type mice of the same age and sex (Fig. 2b). A similar difference between genotypes was found in an independent set of experiments with synaptic vesicles isolated from ageing 14-month old male ( $39.2 \pm 1.29\%$  reduction) or 18-month old female mice ( $39.0 \pm 4.79\%$  reduction), suggesting that the effect is not age- or sex-dependent. Further analysis demonstrated decreased Vmax (9.97 vs 12.29) and Km (117.4 vs 196.1) values for this uptake in the absence of synucleins. These changes were not due to a decrease in either the number of synaptic

vesicles in dopaminergic synapses or levels of VMAT-2 in the striatum of TKO mice [25] and in synaptic vesicles isolated from the striatum of TKO mice (Fig. 2b, inset).

Reduced dopamine uptake efficiency of synuclein-depleted synaptic vesicles and the resultant decrease of the vesicular dopamine pool explains the attenuated response of TKO mice to amphetamine observed in our previous study [25]. Moreover, this functional deficiency should lead to the accumulation of free cytosolic dopamine within the presynaptic terminals because TH activity and dopamine re-uptake by DAT are unaffected in the striatum of TKO mice [25]. The increased striatal DOPAC/dopamine ratio suggests that to prevent toxicity of free cytosolic dopamine, these mice utilise a compensatory increase of dopamine degradation [25].

It is feasible that synucleins differ in their ability to potentiate vesicular uptake of dopamine and other, structurally similar, molecules e.g. MPP<sup>+</sup>. Thus, functional substitution for the loss of a family member(s) by another, more efficacious member might significantly affect both vesicular dopamine uptake and sensitivity of dopaminergic neurons to MPTP toxicity. The resistance of  $\alpha/\gamma$ -synuclein-deficient ([12] and Fig. 3a), but sensitivity of  $\alpha/\beta$ -,  $\beta/\gamma$ - and  $\alpha/\beta/\gamma$ -synuclein-deficient (Figs. 1 and 3a) neurons to this drug suggest that within the family only  $\beta$ -synuclein can efficiently potentiate vesicular uptake. In accordance with this assumption, reduced dopamine uptake, similar to the level of uptake by TKO vesicles, was observed for synaptic vesicles isolated from the striatum of  $\beta$ -synuclein but not  $\alpha$ -synuclein or  $\gamma$ -synuclein null mutant mice (Additional file 2). To confirm the pivotal role of  $\beta$ -synuclein in potentiating the efficiency of vesicular uptake, we first compared the sensitivity of SNpc dopaminergic neurons to subchronic MPTP administration in three groups of  $\beta$ -synuclein deficient mice and in three groups of mice lacking the other two synucleins singularly or in combination. In single  $\beta$ -synuclein and both double  $\beta/\gamma$ -synuclein and  $\alpha/\beta$ -synuclein null mutant mice these neurons were sensitive to the drug to approximately the same degree as neurons of wild type mice (Fig. 3a). In contrast, this protocol of MPTP administration did not cause loss of dopaminergic neurons in the SNpc of  $\alpha$ -synuclein,  $\gamma$ -synuclein and  $\alpha/\gamma$ -synuclein null mutant mice (Fig. 3a), consistent with previous observations [10, 12].

Next, we attempted to restore  $\beta$ -synuclein expression in midbrain neurons of TKO mice using *in vivo* lentiviral delivery of an expression construct. Stereotaxic injection of viral particles into the SNpc region resulted in  $\beta$ -synuclein expression and its transport via the nigrostriatal tract (Additional file 3) to the dorsal striatum where the protein could be detected by Western blotting (Fig. 3b). Vesicular dopamine uptake was significantly higher in vesicles isolated from the ipsilateral than from contralateral striatum (Fig. 3c), suggesting that  $\beta$ -synuclein potentiates this process. To assess whether  $\beta$ -synuclein directly affects vesicular dopamine uptake, we performed the uptake reaction by TKO mouse striata-derived vesicles in the presence of purified recombinant  $\beta$ -synuclein. Preincubation of isolated vesicles (i.e. from P4 fraction) with the recombinant protein before adding <sup>3</sup>H-dopamine did not affect the uptake (Fig. 4a). However, when the vesicle-containing S3 supernatant was incubated with recombinant  $\beta$ -synuclein followed by spinning down synaptic vesicles (i.e. obtaining P4 fraction from  $\beta$ -synuclein preincubated S3 supernatant) and using them to measure dopamine uptake, a statistically significant increase was observed compared to the uptake by vesicles from a mock-incubated S3 supernatant (Fig. 4b). This

effect was not observed for recombinant  $\alpha$ - or  $\gamma$ -synuclein, suggesting that the presence of  $\beta$ -synuclein alongside one or more proteins contained within the S3 supernatant are required for efficient synaptic vesicle dopamine uptake.

In an attempt to provide an insight into how supplementing with exogenous  $\beta$ -synuclein improves dopamine uptake by striatal synaptic vesicles from synuclein-free mice, we assessed the proteome of  $\beta$ -synuclein-bound vesicles by a combination of the crosslink immunoprecipitation (CLIP) and mass spectrometry techniques. Synaptic vesicle-containing S3 supernatant obtained from the striata of TKO mice was preincubated with Strep-tagged human  $\beta$ -synuclein followed by incubation with a cleavable crosslinking agent (DTSSP) and subsequent high-speed centrifugation to spin down synaptic vesicles (for details see Methods). Synaptic vesicles isolated from the same S3 supernatant sample preincubated without  $\beta$ -synuclein but similarly crosslinked were used as a non-specific binding control. Membranes of both control and  $\beta$ -synuclein-bound vesicles were lysed in a non-ionic detergent solution and protein complexes containing  $\beta$ -synuclein were pulled down using Tactin magnetic beads that have high affinity for Strep-tagged proteins. Proteins incorporated in these complexes were eluted from the beads by breaking crosslinking bonds with dithiothreitol, leaving Strep-tagged  $\beta$ -synuclein attached to beads. Protein composition of the resulting CLIP proteome was analysed by mass spectrometry (MS). Proteins identified by the presence of at least two unique peptides in the  $\beta$ -synuclein-CLIP and undetectable in the control CLIP were further filtered using the CRAPome tool ([crapome.org](http://crapome.org)) to remove common non-specific proteins often present in MS immunoprecipitation experiments.

Not surprisingly, around half of the 224 proteins included in the final analysis (thereafter,  $\beta$ -synuclein-CLIP proteome) were proteins with predominant localisation to, or function at, neuronal membranous structures and synaptic vesicles in particular (Fig. 5a). Presynapse (GO:0098793) appeared to be the top Cellular Component with a false discovery rate (FDR) of  $3.44e-27$ . Mechanistically, proteins involved in presynaptic functions were also vastly overrepresented in this  $\beta$ -synuclein-CLIP proteome. Gene Ontology analysis revealed Synaptic Vesicle Cycle (GO:0099504) and Neurotransmitter Transport (GO:0006836) as the most represented Biological Processes with FDR of  $4.91e-12$  and  $3.35e-11$ , respectively. Similarly, Synaptic Vesicle Cycle (MMU4721; FDR  $4.85e-12$ ) appeared to have the highest representation between KEGG Pathways, as well as Transmission Across Chemical Synapse (MMU112315) and Neurotransmitter Release Cycle (MMU112310) – between Reactome Pathways (FDR  $6.88e-12$  and  $5.71e-12$ , respectively).

The improved dopamine uptake after addition of exogenous  $\beta$ -synuclein was observed only when synaptic vesicles were preincubated with  $\beta$ -synuclein in the presence of cytosolic factors but not after these factors were removed by high-speed centrifugation at the final step of vesicle purification (Fig. 4). A logical interpretation of these data is that upon interaction with the synaptic vesicle membrane via its N-terminal lipid-binding domain,  $\beta$ -synuclein attracts certain proteins, principally residing in the cytosol of dopaminergic presynaptic terminals, to the outer surface of these vesicles, and that the resulting molecular complexes improve the efficiency of the VMAT-2 transporter. The absence of VMAT-2 itself between proteins included in the  $\beta$ -synuclein-CLIP proteome provides additional evidence that its direct interaction with  $\beta$ -synuclein is not a plausible reason for this improved efficiency.

A number of proteins, which are not intrinsic constituents of synaptic vesicles at any stage of their presynaptic cycle, have been previously detected in brain synaptic vesicle fractions. It has been suggested that some of these proteins can affect vesicular uptake of dopamine, for example by increasing local availability of ATP in the case of glycolytic enzymes [39] or direct modulation of VMAT-2 activity [40–42]. Amongst the various, principally cytosolic, proteins identified in our analysis of the  $\beta$ -synuclein-CLIP proteome, the most probable candidates for augmentation of synaptic dopamine uptake are TH and AADC (also known as DOPA decarboxylase, DDC). Both proteins were detected in the  $\beta$ -synuclein-CLIP but not in the control CLIP samples by Western blot, confirming the findings of the MS analysis (Fig. 5b). These proteins are not only involved in presynaptic dopamine production but can also form a transient complex with VMAT-2, which creates spatial coupling of dopamine synthesis and loading into synaptic vesicles [43, 44]. Although this coupling cannot explain the improved  $^3\text{H}$ -dopamine uptake in the *in vitro* assay nor improved synaptic vesicle uptake of MPP<sup>+</sup> in the striatum of MPTP-treated mice, the  $\beta$ -synuclein-triggered formation of the TH/AADC/VMAT-2 complex might have an allosteric effect on transporter function.

Although the TH/AADC/VMAT-2 complex is the most obvious scenario, a contribution from other proteins interacting with the vesicle-bound  $\beta$ -synuclein cannot be excluded and it is feasible that improved function of VMAT-2 in the presence of  $\beta$ -synuclein is the result of a cumulative effect of several multiprotein interactions rather than of any one particular complex. Indeed, our data suggest that  $\beta$ -synuclein, via interaction with a number of vesicular and cytosolic proteins, can potentiate formation of various molecular complexes on the surface of synaptic vesicles. This is similar to  $\alpha$ -synuclein, which is known to interact with a variety of proteins and function as a chaperone or scaffold for the assembly of multiprotein complexes on the surface of synaptic vesicles, implicating  $\alpha$ -synuclein in a number of molecular processes at several stages of the synaptic vesicle cycle [1–3, 5, 45–47]. However, these do not include potentiation of vesicular uptake and therefore,  $\alpha$ -synuclein, as well as  $\gamma$ -synuclein, are unable to compensate for the loss of this particular function of  $\beta$ -synuclein. All three members of the synuclein family can interact with phospholipids at the outer surface of synaptic vesicles via their conserved N-terminal repeat domain, but the number of functional multiprotein complexes each member can form on a given vesicle is limited. It is feasible that in the presence of all three synucleins (i.e. in the synaptic terminals of dopamine neurons of WT animals), this competition for space results in a limited number of  $\beta$ -synuclein-triggered complexes formed at the vesicle surface. This is not sufficient to potentiate VMAT-2-dependent uptake to the level that can efficiently sequester MPP<sup>+</sup> in synaptic vesicles and thus, prevent its toxic effect. However, in the absence of one or two other family members,  $\beta$ -synuclein occupies vacant sites on the vesicle surface, triggering the formation of more complexes that efficiently potentiate vesicular uptake of dopamine and MPP<sup>+</sup>, making dopaminergic neurons more robust to MPTP-induced toxicity.

## Conclusions

Our experimental data provide evidence that of the three members of the synuclein family, only  $\beta$ -synuclein can potentiate VMAT-2-dependent uptake of dopamine (and structurally similar molecules) by synaptic vesicles. The increased presence and activity of  $\beta$ -synuclein at the synaptic vesicles, and not the absence of other synucleins per se, explains the decreased sensitivity to MPTP toxicity of SNpc dopaminergic neurons in mice lacking  $\alpha$ -synuclein and/or  $\gamma$ -synuclein.

## Abbreviations

### **SNpc**

substantia nigra pars compacta

### **MPTP**

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

### **MPP+**

1-methyl-4-phenylpyridinium

### **TH**

tyrosine hydroxylase

### **AADC**

aromatic L-amino acid decarboxylase

### **TKO**

triple  $\alpha/\beta/\gamma$ -synuclein null mutant mice

### **WT**

wild type mice

### **DAT**

dopamine transporter

### **VMAT-2**

vesicular monoamine transporter 2

### **VTA**

ventral tegmental area

### **CLIP**

crosslink immunoprecipitation

### **DTSSP**

3,3'-Dithiobis(sulfosuccinimidylpropionate)

### **MS**

mass spectrometry

## Declarations

## Ethics approval and consent to participate

Animal studies were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act (1986).

### **Consent for publication**

Not applicable

### **Availability of data and materials**

All data generated or analysed during this study are included in this published article and its supplementary information files or available from the corresponding author on reasonable request.

### **Competing interests**

Authors declare no competing interests.

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## **Authors' contributions**

NN and VLB conceived and supervised the study. NN, SJM, OMP, NCR, KC, AM, HK and VLB conducted the experiments. NN, DJW and VLB analysed data. VLB wrote the manuscript with input of all authors. All authors read and approved the final version of the manuscript.

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## Figures

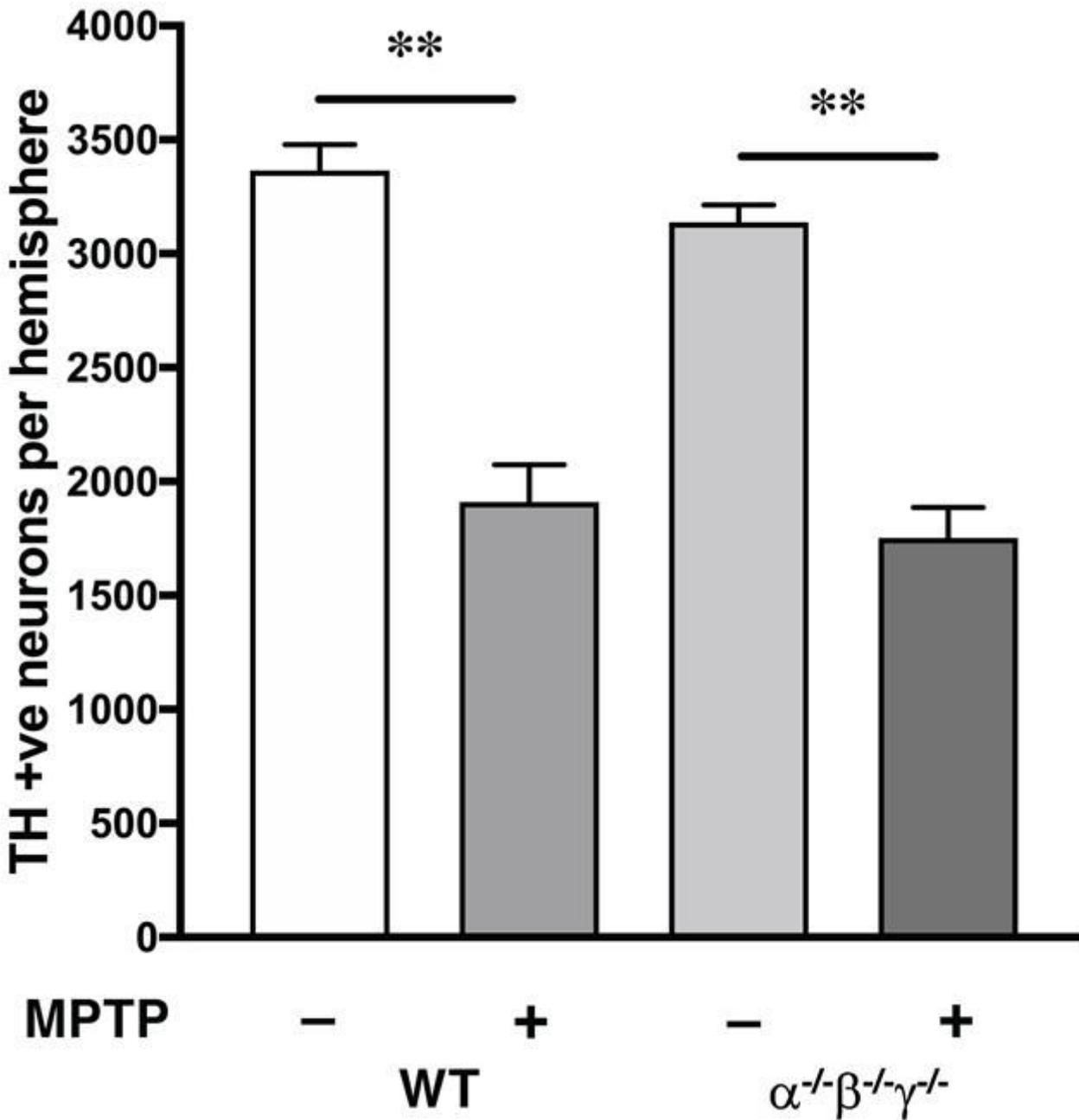
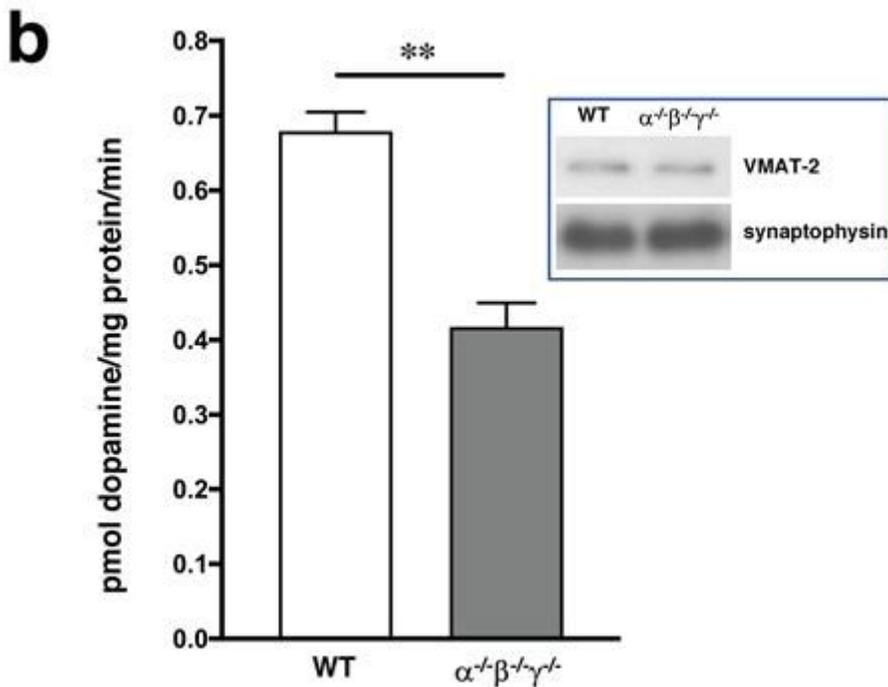
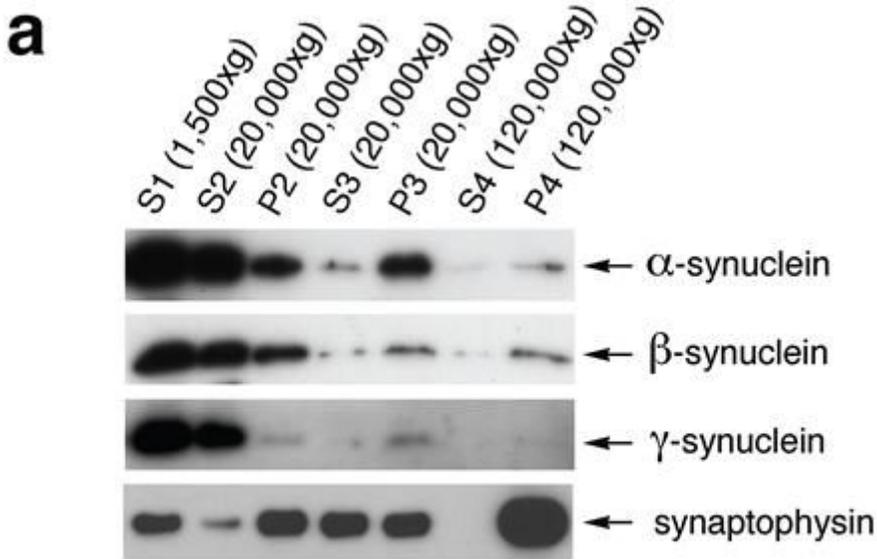


Figure 1

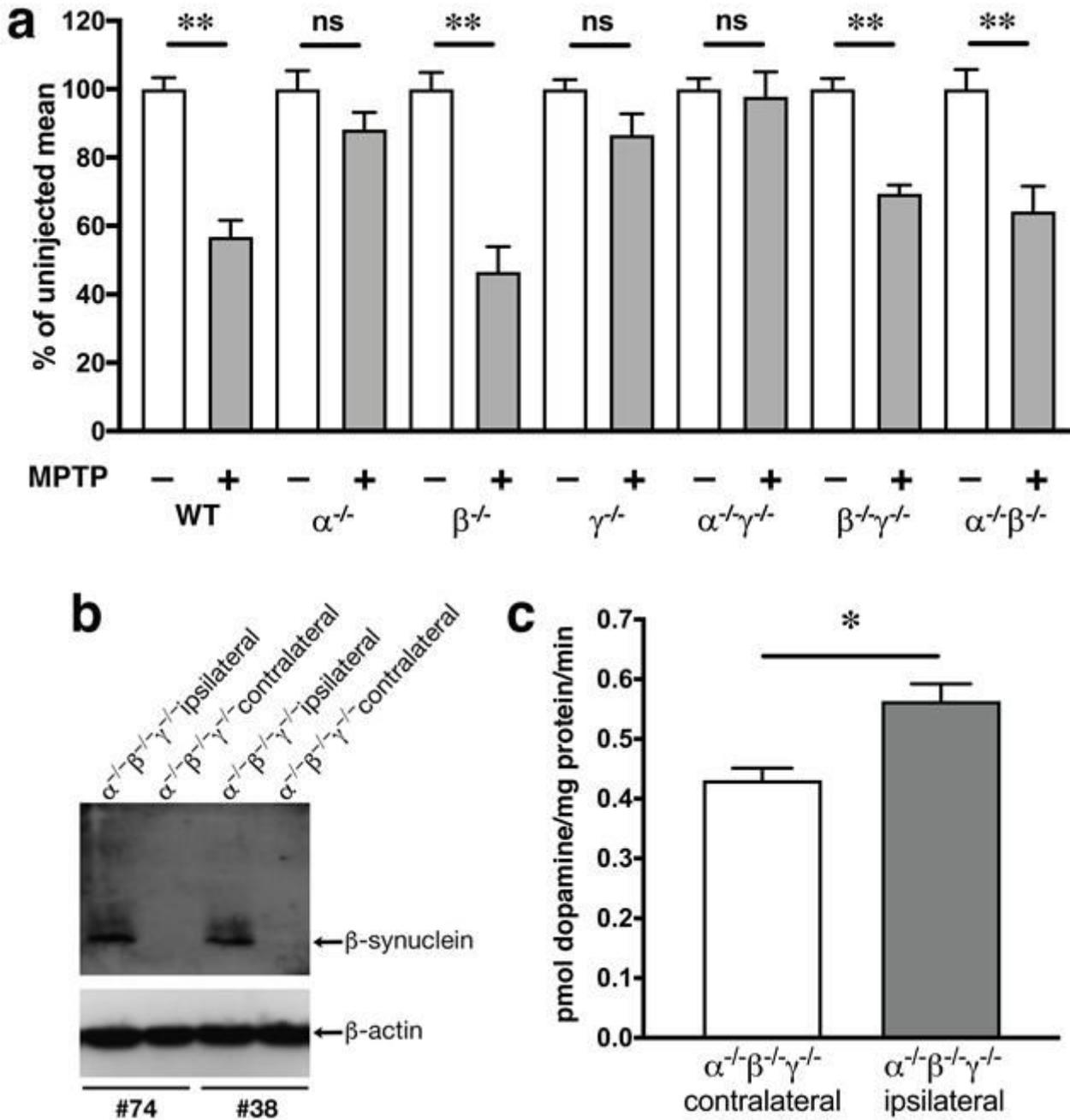
Sensitivity of SNpc dopaminergic neurons of wild type and triple synuclein deficient mice to subchronic MPTP treatment. Bar chart shows means $\pm$ s.e.m. of total number of TH-positive neurons in the SNpc of wild type (WT) and triple synuclein null mutant ( $\alpha^{-/-}\beta^{-/-}\gamma^{-/-}$ ) 5-month old male mice. Significant loss of neurons was observed for both genotypes following MPTP treatment (\*\* $p < 0.0001$ ; one-way ANOVA with Sidak's multiple comparison test;  $n = 14-20$  per genotype/treatment). No difference in the degree of neuronal loss was found between genotypes ( $43.2 \pm 4.87\%$  for WT mice and  $44.1 \pm 4.26\%$  for TKO mice).



**Figure 2**

Synucleins are associated with striatal synaptic vesicles and affect dopamine uptake. (a) Western blot analysis of proteins in fractions of wild type mouse striatum. Post-nuclear supernatant (S1) was centrifuged at 20,000 x g, synaptosomes in the resulting pellet (P2) were lysed by homogenization in hypotonic buffer and again centrifuged at 20,000 x g to separate heavy synaptic membrane fraction (pellet P3) from cytosolic fraction containing small synaptic vesicle (supernatant S3). Synaptic vesicle fraction (P4) was obtained from the supernatant S3 by centrifugation at 120,000 x g. (b) Bar chart shows means $\pm$ s.e.m. of dopamine uptake by synaptic vesicles isolated from the striatum of 5-month old male wild type (WT) and TKO ( $\alpha^{-/-}\beta^{-/-}\gamma^{-/-}$ ) mice (\*\*p<0.0001; unpaired t-test, n=15 for both genotypes from 3

independent experiments). A representative Western blot in the inset illustrates equal amounts of VMAT-2 in vesicular fractions isolated from striata of wild type and TKO mice.



**Figure 3**

$\beta$ -synuclein is pivotal for acquiring MPTP resistance by dopaminergic neurons lacking other synucleins and potentiates vesicular dopamine uptake in vivo. (a) Bar charts show means $\pm$ s.e.m. of the number of TH-positive neurons in the SNpc of wild type (WT), single ( $\alpha^{-/-}$ ,  $\beta^{-/-}$ ,  $\gamma^{-/-}$ ) and double ( $\alpha^{-/-}\beta^{-/-}$ ,  $\alpha^{-/-}\gamma^{-/-}$ ,  $\beta^{-/-}\gamma^{-/-}$ ) synuclein null mutant mice following subchronic administration of MPTP (n=8-14 per genotype) expressed as percent of the number of neurons in control vehicle-injected animals of the same genotype (\*\*p<0.0001, one-way ANOVA with pairwise uncorrected Fisher's LSD test). (b, c) Analysis of dopamine uptake by synaptic vesicles from striata of TKO ( $\alpha^{-/-}\beta^{-/-}\gamma^{-/-}$ ) mice stereotactically injected into SNpc

region with  $\alpha$ -synuclein-expressing lentiviral vector particles. (b) Western blot confirms the presence of  $\alpha$ -synuclein in the ipsilateral but not contralateral striatum of two injected TKO mice. (c) Bar chart shows means $\pm$ s.e.m. of dopamine uptake by synaptic vesicles isolated from the ipsilateral and contralateral striatum of 6 TKO animals (\* $p$ <0.0286, Mann-Whitney U-test).

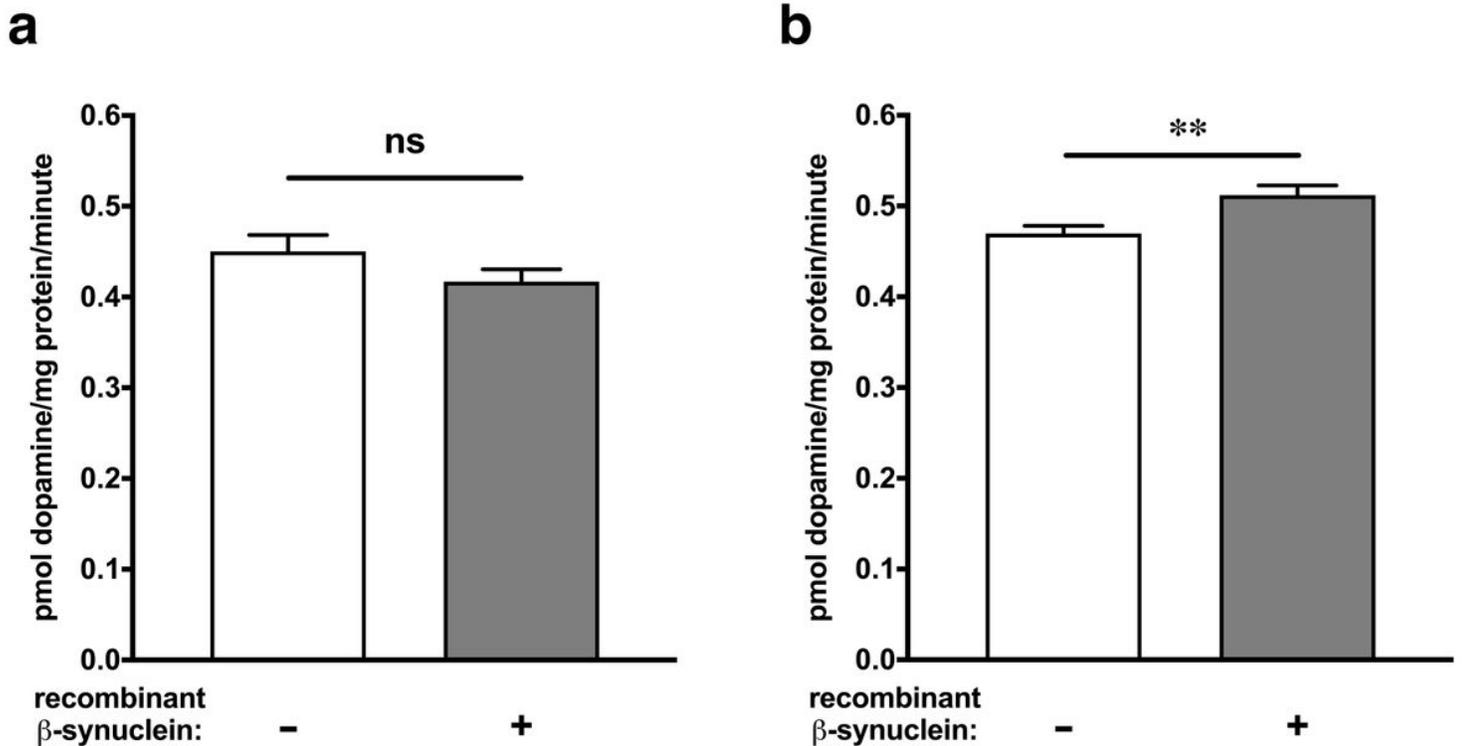
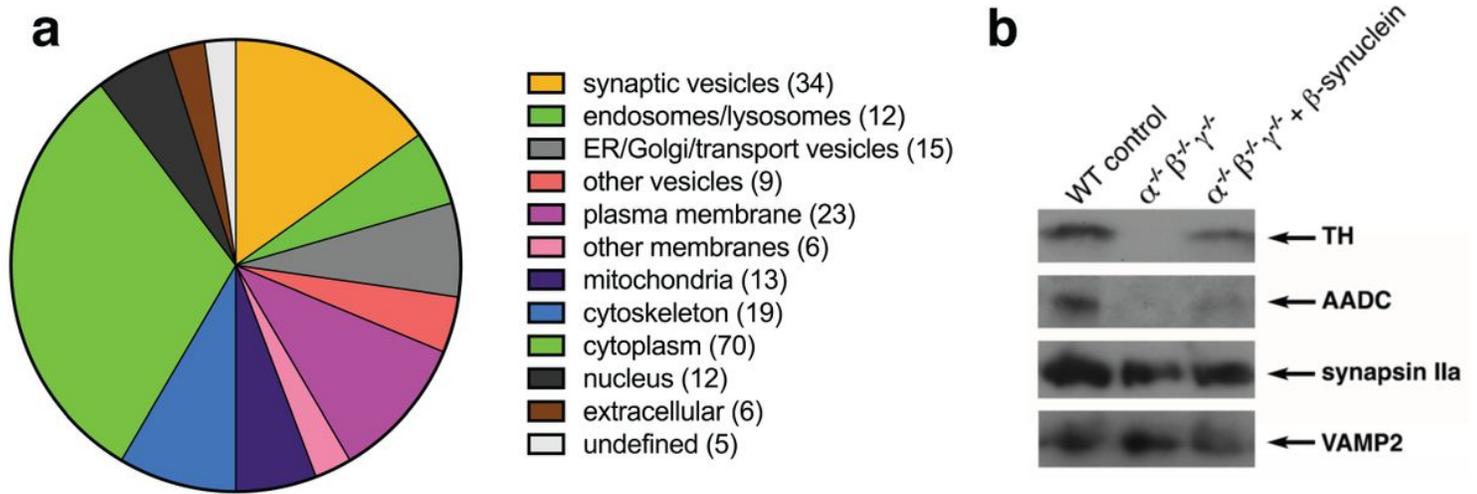


Figure 4

$\alpha$ -synuclein potentiates vesicular dopamine uptake in vitro. Effect of purified recombinant  $\alpha$ -synuclein on dopamine uptake by synaptic vesicles isolated from TKO mice. (a) Bar chart shows means $\pm$ s.e.m. of dopamine uptake in the presence of purified recombinant  $\alpha$ -synuclein added to the uptake reaction mixture to a final concentration of 20  $\mu$ g/ml ( $n$ =7 for each condition from two independent experiments, ns,  $p$ =0.1807, Mann-Whitney U-test). (b) Bar chart shows means $\pm$ s.e.m. of dopamine uptake after preincubation of S3 supernatant with or without  $\alpha$ -synuclein (20  $\mu$ g/ml) followed by high-speed sedimentation of vesicles before use in the uptake reaction ( $n$ =13 for each conditions from three independent experiments, \*\* $p$ <0.0035, Mann-Whitney U-test).



**Figure 5**

Proteins detected in the  $\alpha$ -synuclein-CLIP proteome. (a) Pie-chart shows the distribution of  $\alpha$ -synuclein-CLIP proteome proteins between cell compartments associated with their main compartmentalisation and/or function(s). The full list of these proteins can be found in Additional file 4. (b) Western blot analysis of proteins with principally vesicular (VAMP2 and synapsin IIa) or predominantly cytosolic (AADC and TH) localisation. Equal amounts of total proteins eluted from Tactin beads (see Methods section for details) were loaded on each lane. Proteins were obtained from striatal synaptic vesicles prepared by high-speed centrifugation (P4 fraction) of S3 fraction containing synaptic vesicles and cytosolic proteins of TKO mice. Prior to centrifugation S3 fraction proteins were cross-linked in the presence ( $\alpha$ -synuclein) or absence (control) of Strep-tagged  $\alpha$ -synuclein. Proteins of purified synaptic vesicles from the striata of wild type mice are loaded to the 'WT control' lane.

## Supplementary Files

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