

Induction of α -synuclein aggregate formation by CSF exosomes from patients with Parkinson's disease and dementia with Lewy bodies

Anne Stuendl,^{1,2} Marcel Kunadt,^{1,2,3} Niels Kruse,⁴ Claudia Bartels,¹ Wiebke Moebius,² Karin M. Danzer,⁵ Brit Mollenhauer^{4,6,*} and Anja Schneider^{1,2,3,7,*}

*These authors contributed equally to this work.

Extracellular α -synuclein has been proposed as a crucial mechanism for induction of pathological aggregate formation in previously healthy cells. *In vitro*, extracellular α -synuclein is partially associated with exosomal vesicles. Recently, we have provided evidence that exosomal α -synuclein is present in the central nervous system *in vivo*. We hypothesized that exosomal α -synuclein species from patients with α -synuclein related neurodegeneration serve as carriers for interneuronal disease transmission. We isolated exosomes from cerebrospinal fluid from patients with Parkinson's disease, dementia with Lewy bodies, progressive supranuclear palsy as a non- α -synuclein related disorder that clinically overlaps with Parkinson's disease, and neurological controls. Cerebrospinal fluid exosome numbers, α -synuclein protein content of cerebrospinal fluid exosomes and their potential to induce oligomerization of α -synuclein were analysed. The quantification of cerebrospinal fluid exosomal α -synuclein showed distinct differences between patients with Parkinson's disease and dementia with Lewy bodies. In addition, exosomal α -synuclein levels correlated with the severity of cognitive impairment in cross-sectional samples from patients with dementia with Lewy bodies. Importantly, cerebrospinal fluid exosomes derived from Parkinson's disease and dementia with Lewy bodies induce oligomerization of α -synuclein in a reporter cell line in a dose-dependent manner. Our data suggest that cerebrospinal fluid exosomes from patients with Parkinson's disease and dementia with Lewy bodies contain a pathogenic species of α -synuclein, which could initiate oligomerization of soluble α -synuclein in target cells and confer disease pathology.

- 1 Department of Psychiatry and Psychotherapy, University Medicine Göttingen, Von-Siebold-Str. 5, 37075 Göttingen, Germany
- 2 Max-Planck-Institute for Experimental Medicine, Hermann-Rein-Str. 3, 37075 Göttingen, Germany
- 3 Cluster of Excellence 'Nanoscale Microscopy and Molecular Physiology of the Brain' (CNMPB), Göttingen, Germany
- 4 Department of Neuropathology, University Medicine Göttingen, Robert-Koch-Str. 40, 37075 Göttingen, Germany
- 5 Department of Neurology, Ulm University, Albert-Einstein-Allee 11, 89081 Ulm, Germany
- 6 Paracelsus-Elena Klinik, Klinikstr. 16, 34128 Kassel, Germany
- 7 German Centre for Neurodegenerative Diseases (DZNE), Göttingen, Von-Siebold-Str. 5, 37075 Göttingen, Germany

Correspondence to: Anja Schneider,
DZNE Göttingen, c/o MPI-EM,
Hermann-Rein-Str. 3, 37075 Göttingen,
Germany.
E-mail: anja.schneider@dzne.de

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Abbreviation: ROC = receiver operating curve

Introduction

Parkinson's disease is a neurodegenerative disorder with motor symptoms that affects ~1% of the population over 65 years of age (Pringsheim *et al.*, 2014). It is characterized by the progressive loss of dopaminergic neurons in the substantia nigra and pathological aggregation of the intrinsically disordered protein α -synuclein into Lewy bodies (Spillantini *et al.*, 1998; Braak *et al.*, 2003a). Parkinson's disease and other neurodegenerative diseases with Lewy body pathology, such as Parkinson's disease dementia and dementia with Lewy bodies, are considered as a continuum of a disease spectrum termed Lewy body disorders (Lippa *et al.*, 2007). Clinically, dementia with Lewy bodies is defined by dementia, visual hallucinations and fluctuating attention within 12 months of Parkinson syndrome onset. In contrast, the onset of dementia later than 12 months after initial motor symptoms specifies Parkinson's disease dementia (McKeith *et al.*, 2005b).

The accurate distinction between Parkinson's disease, dementia with Lewy bodies and other non- α -synuclein variants with Parkinson syndrome is challenging due to an overlap of clinical symptoms and neuropathological changes. Several comprehensive studies have identified imaging and fluid biomarkers, including dopamine transporter scans, serum peptide markers, and CSF α -synuclein (Mollenhauer and Schlossmacher, 2010; Suzuki *et al.*, 2015).

Although α -synuclein does not contain a sorting signal for extracellular release, soluble and aggregated α -synuclein was detected in tissue culture medium and body fluids, such as brain interstitial fluid, plasma and CSF (El-Agnaf *et al.*, 2003, 2006; Lee *et al.*, 2005, 2014; Tokuda *et al.*, 2010; Emmanouilidou *et al.*, 2011; Hansson *et al.*, 2014). Extracellular α -synuclein was subsequently studied as a potential diagnostic biomarker, especially in the CSF, where the majority of α -synuclein is derived from the CNS rather than from peripheral blood (Mollenhauer *et al.*, 2012). Most studies have shown a reduction of CSF α -synuclein levels in Parkinson's disease and dementia with Lewy bodies (Tokuda *et al.*, 2006; Hong *et al.*, 2010; Mollenhauer *et al.*, 2011); however, conflicting results with either no differences compared to controls or even increased levels of extracellular α -synuclein were reported (Noguchi-Shinohara *et al.*, 2009; Ohrfelt *et al.*, 2009; Reesink *et al.*, 2010). In addition, the sensitivity and specificity of CSF α -synuclein to distinguish Parkinson's disease or dementia with Lewy bodies from non- α -synuclein related Parkinson syndrome and other neurological controls are low and to date, α -synuclein has not been approved as a biomarker for clinical applications (Gao *et al.*, 2015).

Extracellular α -synuclein was recently implied in the prion-like transmission of pathological α -synuclein from diseased to healthy neurons where misfolded α -synuclein might serve as a seed to induce the aggregation of soluble α -synuclein. This concept of interneuronal spreading is

based on Braak's observation that α -synuclein pathology in the brain propagates caudo-rostrally along axonal projections (Braak *et al.*, 2003b). This hypothesis was further supported by the detection of α -synuclein aggregates in transplanted embryonic neurons in Parkinson's disease patients' brains (Li *et al.*, 2008; Kordower and Brundin, 2009b), the observation of neuron-to-neuron transfer of α -synuclein in mouse brain (Desplats *et al.*, 2009), internalization of exogenous α -synuclein fibrils and induction of neuronal α -synuclein aggregation *in vitro* and *in vivo* (Emmanouilidou *et al.*, 2010; Nonaka *et al.*, 2010; Volpicelli-Daley *et al.*, 2011; Danzer *et al.*, 2012a; Luk *et al.*, 2012a, b; Mougenot *et al.*, 2012). Recently, exosomes have been implicated in the dissemination of misfolded proteins in a variety of neurodegenerative disorders, including Parkinson's disease (Bellingham *et al.*, 2012; Schneider and Simons, 2013). Exosomes are extracellular vesicles of 40–120 nm diameters that are released from various cells including neurons (Faure *et al.*, 2006). Exosomal release and transport of α -synuclein was reported by us and several other groups *in vitro* and *in vivo* (Emmanouilidou *et al.*, 2010; Danzer *et al.*, 2012b), followed by intracellular uptake and the induction of toxicity and cell death.

The aim of our study was to characterize exosomal α -synuclein in CSF from patients with α -synuclein-related neurodegeneration (Parkinson's disease, dementia with Lewy bodies), patients with Parkinson syndrome without α -synuclein related degeneration (progressive supranuclear palsy) and neurological controls.

Materials and methods

Reagents

Primary antibodies were anti-Flotillin-2 (BD Biosciences), anti-Calnexin (Sigma-Aldrich), anti- α -synuclein (Invitrogen), anti- α -synuclein clone 42/ α -synuclein (BD Transduction Laboratories). MJF-1 clone 12.1 was kindly provided by Dr Liyu Wu, Epitomics, Burlingame, USA. Secondary antibodies against mouse, rabbit and human IgG were obtained from Dako, Dianova and Invitrogen.

Plasmids

α -Synuclein hGLuc1 (S1) and α -synuclein hGLuc2 (S2) constructs were described previously (Outeiro *et al.*, 2008).

Cerebrospinal fluid collection

CSF specimens were collected at the Paracelsus-Elena Klinik in Kassel and the Göttingen University Memory Clinic, Department of Psychiatry, Germany [Institutional review board (IRB) approval by the local board of Hessen, Germany, IRB 09/07/04 and 26/07/02 and by the Ethics committee of the University Medical Centre, Göttingen, IRB 02/05/09] between 2009 and 2012 by lumbar puncture between 9 and 12 am. Specimens were collected in polypropylene tubes

and centrifuged at 2000g for 10 min at room temperature, aliquoted and frozen at -80°C within 30 min of completion of the procedure (Mollenhauer *et al.*, 2011). Samples with erythrocyte counts $>50/\text{mm}^3$ were excluded. All samples were obtained in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

The cross-sectional Kassel cohort

The cohort is described in detail in Mollenhauer *et al.* (2011). All patients with Parkinson's disease fulfilled UK Brain Bank criteria. Patients with dementia with Lewy body were diagnosed according to McKeith consensus criteria (McKeith *et al.*, 2005a). Patients with progressive supranuclear palsy fulfilled the National Institute of Neurological Disorders and Stroke-Society for Progressive Supranuclear Palsy (NINDS-SPSP) criteria for possible or probable disease (Litvan *et al.*, 1996). None of the neurological control patients suffered from Parkinson's disease or dementia.

The DeNoPa cohort

Recruitment and diagnostic assessments of the longitudinal *de novo* Parkinson (DeNoPa) cohort have been published (Mollenhauer *et al.*, 2013a). In brief, 159 patients had been diagnosed with Parkinson's disease according to the UK Brain Bank criteria and were drug naïve at enrolment. One hundred and ten neurologically healthy controls matched according to age, gender and educational level without family history of Parkinson's disease were enrolled in parallel. Clinical diagnosis at baseline was confirmed after a 2-year follow-up. Ninety-four per cent of subjects returned for follow-up investigations after 24 months; in 84% the clinical diagnosis was confirmed by follow-up. CSF was gained by lumbar puncture in the morning (between 8 and 9 am with the patients fastened in a sitting position). CSF (14 ml) was drawn in polypropylene tubes and processed by centrifugation and freezing within 30 min after lumbar puncture according to the published standard operating procedure (Mollenhauer *et al.*, 2010). Baseline CSF samples of 76 patients with Parkinson's disease and 58 healthy controls who had finalized the first follow-up with normal routine CSF parameters (i.e. haemoglobin, protein content), were randomly selected from this cohort.

Purification of exosomes from cerebrospinal fluid

Exosomes were isolated as described previously (Strauss *et al.*, 2010). CSF was thawed on ice and subjected to subsequent centrifugation steps at 4°C : 3500g for 10 min, two times 4500g for 10 min, 10 000g for 30 min and 100 000g for 60 min (Fig. 1A). The 100 000g pellet was washed once with phosphate-buffered saline (PBS) at 100 000g for 60 min before resuspension. Exosomes from human CSF for western blot analysis were prepared from 2.5 ml CSF and resuspended in Lämmli buffer. Exosomes for electrochemiluminescence measurements were dissolved in 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 5 mM EDTA, 50 mM Tris-HCl, pH 8.0 lysis buffer. Total CSF was diluted in $2\times$ lysis buffer.

Nanoparticle tracking analysis

Exosomes in CSF or in the ultracentrifugation pellet were analysed by nanoparticle tracking analysis with a NanoSight LM10 instrument and a LM14 viewing unit equipped with a 532 nm laser (NanoSight Ltd). Total CSF samples were diluted 1:4 to 1:40 in PBS (Gibco) to a final volume of 400 μl prior to analysis. Pellets from 100 000g centrifugation derived from 0.5 ml total CSF were resuspended in 50 μl PBS and diluted 1:40 in PBS. Samples were recorded in triplicates (DeNoPa cohort) or six times (Kassel cohort) for 30 s. Particle numbers were then analysed with the Nanoparticle Tracking Analysis (NTA) 2.3 software.

Electrochemiluminescence assay for α -synuclein quantification

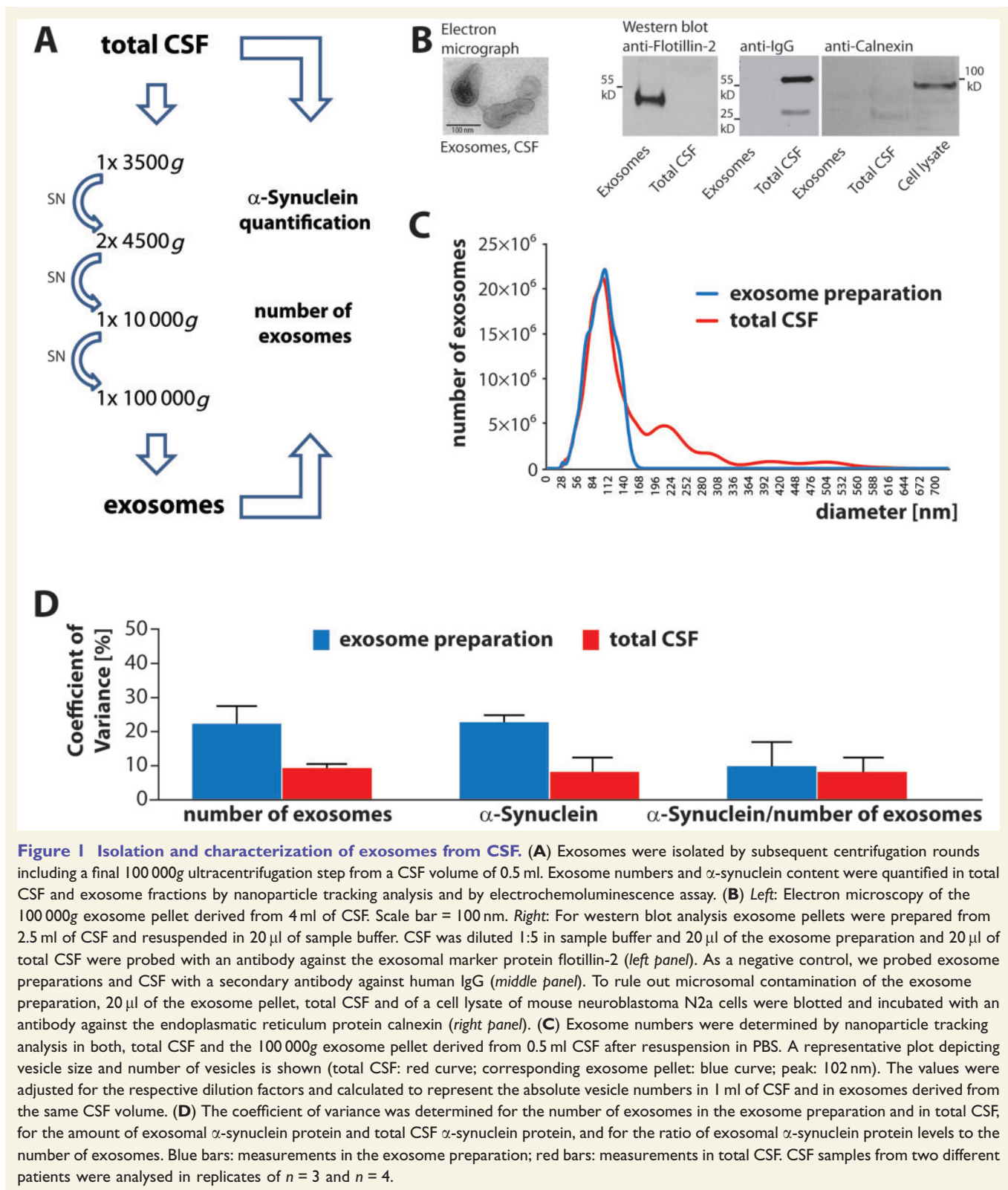
Quantification of α -synuclein protein in CSF and in exosomes prepared from CSF was performed as described (Kruse *et al.*, 2012). Standard 96-well plates (Meso Scale Discovery) were coated overnight at 4°C with 3 $\mu\text{g}/\text{ml}$ antibody MJF-1 clone 12.1 in PBS. After washing three times with 150 μl PBS + 0.05% Tween-20, plates were blocked with 150 μl 1% bovine serum albumin (Meso Scale Discovery) for 1 h under shaking at 300 rpm at room temperature. After washing three times with recombinant α -synuclein standards (kindly provided by Dr Omar El-Agnaf, Hamad Bin Khalifa University, Doha, Qatar), samples were applied in duplicate for 1 h at room temperature and under shaking at 700 rpm. After washing three times, Sulfo-TAG-labelled anti- α -synuclein clone 42 (1 $\mu\text{g}/\text{ml}$) was added for 1 h at room temperature and 700 rpm shaking. After washing three times $2\times$ Read Buffer T (Meso Scale DiscoveryTM) was applied to each well and plates were measured in a Sector Imager 6000 (Meso Scale DiscoveryTM). Data analysis was performed with the MSD Discovery Workbench 3.0 Data Analysis Toolbox.

Quantification of CSF tau levels

The concentrations of total CSF tau were determined by a commercially available validated enzyme-linked immunosorbent assay (ELISA) (Innogenetics) according to the manufacturer's instructions.

Cell culture and transfections

For oligomerization induction experiments, human H4 neuroglioma cells (Cell Lines Service) were maintained in Opti-MEMTM medium supplemented with 10% foetal bovine serum (both from Life Technologies) and incubated at 37°C and 5% CO_2 . Cells were plated 24 h prior to transfection. Transfection was performed at 80–90% confluency using Superfect (Qiagen) with equimolar ratios of plasmids according to the manufacturer's instructions. After 2 h transfection mix was removed from cells and replaced by fresh Opti-MEMTM medium supplemented with 10% foetal bovine serum containing purified exosomes derived from CSF from patients with dementia with Lewy body, patients with Parkinson's disease or neurological controls. After 12 h, the oligomerization of α -synuclein was measured using *Gaussia* luciferase protein-fragment complementation assay as described in Danzer *et al.* (2012a) and below.



Gaussia luciferase protein-fragment complementation assay

Complementation pairs of α -synuclein S1 and S2 wild-type were transfected into H4 cells in a 96-well plate format as

described above. Twelve hours after transfection cells were washed with PBS and replaced with serum- and phenol-red free media. Luciferase activity from protein complementation was measured in live cells in an automated plate reader at 480 nm following the injection of the cell permeable substrate,

Coelenterazine (20 μ M) (p.j.k) with a signal integration time of 2 s.

Electron microscopy

Exosomes were prepared from CSF as described above, pelleted to glow-discharged Formvar-carbon-coated copper grids and fixed with 4% paraformaldehyde. The grids were negatively stained with 2% uranyl acetate containing 0.7 M oxalate, pH 7.0, and imaged with a LEO EM912 Omega electron microscope (Zeiss). Digital micrographs were obtained with an on-axis 2048 CCD camera (Proscan).

Data and statistical analysis

Data were analysed blinded to the diagnosis. Statistical analysis was performed using SPSS statistics software (SPSS-Statistics 17.0) and Microsoft Excel software. Correlation analysis was performed by using Pearson's correlation. Receiver operating characteristic (ROC) curves were used to evaluate sensitivity and specificity relationships to determine the diagnostic performance of exosomal α -synuclein as a diagnostic test.

Results

Our analysis included the quantification of CSF exosome numbers by nanoparticle tracking, measurement of CSF exosomal α -synuclein by a well-established electrochemiluminescence assay (Kruse *et al.*, 2012) and determination of the potential of CSF exosomes to induce the oligomerization of α -synuclein in a split luciferase reporter cell assay (Danzer *et al.*, 2009).

Isolation of exosomes from cerebrospinal fluid

We have previously shown that exosomes can be prepared from CSF by subsequent centrifugation rounds followed by a final 100 000g ultracentrifugation step (Kunadt *et al.*, 2015) (Fig. 1A and B). The ultracentrifugation pellet contained vesicles of 40 to 120 nm diameters with the typical cup-shaped morphology of exosomes (Fig. 1B). The CSF exosome fraction was immunoreactive for the exosomal marker protein flotillin-2 (Fig. 1B) whereas in total CSF, flotillin-2 was only detectable at a much higher exposure time (data not shown). In contrast, the heavy and light chains of immunoglobulin G (IgG) were abundant in total CSF but absent from the CSF exosome fraction (Fig. 1B). In addition, the endoplasmic reticulum protein calnexin was not detected in the CSF exosome preparation, indicating the absence of microsomal contamination (Fig. 1B). We have already demonstrated by western blot that CSF exosome preparations from 5 ml starting volume contain α -synuclein (Kunadt *et al.*, 2015). However, for quantification of exosomal α -synuclein from smaller CSF volumes as low as 0.5 ml a more sensitive detection method was needed. We have previously described an

optimized ELISA that is based on an electrochemiluminescence (ECL) platform and allows for CSF α -synuclein quantification with a large dynamic range and high sensitivity (Kruse *et al.*, 2012). Exosomes were prepared from 0.5 ml CSF and the number and size distribution of vesicles in total CSF and in the CSF exosome preparations were measured by nanoparticle tracking analysis (NTA) (Sokolova *et al.*, 2011; Oosthuyzen *et al.*, 2013; van der Pol *et al.*, 2014). A representative graph is depicted in Fig. 1C and shows vesicles in the range from 0 to 550 nm in total CSF (red line). In contrast, the exosome preparation predominantly consists of vesicles in the size range of exosomes (blue line). In addition, we quantified α -synuclein protein levels in CSF and CSF exosome preparations by an electrochemiluminescence assay using MJF-1 clone 12.1 as a capture and SULFO-TAG-labelled anti- α -synuclein clone 42 as a detection antibody (Kruse *et al.*, 2012). This assay has an average lower limit of detection of 5 pg/ml α -synuclein, which allows the quantification of exosomal α -synuclein levels from a CSF starting volume as low as 0.5 ml. We next verified the reproducibility of these quantification methods (Fig. 1D). Exosomes were prepared from several aliquots of the same sample and exosome numbers as well as α -synuclein protein levels were measured in total CSF and in the exosome preparation. This was repeated with a second series of aliquots from an independent CSF sample. Coefficient of variance (CV) was <10% for the number of exosomes (9.4%) and for α -synuclein protein (8.2%) in total CSF. Coefficient of variance values for the same measurements carried out in the exosome preparations were 22.3% for both the number of exosomes and for exosomal α -synuclein (Fig. 1D). Coefficient of variance values were also calculated for the ratio of exosomal α -synuclein protein levels to the number of exosomes (9.9% for the determination of exosome numbers in the exosome pellet and 8.3% for the quantification of exosome numbers in the starting volume of total CSF). These data show that our exosome preparation is robust and suitable for quantitative analysis in clinical samples.

Quantification of exosomal α -synuclein in cerebrospinal fluid samples from early stage Parkinson's disease

We first characterized exosomal α -synuclein levels in early stage Parkinson's disease. Our single centre, prospective and longitudinal study cohort 'DeNoPa' comprises 159 early, at enrolment drug-naïve Parkinson's disease subjects and 110 healthy controls (Mollenhauer *et al.*, 2013b). From this cohort we selected 76 patients with Parkinson's disease and 58 age- and gender-matched healthy control subjects (Supplementary Table 1). We prepared exosomes from 0.5 ml baseline CSF samples and quantified α -synuclein levels in the total CSF (Fig. 2A) as well as in the exosome preparation (Fig. 2B). As previously described,

total α -synuclein levels in CSF were significantly lower in the Parkinson's disease group compared to healthy controls (Parkinson's disease: mean α -synuclein = 647.88 pg/ml, SEM = 26.95 pg/ml, $n = 76$; healthy controls: mean α -synuclein = 774.64 pg/ml, SEM = 36.13 pg/ml, $n = 58$; $*P < 0.05$, Student's two-tailed t -test) (Fig. 2A). When we compared the absolute amounts of CSF exosomal α -synuclein between Parkinson's disease and healthy control samples (Fig. 2B), we found a small but significant difference, with lower levels in the Parkinson's disease group (Parkinson's disease mean = 13.04 pg in exosomes derived from 1 ml CSF, SEM = 0.61 pg, $n = 76$; healthy controls mean = 16.01 pg in exosomes prepared from 1 ml CSF, SEM = 0.98 pg, $n = 58$, $*P < 0.05$, Student's two-tailed t -test) (note that exosome preparations were performed from 0.5 ml CSF in this and all subsequent experiments; all numbers were normalized to 1 ml CSF starting volume after quantification). Next, we calculated the ratio of exosomal α -synuclein to total α -synuclein present in the starting volume of CSF (i.e. α -synuclein encapsulated within exosomes plus vesicle-free CSF α -synuclein). Interestingly, we found that only 2.17% (SEM = 0.12%) of CSF α -synuclein is present in exosomes in CSF from Parkinson's disease patients and healthy controls (Parkinson's disease mean = 2.17%, SEM = 0.12%, $n = 76$; healthy controls mean = 2.17%, SEM = 0.12%, $n = 58$, Student's two-tailed t -test) (Fig. 2C).

CSF exosomal α -synuclein distinguishes between Parkinson's disease, dementia with Lewy bodies and neurological controls and correlates with cognitive impairment

We next examined CSF exosomal α -synuclein in more progressed disease stages. To this end we prepared CSF exosomes from 37 clinically diagnosed patients with Parkinson's disease with different grades of motor symptoms as assessed by Hoehn and Yahr staging (mean Hoehn and Yahr stage = 3.9, SEM = 0.20). All samples were randomly selected from the 'Kassel cohort' and complemented with two different neurological control groups (Supplementary Table 2 and Supplementary Fig. 1) (Mollenhauer *et al.*, 2011). One group consisted of 15 patients with gait problems due to polyneuropathy but without Parkinson's disease. A second group contained 25 patients with progressive supranuclear palsy, which is characterized by a Parkinson syndrome without underlying α -synuclein but known tau pathology. When we quantified absolute amounts of α -synuclein protein in the exosome fraction of equalized CSF starting volumes we found no significant differences between this more advanced Parkinson's disease, polyneuropathy and progressive supranuclear palsy groups (one-way ANOVA, $P = 0.000691$. Parkinson's disease mean = 17.20 pg in exosomes derived

from 1 ml CSF, SEM = 1.50 pg, $n = 36$; polyneuropathy control mean = 17.20 pg in exosomes derived from 1 ml CSF, SEM = 2.97 pg, $n = 15$, progressive supranuclear palsy control mean = 20.60 pg in exosomes derived from 1 ml CSF, SEM = 4.56 pg, $n = 25$, no significant differences, Games-Howell *post hoc* test) (Fig. 3A). This finding is in contrast to the small difference in exosomal α -synuclein observed between very early Parkinson's disease and healthy controls in the DeNoPa cohort. It is possible that CSF exosomal α -synuclein levels could increase during disease progression. However, in the Kassel cohort with more advanced disease stages, no correlation was observed between exosomal α -synuclein and the severity of motor symptoms as assessed by Hoehn and Yahr staging (Pearson's coefficient, $r = -0.011$, $P = 0.950$) (Fig. 3B). Another possible reason for the discrepancy between the two cohorts could be the differences in the control groups which consisted of neurologically healthy controls in one (Fig. 2) and patients with polyneuropathy in the second group (Fig. 3), who were referred to the movement disorder clinic in Kassel due to gait disturbances and symmetric positional tremor mimicking Parkinson's disease. Although a Parkinson's disease was ruled out by standardized levodopa testing, smell test and brainstem ultrasound, it is still possible that some of these patients had an early stage of Parkinson's disease. Furthermore, we cannot rule out that the lack of a difference in exosomal α -synuclein between Parkinson's disease and neurological controls is caused by the relatively smaller number of samples tested.

We additionally analysed exosomal α -synuclein levels in CSF from 35 patients with dementia with Lewy bodies from both the Kassel and the Göttingen cohorts, as another disease with α -synuclein-related Parkinson syndrome. As shown in Fig. 3A, we found significantly lower CSF exosomal α -synuclein from patients with dementia with Lewy bodies as compared to Parkinson's disease, polyneuropathy and progressive supranuclear palsy groups [dementia with Lewy bodies mean = 7.92 pg in exosomes derived from 1 ml, SEM = 0.78 pg, $n = 35$, ($*)P = 0.05$, $*P < 0.05$, $***P < 0.0005$, Games-Howell *post hoc* test]. Importantly, exosomal α -synuclein levels correlated inversely with the dementia with Lewy bodies patients' Mini-Mental State Examination (MMSE) scores (Pearson's coefficient, $r = -0.382$, $n = 32$ for whom MMSE data were available, $P = 0.028$, two-tailed probability) (Fig. 3C). These data indicate that higher amounts of exosomal α -synuclein in CSF are associated with a lower MMSE performance and therefore with cognitive impairment. It is feasible to assume that during disease progression, an increasing number of neurons contain pathological α -synuclein aggregates, which could be released with exosomes. This notion is further supported by the strong positive correlation of exosomal α -synuclein levels with tau protein levels rising in CSF (Pearson's coefficient, $r = 0.751$, $n = 17$ for whom CSF tau data were available, $P = 0.0005$, two-tailed probability) (Fig. 3D). Tau is an intraneuronal protein required to maintain the structural

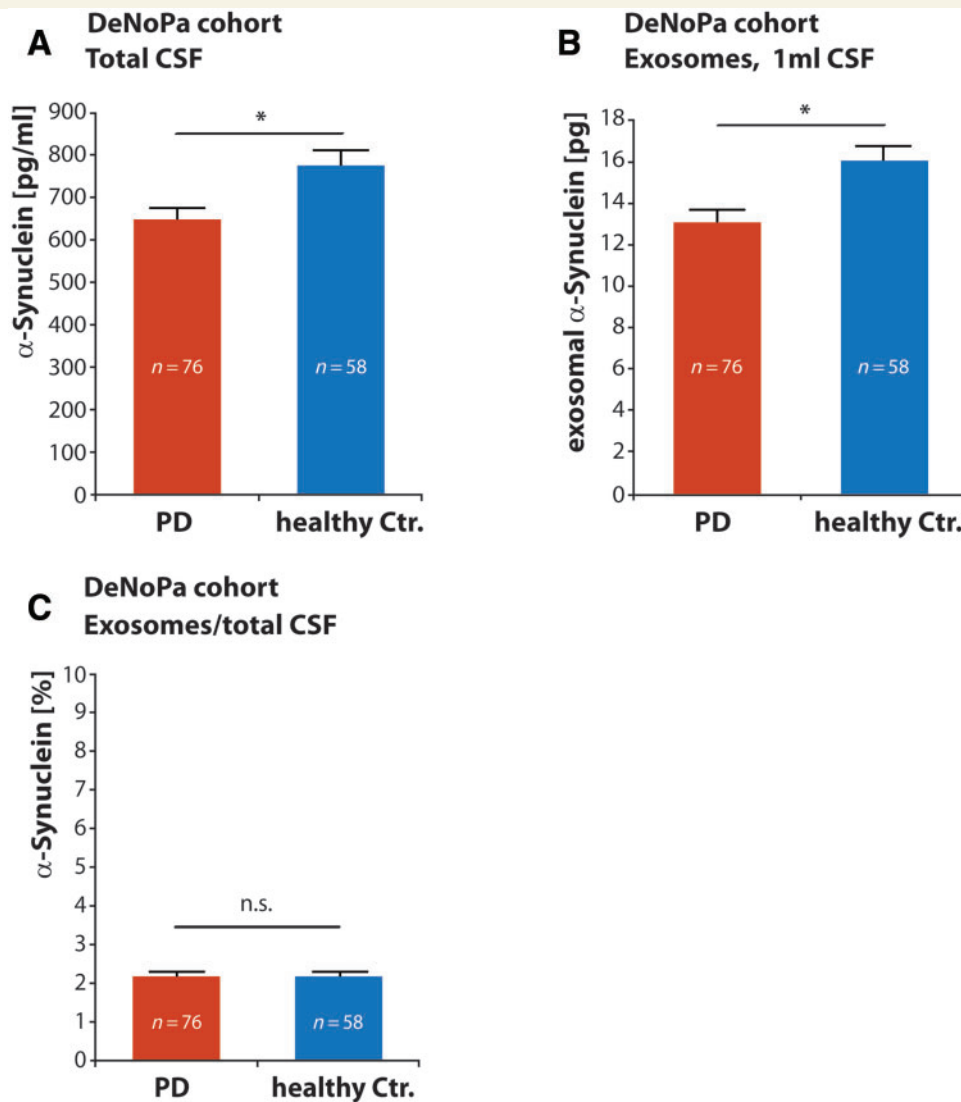


Figure 2 Quantification of exosomal α -synuclein in CSF samples from the DeNoPa cohort. **(A)** CSF concentrations of α -synuclein in total CSF were determined [red bar: Parkinson's disease (PD) patients, blue bar: healthy control patients], $n = 76$ Parkinson's disease patients, $n = 58$ healthy control patients, $*P < 0.05$, two-sided Student's t -test. **(B)** Exosomal α -synuclein protein levels were quantified in exosomes prepared from 0.5 ml CSF starting material and levels were normalized to 1 ml (red bar: Parkinson's disease patients, blue bar: healthy control patients), $n = 76$ Parkinson's disease patients, $n = 58$ healthy control patients, $*P < 0.05$, two-sided Student's t -test. **(C)** The ratio of exosomal α -synuclein (prepared from 0.5 ml CSF and normalized to 1 ml) to α -synuclein in total CSF (normalized to 1 ml) was determined in samples from patients with Parkinson's disease (red bar) and healthy controls (blue bar) and is given in %, $n = 76$ Parkinson's disease patients, $n = 58$ healthy control patients, n.s. = not significant, two-sided Student's t -test.

integrity of neurons by stabilizing the microtubule cytoskeleton. It is assumed that neuronal damage leads to the release of tau from disintegrating neurons to the CSF. Thus, an increase in CSF total tau levels is generally considered as an unspecific marker of neurodegeneration, e.g. in Alzheimer's disease and dementia with Lewy bodies (Mollenhauer *et al.*, 2005; Montine *et al.*, 2010; Musiek and Holtzman, 2012; Kang *et al.*, 2013). Therefore, higher levels of exosomal α -synuclein in progressed stages of dementia with Lewy bodies could likely reflect an increased disease activity, although it is not clear why such a correlation between disease progression and exosomal

α -synuclein is not observed in Parkinson's disease. The correlation between increasing exosomal α -synuclein with clinical disease progression and the rise in the neuronal injury marker tau contrasts with the overall lower exosomal α -synuclein levels in the group of patients with dementia with Lewy bodies compared to the other groups.

We next performed a preliminary evaluation of the performance of exosomal α -synuclein as a potential diagnostic biomarker by ROC curve analysis (Supplementary Fig. 2 and Supplementary Table 3A). The sensitivity and specificity of CSF exosomal α -synuclein to distinguish dementia with Lewy bodies from Parkinson's disease were 85.7%

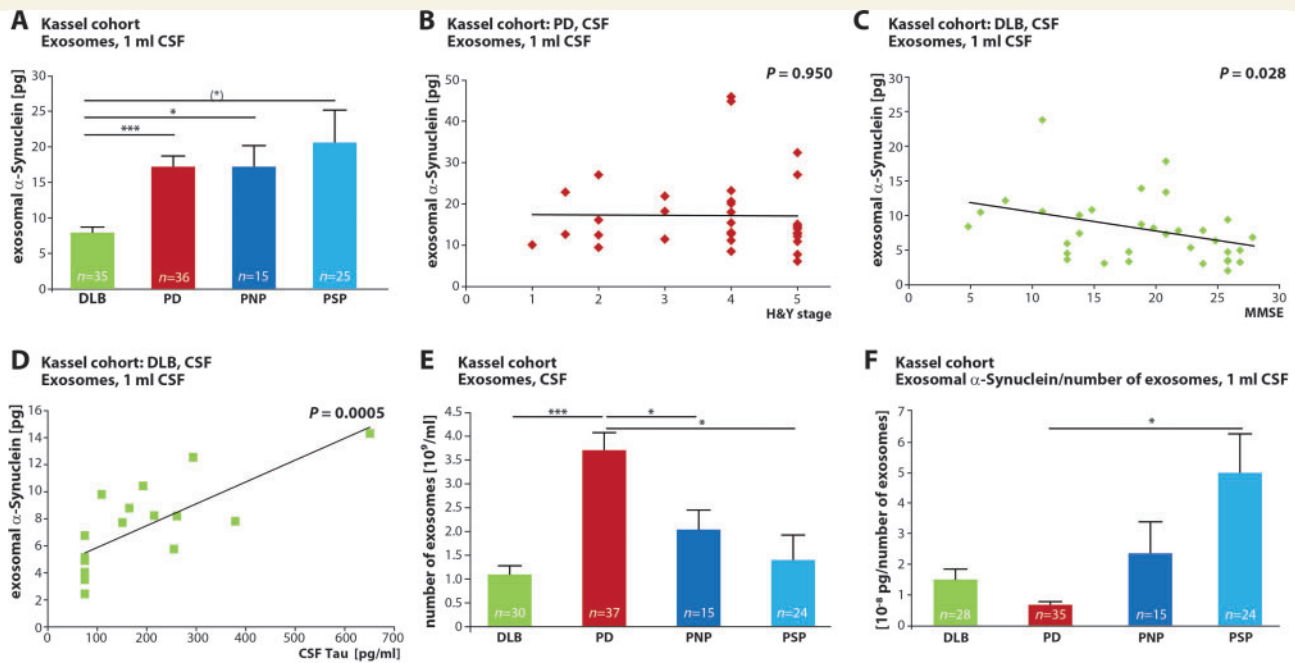


Figure 3 Characterization of exosomal α -synuclein in dementia with Lewy bodies, Parkinson's disease and non α -synuclein-related disease controls in the Kassel cohort. **(A)** Quantification of exosomal α -synuclein protein content. Exosomes were prepared from CSF samples from patients with dementia with Lewy bodies (DLB, green bar), Parkinson's disease (PD, red bar), polyneuropathy (PNP, dark blue bar) and progressive supranuclear palsy (PSP, light blue bar), $n = 35$ DLB, $n = 36$ PD, $n = 15$ PNP control, $n = 25$ PSP control. (*) $P = 0.05$, * $P < 0.05$, *** $P < 0.0005$, Games-Howell *post hoc* test. One-way ANOVA $P = 0.000691$. For ROC curve analysis, see Supplementary Fig. 2 and Supplementary Table 3A. **(B)** Exosomal α -synuclein amounts do not correlate with the severity of motor symptoms determined by Hoehn and Yahr staging (H&Y) in Parkinson's disease ($n = 36$, Pearson's correlation coefficient $r = -0.011$, $P = 0.950$, two-tailed probability). **(C)** Correlation of CSF exosomal α -synuclein with impaired cognitive function in patients with dementia with Lewy bodies. Cognitive state was measured by MMSE (lower scores indicate a decrease in cognitive function) ($n = 32$, Pearson's correlation coefficient $r = -0.382$, $P = 0.028$, two-tailed probability). **(D)** Correlation of CSF exosomal α -synuclein with increasing levels of CSF total tau indicating more progressed neuronal degeneration in patients with dementia with Lewy bodies ($n = 17$, Pearson's correlation coefficient $r = 0.751$, $P = 0.0005$, two-tailed probability). **(E)** The number of exosomes per ml CSF was quantified by nanoparticle tracking analysis in CSF from patients with dementia with Lewy bodies (green bar), Parkinson's disease (red bar), polyneuropathy (dark blue bar) and progressive supranuclear palsy (light blue bar) ($n = 30$ DLB, $n = 37$ PD, $n = 15$ PNP, $n = 24$ PSP, * $P < 0.05$, *** $P < 0.0005$, Games-Howell *post hoc* test. One-way ANOVA $P < 0.0001$). **(F)** Ratio of exosomal α -synuclein to number of exosomes in CSF from patients with dementia with Lewy bodies (green bar), Parkinson's disease (red bar), polyneuropathy (dark blue bar) and progressive supranuclear palsy (light blue bar) ($n = 28$ DLB, $n = 35$ PD, $n = 15$ PNP, $n = 24$ PSP, * $P < 0.05$, Games-Howell *post hoc* test. One-way ANOVA $P = 0.000377$). For ROC curve analysis, see Supplementary Fig. 2 and Supplementary Table 3B.

and 80.6% with a positive and negative predictive value of 81.1% and 85.3%, respectively. The sensitivity and specificity for the distinction of dementia with Lewy bodies from polyneuropathy were 85.7% and 66.7%; that for dementia with Lewy bodies versus progressive supranuclear palsy 71.4% and 92.0% (Supplementary Fig. 2 and Supplementary Table 3A).

As depicted in Fig. 3E, the number of exosomes in CSF differs highly between the various diagnostic groups, with nearly 4-fold higher values in Parkinson's disease as compared to dementia with Lewy bodies (one-way ANOVA, $P < 0.0001$. Parkinson's disease mean = 3.69×10^9 exosomes/ml CSF, SEM = 1.83×10^8 exosomes/ml CSF, $n = 37$; dementia with Lewy bodies mean = 1.08×10^9 exosomes/ml CSF, SEM = 1.83×10^8 exosomes/ml CSF, $n = 30$, $P < 0.000$, Games-Howell *post hoc* test) and ~ 2 times higher numbers as compared to polyneuropathy and progressive supranuclear palsy (polyneuropathy

mean = 2.03×10^9 exosomes/ml CSF, SEM = 4.09×10^8 exosomes/ml CSF, $n = 15$; progressive supranuclear palsy mean = 1.39×10^9 exosomes/ml CSF, SEM = 5.23×10^8 exosomes/ml CSF, $n = 24$, * $P < 0.05$, *** $P < 0.0005$ Games-Howell *post hoc* test). In contrast, no significant differences were observed between the polyneuropathy and progressive supranuclear palsy groups (Fig. 3E).

We next calculated the ratio of exosomal α -synuclein per number of exosomes in the different diagnostic groups (Fig. 3F). Interestingly, an approximately two to three times lower ratio was detected in patients with Parkinson's disease compared to all other groups (one-way ANOVA $P = 0.000377$; Parkinson's disease mean = 6.74×10^{-9} pg α -synuclein/number of exosomes, SEM = 1.04×10^{-9} pg α -synuclein/number of exosomes, $n = 35$; dementia with Lewy bodies mean = 1.50×10^{-8} pg α -synuclein/number of exosomes, SEM = 3.37×10^{-9} pg α -synuclein/number of exosomes, $n = 28$; polyneuropathy mean = 2.63×10^{-8} pg

α -synuclein/number of exosomes, SEM = 1.03×10^{-8} pg α -synuclein/number of exosomes, $n = 15$; progressive supranuclear palsy mean = 4.99×10^{-8} pg α -synuclein/number of exosomes, SEM = 1.27×10^{-8} pg α -synuclein/number of exosomes, $n = 24$, $*P < 0.05$, Games-Howell *post hoc* test). In contrast, no significant differences were found between polyneuropathy and progressive supranuclear palsy groups. ROC curve analysis of this marker for the distinction of Parkinson's disease versus dementia with Lewy bodies, polyneuropathy and progressive supranuclear palsy is shown in Supplementary Fig. 2 and in Supplementary Table 3B. The ratio of CSF exosomal α -synuclein to CSF exosome numbers distinguishes Parkinson's disease from dementia with Lewy bodies with a sensitivity of 74.3% and a specificity of 60.7% (Supplementary Table 3B).

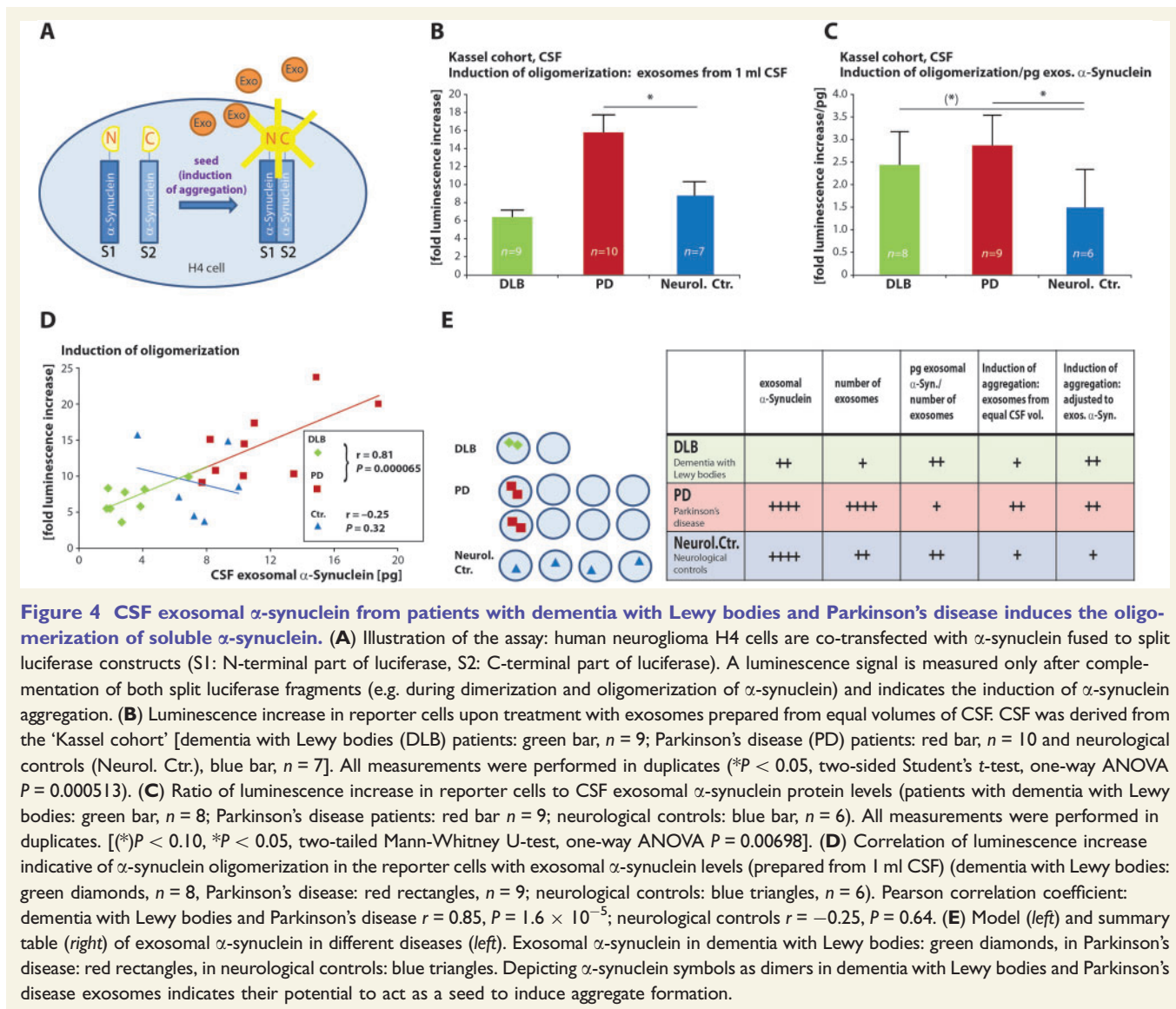
Taken together, exosomal α -synuclein is significantly decreased in CSF from patients with dementia with Lewy bodies compared to neurological controls, mainly due to a lower absolute number of CSF exosomes. Disease progression in dementia with Lewy bodies, defined by the severity of cognitive dysfunction and increasing concentrations of the neuronal injury marker CSF tau, is paralleled by higher exosomal α -synuclein levels (Fig. 3C and D). In Parkinson's disease, exosome numbers are significantly increased compared to dementia with Lewy bodies and neurological controls with comparable levels of total exosomal α -synuclein and a lower ratio of α -synuclein per exosomal particle (for a graphic summary and model see Fig. 4E).

CSF exosomal α -synuclein from patients with Parkinson's disease and dementia with Lewy bodies induces the oligomerization of α -synuclein

Exosomes could contribute to spreading of α -synuclein disease pathology by the transfer of α -synuclein oligomers to healthy neurons where they could induce the oligomerization of soluble intracellular α -synuclein. We have previously shown that exosomal α -synuclein oligomers are efficiently internalized into target cells (Danzer *et al.*, 2012b). We therefore quantified the seeding potential of CSF exosomes to induce oligomerization of α -synuclein in a cell line. Exosomes were prepared from CSF from patients with dementia with Lewy bodies, Parkinson's disease and neurological controls. Controls included six patients with polyneuropathy, one patient with normal pressure hydrocephalus and one with corticobasal degeneration. All CSF samples were randomly selected from the 'Kassel cohort'. Induction of oligomerization was measured by a split luciferase fragment complementation technique, which is one of the most sensitive and dynamic detection methods to study protein-protein interactions (Luker and Piwnicka-Worms, 2004; Danzer *et al.*, 2012b). In short, luciferase is split into two non-bioluminescent fragments S1 and S2, and

fused with a short linker to the C-terminus of α -synuclein (Fig. 4A). Interaction of α -synuclein molecules, e.g. during oligomerization and higher order aggregation, brings both luciferase fragments into close proximity. The resulting luciferase complementation can be measured as a luminescence signal in the presence of the substrate (Fig. 4A). Unspecific complementation of both luciferase fragments had previously been excluded by a series of experiments where no complementation was shown for the combination of α -synuclein S1 with amyloid- β fused to the C-terminal fragment of luciferase nor for α -synuclein S2 and amyloid- β fused to the N-terminal fragment of luciferase (Danzer *et al.*, 2011). As a reporter cell line we used H4 neuroglioma cells co-transfected with the split luciferase α -synuclein constructs S1 and S2. The reporter assay was previously characterized by size exclusion chromatography and revealed the detection of α -synuclein S1/S2 multimers in the size range of dimers to 30mers (Danzer *et al.*, 2011). The activity change of the bioluminescence signal induced by treatment with CSF-derived exosomes was quantified as 'fold luminescence increase' compared to mock treated control, e.g. cells that were exposed to exosomes but were treated with transfection reagent without the plasmids coding for S1/S2 α -synuclein. Incubation of the reporter cell line with exosomes prepared from equal amounts of either dementia with Lewy bodies, Parkinson's disease or neurological control CSF resulted in ~ 2 -fold increase of the bioluminescence signal in cells treated with Parkinson's disease-derived exosomes compared to neurological control or dementia with Lewy bodies exosomes (one-way ANOVA, $P = 0.000513$; dementia with Lewy bodies mean = 6.40-fold increase, SEM = 0.77-fold increase, $n = 9$; Parkinson's disease mean = 15.79-fold increase, SEM = 1.73-fold increase, $n = 10$; control mean = 8.79-fold increase, SEM = 1.58-fold increase, $n = 7$, $*P < 0.05$, Student's two-tailed *t*-test) (Fig. 4B). This indicates that exosomes in the CSF of Parkinson's disease patients can serve as seeds to induce the oligomerization of α -synuclein in recipient cells.

As shown above, the overall amount of exosomal α -synuclein and the number of exosomes in CSF from patients with dementia with Lewy bodies is significantly lower compared to Parkinson's disease and neurological controls. We therefore analysed the potential of CSF-derived exosomes to induce oligomerization after adjusting for CSF exosomal α -synuclein levels (Fig. 4C). The ratio of luminescence increase as a measure for oligomer inducing potential to CSF exosomal α -synuclein protein levels was calculated for each preparation. Interestingly, when adjusted to exosomal α -synuclein content, CSF exosomes derived from patients with dementia with Lewy bodies and Parkinson's disease had a higher potential to induce α -synuclein oligomerization as CSF exosomes from neurological controls [one-way ANOVA $P = 0.00698$; dementia with Lewy bodies mean = 2.43-fold increase/pg exosomal α -synuclein, SEM = 1.05-fold increase/pg exosomal α -synuclein, $n = 8$; Parkinson's disease mean = 2.87-fold increase/pg exosomal α -synuclein, SEM = 0.95-fold increase/pg exosomal



α -synuclein, $n = 9$; neurological controls mean = 1.50-fold increase/pg exosomal α -synuclein, SEM = 1.19-fold increase/pg exosomal α -synuclein, $n = 6$, ($*P < 0.1$ indicates strong statistical tendency due to small sample sizes, Mann-Whitney U-Test, $*P < 0.05$, Mann-Whitney U-Test]. When we plotted the oligomerization inducing activity versus exosomal α -synuclein content we found a strong correlation for CSF exosome preparations derived from dementia with Lewy bodies and Parkinson's disease patients but not for preparations from the neurological control group (Fig. 4D) (dementia with Lewy bodies and Parkinson's disease group $r = 0.85$, $n = 17$, $P = 1.6 \times 10^{-5}$, neurological control group $r = -0.25$, $n = 6$, $P = 0.64$, Pearson's correlation).

Discussion

The findings from our work address two major aspects: (i) we provide the first comprehensive analysis and

quantification of exosomal α -synuclein in CSF from patient cohorts; and (ii) we show that CSF exosomes from patients with Parkinson's disease and dementia with Lewy bodies contain a pathogenic α -synuclein species which serves as a seed to induce the oligomerization of soluble α -synuclein in recipient cells. These findings may shed light on several aspects of α -synuclein spreading and clearance in α -synuclein-related disorders. Moreover, replication provided, CSF exosomal α -synuclein may well be suited as a diagnostic marker in the future.

CSF exosomes from patients with dementia with Lewy bodies and Parkinson's disease induce α -synuclein oligomerization

We and others have demonstrated previously *in vivo* uptake and functional recovery of exosomal proteins and RNA in neurons (Fruhbeis *et al.*, 2012; El Andaloussi *et al.*,

2013). Exosomal release and transfer of neuron-derived oligomeric α -synuclein was reported *in vitro* (Danzer *et al.*, 2012a) and we have recently shown first *in vivo* evidence of exosomal α -synuclein in the CNS (Kunadt *et al.*, 2015). Our study revealed that exosomal α -synuclein can be isolated from human CSF. Interestingly, here we find that CSF exosomes from patients with Parkinson's disease induce α -synuclein oligomerization in a reporter cell line. CSF from patients with dementia with Lewy bodies contains lower numbers of exosomes compared to Parkinson's disease and neurological controls. As a result, exosomes isolated from equal CSF volumes from dementia with Lewy bodies and neurological controls do not differ in their activity to induce α -synuclein oligomerization. However, after adjusting to exosomal α -synuclein protein levels, the oligomerization-inducing potential of dementia with Lewy bodies and Parkinson's disease CSF exosomes was approximately two times higher compared to neurological controls. Furthermore, a linear correlation between exosomal α -synuclein levels and their activity to induce α -synuclein oligomerization was observed for dementia with Lewy bodies and Parkinson's disease but not for control CSF exosomes. Our results thus suggest that exosomes derived from Parkinson's disease and dementia with Lewy bodies CSF may contain a pathogenic species of α -synuclein that is distinct from neurological controls (Fig. 4E). It is tempting to speculate that exosomes are enriched for misfolded oligomers of α -synuclein since oligomerization was described to target proteins for exosomal release (Fang *et al.*, 2007). In Parkinson's disease and dementia with Lewy bodies, pathogenic α -synuclein oligomers may be preferentially sorted into exosomes and act as a seed for α -synuclein fibril growth in recipient cells. Interneuronal transfer of exosomal α -synuclein may thus explain the propagation of pathology along interconnected neuronal tracts, which is observed in α -synuclein-related neurodegeneration (Muller *et al.*, 2005; Kordower and Brundin, 2009a; Li *et al.*, 2010; Lee *et al.*, 2012; Luk *et al.*, 2012a; Ulusoy *et al.*, 2013).

Exosomal secretion of superfluous or toxic cellular content has been described under various physiological and pathophysiological conditions previously (Vidal *et al.*, 1997; Strauss *et al.*, 2010). Therefore, exosomal release of pathological α -synuclein intermediates may represent a cellular response to remove neurotoxic substances, especially in cells with low degradative capacity. Supporting this notion, increased exosomal secretion of α -synuclein was observed after lysosomal inhibition *in vitro* (Alvarez-Erviti *et al.*, 2011; Danzer *et al.*, 2012b). Interestingly, exosomes are most efficiently internalized into microglia whereas neuronal uptake rates are much lower (Fitzner *et al.*, 2011; Zhuang *et al.*, 2011; Fruhbeis *et al.*, 2013). This suggests that pathogenic α -synuclein may be released by exosomes and subsequently degraded by microglia. However, insufficient microglial clearance function, as observed in many neurodegenerative diseases, may pave the way for interneuronal propagation of pathogenic

exosomal α -synuclein species (Luo *et al.*, 2010). Interestingly, the levels of exosomal α -synuclein rise in later disease stages of dementia with Lewy bodies as reflected by deteriorating cognitive function and increasing levels of the neuronal injury marker tau. On a more speculative basis we suggest that the initially decreased levels of exosome numbers and hence exosomal α -synuclein in dementia with Lewy bodies CSF might mirror a clearance deficit for α -synuclein, which might explain the pan-cortical presence of α -synuclein aggregates. It is feasible to assume that during disease progression, more neurons contain pathological α -synuclein aggregates, which could be released with exosomes. Therefore, higher levels of exosomal α -synuclein in progressed stages of dementia with Lewy bodies could likely reflect an increased disease activity and the higher number of affected neurons could contribute to the rise of exosomal α -synuclein levels in CSF. Clearly, this assumption needs to be corroborated by further experimentation, including a comparison of exosomal release rates from cortical neurons in dementia with Lewy bodies and controls, e.g. by using induced pluripotent stem cell-derived cortical neurons from the respective patient groups or by using neuronal subtype-specific exosomal marker proteins, which would allow us to quantify exosomes in CSF, dependent on their cellular origin.

Preliminary evaluation of CSF exosomal α -synuclein as a potential biomarker in α -synuclein-related disorders

We demonstrate that exosomal α -synuclein is present in CSF where it is accessible for quantification as a potential biomarker to detect α -synuclein-related pathology (Fig. 4E). Preliminary ROC curve analysis revealed a high sensitivity and specificity for the discrimination of dementia with Lewy bodies, Parkinson's disease and neurological controls. Moreover, we detected a highly significant correlation of CSF exosomal α -synuclein with cognition and the neuronal injury marker tau in dementia with Lewy bodies. Thus, CSF exosomal α -synuclein could additionally serve as a surrogate marker to monitor disease progression. Such markers have not been available in the past and could aid future interventional studies in dementia with Lewy bodies. Clearly, further experiments including larger cohorts and independent validation samples are needed to verify the potential of CSF exosomal α -synuclein as a biomarker in Parkinson's disease and dementia with Lewy bodies.

Interestingly, exosomal α -synuclein was recently characterized in plasma samples from a large cohort of patients with Parkinson's disease and healthy controls. There, exosomes derived from the CNS were immune-captured by an antibody directed against the neural L1 cell adhesion molecule L1CAM (Shi *et al.*, 2014). Using this approach and in contrast to our findings, Shi *et al.* (2014) report increased

amounts of exosomal α -synuclein in Parkinson's disease compared to healthy controls. However, this may be caused by increased rates of transport from the CNS to the blood that are described in Parkinson's disease (Popescu *et al.*, 2009).

In summary, our study is the first detailed analysis of α -synuclein in CSF exosomes from different diseases with α -synuclein-related neurodegeneration. CSF exosomes from patients with α -synuclein-related neurodegeneration possess the capacity to induce oligomerization of soluble α -synuclein in an *in vitro* model. These data are therefore highly relevant for disease pathogenesis. In addition, upon independent validation in larger cohorts, we propose exosomal α -synuclein as a potential future biomarker in α -synuclein-related neurodegenerative disorders.

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Supplementary material

Supplementary material is available at *Brain* online.

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