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Pharmacological inhibition of nSMase2 reduces brain exosome release and α-synuclein pathology in a Parkinson's disease model

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Abstract

Aim: We have previously reported that cambinol (DDL-112), a known inhibitor of neutral sphingomyelinase-2 (nSMase2), suppressed extracellular vesicle (EV)/exosome production in vitro in a cell model and reduced tau seed propagation. The enzyme nSMase2 is involved in the production of exosomes carrying proteopathic seeds and could contribute to cell-to-cell transmission of pathological protein aggregates implicated in neurodegenerative diseases such as Parkinson's disease (PD). Here, we performed in vivo studies to determine if DDL-112 can reduce brain EV/exosome production and proteopathic alpha synuclein (αSyn) spread in a PD mouse model.

Methods: The acute effects of single-dose treatment with DDL-112 on interleukin-1β-induced extracellular vesicle (EV) release in brain tissue of Thy1-αSyn PD model mice and chronic effects of 5 week DDL-112 treatment on behavioral/motor function and proteinase K-resistant αSyn aggregates in the PD model were determined.

Results/discussion: In the acute study, pre-treatment with DDL-112 reduced EV/exosome biogenesis and in the chronic study, treatment with DDL-112 was associated with a reduction in αSyn aggregates in the substantia nigra and improvement in motor function. Inhibition of nSMase2 thus offers a new approach to therapeutic development for neurodegenerative diseases with the potential to reduce the spread of disease-specific proteopathic proteins.

Keywords: Parkinson's disease, Alpha-synuclein, Extracellular vesicles, Exosomes, Neutral sphingomyelinase-2

Neurodegenerative diseases such as tauopathies and synucleinopathies [1–3] are typically characterized by the spread of proteopathic aggregates throughout the brain [4, 5]. Pathological protein aggregates comprising tau in tauopathies or alpha-synuclein (αSyn) in Parkinson's disease (PD) [6] first appear in a specific brain region and, as disease progresses, spread to other areas of the brain following neuroanatomical pathways.

Exosomes, small (30–150 nm in diameter) extracellular vesicles (EVs) of endocytic origin [7, 8], have been implicated in the spread of protein aggregates throughout the brain [9] and specifically in the the propagation of αSyn pathology [10–16]. A subset of exosomes generated by a pathway independent of the major canonical endosomal sorting complexes required for transport (ESCRT) [17], is dependent on the activity of neutral sphingomyelinase 2 (nSMase2) and plays a key role in the spread of proteopathic seeds [18]. nSMase2 inhibition has been shown to also be associated with reduction in amyloid plaque load and tau pathology in murine models of Alzheimer's disease [19] and tauopathy [20], respectively.

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Fig. 1 Inhibition of EV release by DDL-112 in an acute study and improvement in motor function as well as reduction of PK-resistant αSyn aggregates in the SN with chronic DDL-112 treatment of PD model mice. Acute study: **a** A scheme of the acute study protocol wherein mice were pre-treated with DDL-112 then received ICV injection of IL-1β before tissue collection is shown. **b** A representative transmission electron microscopy (TEM) image of the brain EV fraction. **c** Average concentrations of 50–200 nm size EVs from each treatment condition compared by Tunable Resistive Pulse Sensing (TRPS) analysis and levels. **d** Levels of DDL-112 in brain tissue of mice represented by the mean and SEM. **e** Representative images of immunoblot (IB) analysis of EV fractions from individual animals are shown; membranes were probed against exosomal markers (CD63 and Synt-1) with Ponceau S (PS) as the loading control. **f** Densitometry analysis of CD63 IB images. **g** Densitometry analysis of Syntenin-1 (Synt-1) IB images. Optical density (OD) is shown as percent of control. N = 4 animals per group. Statistical analysis was performed using one-way ANOVA with post-hoc Tukey comparison tests: *p < 0.05 and **p < 0.01. Chronic study: Behavioral/motor function assessment of **h** Challenging Beam (CB) error-step (Veh n = 8, DDL-112 n = 7), **i** pole test time to descend (Veh n = 7, DDL-112 n = 6; one mouse in each group was a non-performer), and **j** Open Field (OF) distance (cm) traveled per episode of movement (Veh n = 8, DDL-112 n = 6; on mouse in the DDL-112 group was a non-performer). **k** Animal weight with and without DDL-112 treatment is shown. **l** The area in percent with proteinase K (PK)-resistant αSyn aggregates in the global SN is graphed. (Veh n = 8, DDL-112 n = 7). **m** Brain levels of DDL-112 at the time of euthanasia (4 h after dosing) are shown. All data plotted minimum to maximum. Statistics performed using Student's unpaired t-test (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001)

In Bilousova et al. 2018, we reported on identification of a small molecule inhibitor of nSMase2, cambinol (DDL-112), that decreased tau propagation, through screening of a small compound library in a cell model [21]. We showed that DDL-112, inhibits nSMase2 activity with an IC50 ~ 7.7 μM in vitro and also inhibits the nSMase2 activity in the brain after a single oral dose [22]. nSMase2 hydrolyzes sphingomyelin to produce ceramide and thereby contributes to EV/exosome formation in the brain [23]. The role of nSMase2 in the development or progression of neurodegenerative disorders is evidenced by increased ceramide levels in the brain, serum and/or plasma that have been reported as early predictors of such disorders [24] and memory impairment in PD [25].

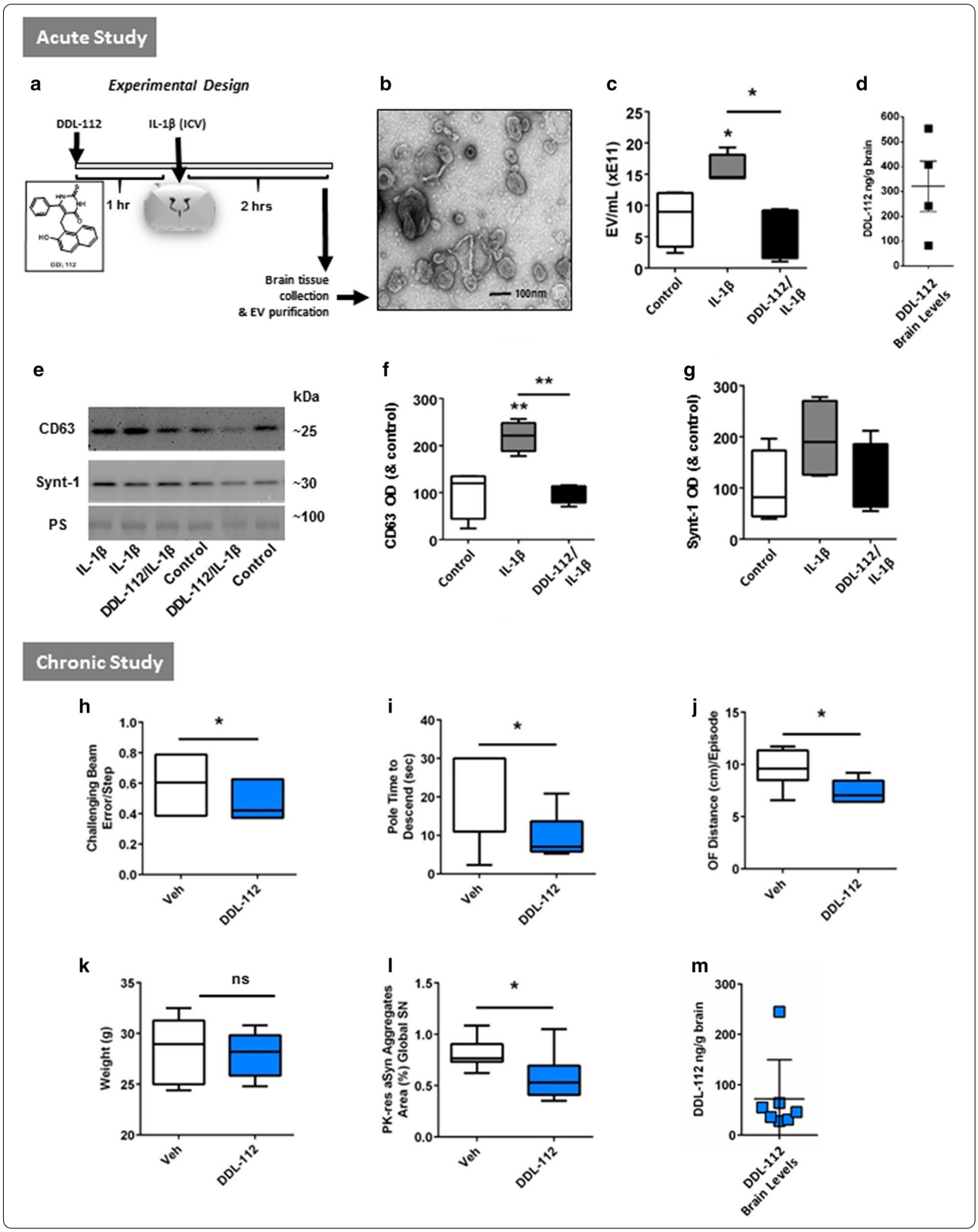
Here, we extend our in vitro findings by assessment of the in vivo effects of DDL-112 on EV biogenesis in an acute study, and on behavior/motor function and αSyn agregates in the Thy1-αSyn PD mouse model [26–28] in a chronic study.

In the studies described below, sucrose gradient purification was used for EV/exosome isolation from brain tissue based on published protocols [29–33] with minor modifications. Characterization of sucrose gradient F1, F2, and F3 fractions is presented in Additional file 1: Fig. S1. Immunoblot analysis confirmed the prevalence of exosomal markers CD63 and syntenin-1 (Synt-1), but no negative control marker calnexin (CNX), in the F2 fraction as compared to the F1 and F3 fractions (Additional file 1: Fig. S1A). Transmission electron microscopy (TEM) of F1, F2, and F3 fractions from mouse brain EV/exosome purification shows an abundance of small EVs with the characteristic 'cup' shape and size of ~ 30–150 nm in the F2 fraction (Fig. 1b and Additional file 1: Fig. S1B). Small EVs (< 50 nm) were also found in the F3 fraction, but at a very low concentration and the F1 fraction largely consisted of membranous debris (Additional file 1: Fig. S1B).

The sucrose gradient characterization data confirm the enrichment of EV/exosomes in the F2 sucrose gradient fractions. To normalize these EV-containing F2 fractions for analyses, F2 pellets were resuspended in volumes of cryopreservation solution based on the original brain tissue weight (0.4 g of tissue/150 μl solution). Further details of the in vivo experimental methods done under protocols approved by the Animal Care and Use Committee can be found in Additional file 1.

To inform design of both the acute and chronic studies, we performed a preliminary study using Thy1-αSyn (Tg) and non-transgenic (NTg) littermate mice wherein mice were dosed orally with 100 mg/kg DDL-112 and brain tissue collected 3 h later for the determination of DDL-112 brain levels and analysis of EV/exosomes. The mean DDL-112 brain tissue level for both Tg and NTg mice was ~ 650 ng/g (Additional file 1: Fig. S1C). The means were lower for EV/exosome levels in brain tissue from DDL-112-treated Tg (but not NTg) compared to DMSO vehicle-treated mice. Furthermore, EV αSyn (pS129) levels from DDL-112-treated Tg mice were lower when compared to vehicle, although the differences were not statistically significant (Additional file 1: Fig. S1D). Levels of exosomal marker CD63 in F2 fractions from DDL-112-treated Tg mice were significantly lower compared to vehicle (Additional file 1: Fig. S1E, F).

The findings from the preliminary study (Additional file 1: Fig. S1) suggest that EV/exosome biogenesis in Thy1-αSyn Tg mice is nSMase2-dependent, but also revealed that evaluation of nSMase2 inhibitors in an acute setting would benefit from additional nSMase2 stimulation in Thy1-αSyn mice. Thus, in the acute study, after oral dosing pretreatment with DDL-112, mice were injected intracerebroventricularly (ICV) with interleukin-1β (IL-1β) known to activate nSMase2 [34, 35] and elicit release of EVs [36, 37]. We chose this method of eliciting EV release because of the reported


Acute Study
a
Experimental Design
DDL-112
IL-1β (ICV)
1 hr
2 hrs
Brain tissue collection & EV purification
b
100nm
c
EV/mL (xE11)
Control
IL-1β
DDL-112/ IL-1β
d
DDL-112 ng/g brain
DDL-112 Brain Levels
e
kDa
CD63
~25
Synt-1
~30
PS
~100
IL-1β
IL-1β
DDL-112/IL-1β
Control
DDL-112/IL-1β
Control
f
CD63 OD (& control)
g
Synt-1 OD (& control)
Chronic Study
h
Challenging Beam Error/Step
Veh
DDL-112
i
Pole Time to Descend (sec)
j
OF Distance (cm)/Episode
k
Weight (g)
ns
l
PK-res aSyn Aggregates Area (%) Global SN
m
DDL-112 ng/g brain
DDL-112 Brain Levels

effect of IL-1β to activate nSMase2, polymorphisms in IL-1β are associated with increased risk for PD [38], and neuroinflammation mediated by IL-1β increases suseptability of dopaminergic neurons to degeneration in animal models [39]; thus IL-1β could induce an PD phenotype [40].

In the chronic study, Thy1-αSyn mice were treated orally with DDL-112 for 5 weeks but without any IL-1β stimulation as the goal of this study was to assess the long-term (5 week) effects of nSmase2 inhibition on behavior/motor function and proteinase K-resistant αSyn aggregate load in a key area of brain affected by αSyn, the *substantia nigra* (SN).

As indicated in Fig. 1a showing the experimental design of the acute study, male Thy1-αSyn PD model mice [26] received a single oral gavage dose of DDL-112 at 100 mg/kg, then one hour later were deeply anesthetized to receive 2 ng IL-1β ICV; two hours later, this group (DDL-112) mice were euthanized and brain tissue collected for the isolation of EVs for analysis. Other groups in the study included vehicle-only ICV injection (Control) and IL-1β ICV injection without DDL-112 pre-treatment (IL-1β). Further details of the experimental methods used in the acute in vivo study, performed using protocols approved by the Animal Care and Use Committee, can be found in Additional file 1.

EVs of 50–200 nm in size from each treatment condition were compared by Tunable Resistive Pulse Sensing (TRPS) analysis, which revealed that IL-1β ICV injection significantly increased EV release ($p < 0.05$) and that DDL-112 pre-treatment significantly ($p < 0.05$) suppressed the IL-1β-induced increase in EV/exosomes as shown in Fig. 1c. In the acute study, DDL-112 inhibition of IL-1β-induced EV/exosome release was seen in the presence of mean brain level of ~ 320 ng/g measured 3 h after dosing (Fig. 1d).

The EV/exosomes from the acute study mice were also analyzed by immunoblot, using probes for Synt-1 and pan-exosomal marker CD63. Representative blots are shown in Fig. 1e and Additional file 1: Figure S2A–C. CD63 was significantly increased in EV fractions by IL-1β injection ($p < 0.01$), and this increase was significantly decreased ($p < 0.01$) by DDL-112 pretreatment (Fig. 1f). Levels of Synt-1, a marker more specific for a subpopulation of exosomes generated through the syndecan-syntenin pathway [41], while not significantly different between groups, showed a similar pattern of mean levels being higher in the IL-1β treated group as compared to DDL-112 treated but with greater variability amongst mice (Fig. 1g).

In the collected EV/exosomes, while the αSyn levels were lower we did not see a significant difference among the fractions. A representative immunoblot for EV fractions probed with anti-human αSyn and densitometry analysis, and Ponceau S staining of the membrane are shown in Additional file 1: Fig. S2D–F.

The results of the acute study showing that EV/exosome release was suppressed by DDL-112 in Thy1-αSyn mouse brain tissue (Fig. 1c) prompted us to proceed with a chronic, 5-week study of daily oral treatment of Thy1-αSyn mice with DDL-112 that included behavioral/motor analysis and determination of affects on proteinase K-resistant (PK-res) αSyn aggregation in the SN.

In the chronic study, male Thy1-αSyn mice of ~ 3 months of age received 100 mg/kg/day DDL-112 ($n = 9$) orally or vehicle ($n = 8$) for 5 weeks. During the course of the study, one mouse in each group was euthanized due to the progression of motor dysfunction that is characteristic of this model. In the last week of treatment, mice underwent behavioral/motor function assessment in Open Field (OF) [42], pole [43], and Challenging Beam (CB) [44, 45] tests. Mice were then deeply anesthetized and perfused with saline before collection of brain tissue for IHC analyses [46, 47].

After 5 weeks of treatment, the DDL-112 group made significantly ($p < 0.05$) fewer errors/step in the CB test than the vehicle Tg group as shown Fig. 1h. In the pole test, DDL-112 treated Thy1-αSyn took significantly less time to descend (Fig. 1i), and in OF, the distance traveled per episode of movement—indicative of the hyperactivity that is characteristic of this model [26]—was reduced for DDL-112 treated mice (Fig. 1j). Other parameters typically measured as part of these motor tests were not signficantly different between vehicle- and DDL-112-treated mice. At the end of the 5 week treatment with DDL-112, there was no significant difference in mean weight between the DDL-112 treated and vehicle groups (Fig. 1k).

The presence of PK-res αSyn aggregates in the SN of Thy1-αSyn mice is a key distinguishing characteristic of brain tissue in this model and, more importantly, in human PD with Lewy bodies [48, 49]; thus any effects of DDL-112 treatment on the levels of these aggregates [50] mediated by EV-mediated proteoapathic spread [51] was the focus of our IHC analyses. The percent area comprising PK-res αSyn aggregates in the SN globally was significantly lower ($p < 0.05$) in DDL-112 treated mice as shown in Fig. 1l and Additional file 1: Fig. S3A, B. Analysis of correlation between PK-res αSyn aggregates in the SN and motor assessments in CB, pole, and OF tests (Additional file 1: Fig. S3C–3H) show positive correlations that they were greater for DDL-112 treated mice in all instances. These data suggest DDL-112 treatment was able to decrease further development of pathology within the dynamic range of pathology and motor performance relationships in this

model; for vehicle treated mice, pathology was beyond the dynamic range.

In the chronic study, we did not assess EV release in brain tissue due to the limitation of tissue available. EV isolation would have required too much tissue, preventing IHC analysis of αSyn aggregates. We also believe that determination of EV levels at a single time point without IL-1β stimulation of release would show only small differences between the treated and untreated groups and statistics would only be powered by use of very high n numbers.

The current in vivo studies support our previous in vitro findings [21] that the nSMase2 inhibitor, DDL-112, can suppress EV/exosome release and affect proteopathic seed propagation. The acute in vivo study shows that DDL-112 treatment results in suppression of EV release in the brain after IL-1β ICV injection, used to stimulate EV release. The chronic study demonstrated that 5-week DDL-112 treatment of Thy1-αSyn mice resulted in reduction of of PK-resistant αSyn aggregate accumulation and improved some aspects of motor function.

While our focus here is on nSMase2 inhibition by DDL-112 (cambinol), cambinol is also a known sirtuin 1 and 2 (SirT1/2) inhibitor [52], thus this mechanism and any potential effects on motor function and αSyn accumulation in the chronic study has to be considered. SirT1 has been demonstrated to affect lysosomal function and exosome secretion [53] as reported by Latifkar et al. who found that a reduction of SirT1 expression increased secretion of pro-tumorigenic exosomes [54]. Lee et al. showed that loss of SirT2 expression also increased the total number of EVs, albeit by a separate suggested mechanism than that of SirT1 [55]. Others have also reported an association between loss of SirT1 and increased EV/exosome release [56]. Based on these studies, if inhibition of SirT1 and/or 2 was implicated in treatment with DDL-112 then it could lead to increased EV release and might then be expected to exacerbate the spread of αSyn pathology, rather than ameliorate its spread as we observe in our chronic testing.

The role of SirT2 in PD is complicated. While reduction of its expression can increase EV release and potentiate disease pathology, sirtuin 2 inhibitors have been shown to block αSyn- mediated toxicity in PD models and thus could be a target for PD therapy [57, 58]. Conversely, SirT1 activation—not inhibition—has been found to be protective against αSyn-mediated toxicity, at least in cell models [59].

The potential for DDL-112 inhibition of SirT1/2 playing a role in the observed improvements in motor function and suppression of αSyn pathology is not supported by the brain levels of DDL-112 both in the acute study (Fig. 1d), as well as in the chronic study (Fig. 1m). Based on the reported inhibition potency of DDL-112 for these enzymes, the measured brain levels would likely cause a greater inhibition of brain nSMase2 enzyme activity (IC50 = 7.7 μM) [21], compared to inhibition of the enzymes SirT1 (IC50 = 56 μM) or SirT2 (IC50 = 59 μM) [60], making the role of sirtuin-mediated mechanism in DDL-112 in vivo effects unlikely.

Additional in vitro studies were performed to compare DDL-112 to potent SirT1 and SirT2 inhibitors used at 20 μM. While DDL-112 reduced EV levels, the SirT2 inhibitor increased EV release (Additional file 1: Fig. S4) providing further support to nSMase2, not SirT2, inhibition as the mechanism of action for DDL-112. The results from SirT1 inhibitor testing were inconclusive due to cell toxicity induced by the inhibitor. These findings support the likelihood that the mechanism by which DDL-112 reduced αSyn aggregates in the chronic in vivo study was due, at least in part, to inhibition of brain nSMase2 and EV release.

Others have shown that inhibition of nSMase2 decreases the transfer of oligomeric aggregates of αSyn in vitro between neurons and reduces accumulation/aggregation of high-molecular-weight α-Syn [61]. While nSMase2 inhibition has been reported to decrease tau propagation in vivo in a mouse model [20], the effects of nSMase2 inhibition on α-Syn propagation in brain in a PD model have not previously been reported.

nSMase2 is highly expressed in brain [62], with nSMase2 mRNA expression being reported to be highest in the striatum [63]. Normal phyiological levels of nSMase2 are thought to be important for protein clustering in lipid rafts. nSMase2 activity is upregulated with age [64] along with increases in long chain C24:1 ceramide levels in circulating serum EVs which can induce senescence in mesenchymal stem cells [65]. Senescence of dopaminergic neurons accompanied by a senescence-associated secretory phenotype (SASP) is suggested to be a contributing factor in the pathology of PD [66]. Increased exosome release is an integral part of SASP [67].

Mutations in the αSyn, E3 ubiquitin ligase Parkin, leucine-rich repeat kinase 2 (LRRK2), glucocerebrosidase (GBA), and acidic sphingomyelinase (SMPD1) genes—all known causes or risk factors for PD—have been linked to autophagy-lysosomal dysfunction, enhanced exosome biogenesis and exosomal αSyn load [68–73]. Manganese (Mn^{2+}) exposure, an environmental risk factor of Parkinsonism, was shown to enhance αSyn-bearing exosome release, which promotes cell-to-cell propagation of pathological αSyn species including by microglia [74]. Uptake of PD-patient plasma EVs by mouse microglia cells both in vitro and in vivo results

in the microglia-mediated release of αSyn-bearing exosomes, which then mediate spread of pathologic αSyn to neuronal cells [75]. Interestingly, contrary to the above examples of enhanced exosome release in PD, intracellular αSyn aggregates may increase degradation of Charged Multivesicular Body Protein 2B (CHMP2B/ESCRT-III), leading to disruption of ESCRT functions [76]. Loss-of-function mutations in ATPase cation transporting 13A2 (PARK9) also decrease intraluminal vesicle formation and exosomal release [12, 77], potentially through the ESCRT-dependent pathway. Collectively, this suggests a switch from the canonical ESCRT-dependent to a stress-induced nSMase2-dependent pathway of exosome biogenesis in PD. In agreement with this hypothesis, we demonstrate here that acute treatment with an nSMase2 inhibitor, DDL-112, affects EV release in the Tg more than in NTg mice (Additional file 1: Fig S1), decreases levels of αSyn aggregates in the SN, and improves motor functions in a PD mouse model.

Our acute in vivo study with the nSMase2 inhibitor DDL-112 shows that targeting this brain enzyme resulted in a reduction in IL-1β-mediated EV release and a trend to reduction in αSyn in the EV fraction (Additional file 1: Fig S2E). Our chronic study shows DDL-112 treatment is associated with a reduction in αSyn aggregates in the SN and improvement of motor function. These studies provide initial proof-of-concept and suggest inhibition of brain nSMase2 with molecules having improved potency and brain permeability could be a therapeutic strategy for treatment of PD. In the acute study we show that treatment with DDL-112 results in decreased EV levels and lowering of αSyn levels in EV fractions compared to vehicle, although these did not reach significance possibly due to limited animal numbers. While we did not measure EV levels in the chronic study due to the amount of tissue needed to analyse EVs, we detected DDL-112 levels in brain (Fig. 1m) that were similar to those we reported in Bilousova et al. [21] that were associated with inhibition of nSMase2 brain activity and thus could lead to a reduction of αSyn aggregates and improvement in motor function. In future studies, we will repeat and expand our acute IL-1β ICV mediated EV release testing paradigm to optimize the protocol for screening of additional brain permeable nSMase2 inhibitors that can decrease EV release and be used as drug candidates to suppress the spread of disease-specific proteopathic proteins.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13041-021-00776-9.

Additional file 1. Additional file.

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Authors' contributions
Participated in research design: CZ, TB, JC, AH, KG, VJ. Conducted experiments: CZ, TB, SF, MJ, CJE, MM, AH. Performed data analysis: CZ, TB, PS, CJE, SC, AH. Wrote or contributed to the writing of the manuscript: CZ, TB, PS, SF, JC, VJ. All authors read and approved the final manuscript.

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Availability of data and materials
All the data generated or analysed during this study are included in the manuscript or Additional file 1.

Declarations

Ethics approval and consent to participate
Animal care was performed in accordance with the United States Public Health Service Guide for the Care and Use of Laboratory Animals, with approval to the Drug Discovery Lab by the Institutional Animal Care and Use Committee at the University of California Los Angeles (UCLA).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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