Brain propagation of transduced α-synuclein involves non-fibrillar protein species and is enhanced in α-synuclein null mice

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Aggregation and neuron-to-neuron transmission are attributes of α-synuclein relevant to its pathogenetic role in human synucleinopathies such as Parkinson’s disease. Intraparenchymal injections of fibrillar α-synuclein trigger widespread propagation of amyloidogenic protein species via mechanisms that require expression of endogenous α-synuclein and, possibly, its structural corruption by misfolded conformers acting as pathological seeds. Here we describe another paradigm of long-distance brain diffusion of α-synuclein that involves inter-neuronal transfer of monomeric and/or oligomeric species and is independent of recruitment of the endogenous protein. Targeted expression of human α-synuclein was induced in the mouse medulla oblongata through an injection of viral vectors into the vagus nerve. Enhanced levels of intra-neuronal α-synuclein were sufficient to initiate its caudo-rostral diffusion that likely involved at least one synaptic transfer and progressively reached specific brain regions such as the locus coeruleus, dorsal raphae and amygdala in the pons, midbrain and forebrain. Transfer of human α-synuclein was compared in two separate lines of α-synuclein-deficient mice versus their respective wild-type controls and, interestingly, lack of endogenous α-synuclein expression did not counteract diffusion but actually resulted in a more pronounced and advanced propagation of exogenous α-synuclein. Self-interaction of adjacent molecules of human α-synuclein was detected in both wild-type and mutant mice. In the former, interaction of human α-synuclein with mouse α-synuclein was also observed and might have contributed to differences in protein transmission. In wild-type and α-synuclein-deficient mice, accumulation of human α-synuclein within recipient axons in the pons, midbrain and forebrain caused morphological evidence of neuritic pathology. Tissue sections from the medulla oblongata and pons were stained with different antibodies recognizing oligomeric, fibrillar and/or total (monomeric and aggregated) α-synuclein. Following viral vector transduction, monomeric, oligomeric and fibrillar protein was detected within donor neurons in the medulla oblongata. In contrast, recipient axons in the pons were devoid of immunoreactivity for fibrillar α-synuclein, indicating that non-fibrillar forms of α-synuclein were primarily transferred from one neuron to the other, diffused within the brain and led to initial neuronal injury. This study elucidates a paradigm of α-synuclein propagation that may play a particularly important role under pathophysiological conditions associated with enhanced α-synuclein expression. Rapid long-distance diffusion and accumulation of monomeric and oligomeric α-synuclein does not necessarily involve pathological seeding but could still result in a significant neuronal burden during the pathogenesis of neurodegenerative diseases.

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**Introduction**

The protein α-synuclein (α-syn, encoded by SNCA) is implicated in the pathogenesis of several neurodegenerative diseases that include Parkinson’s disease and are collectively referred to as synucleinopathies (Halliday et al., 2011). While the precise mechanisms underlying α-syn pathology in these diseases have yet to be fully defined, the tendency of α-syn to assemble into oligomeric and fibrillar aggregates and its ability to pass from donor to recipient neurons are likely to play important pathogenetic roles (Desplats et al., 2009; Uversky and Eliezer, 2009; Guo and Lee, 2014). Protein aggregation may ultimately lead to the formation of intracellular α-syn-containing inclusions, such as Lewy bodies and Lewy neurites, which are characteristic of human synucleinopathies (Spillantini et al., 1997; Tu et al., 1998). Neuron-to-neuron protein transmission could contribute to the spreading of α-syn pathology and explain, at least in part, the caudo-rostral pattern of α-syn accumulation first described in Parkinson’s disease brain by Braak and colleagues (Braak et al., 2003a, b); α-syn lesions are initially seen in the lower brainstem and subsequently diffuse toward mesocortical and neocortical areas via anatomically interconnected pathways.

Animal models have been developed in an attempt to mimic Parkinson’s disease-like α-syn spreading, to assess its relationship with protein aggregation and unravel mechanisms of progressive pathology. At least two experimental strategies have succeeded in triggering long-distance α-syn propagation in rodents. The first approach involves direct injections of α-syn-containing preparations into the mouse brain (e.g. into the striatum or substantia nigra). More precisely, inoculations consisted of: (i) tissue homogenates containing insoluble α-syn from the brains of synucleinopathy patients; (ii) pathological brain lysates from α-syn transgenic mice; or (iii) solutions of α-syn fibrils generated from recombinant protein (Luk et al., 2012a, b; Mougenot et al., 2012; Masuda-Suzukake et al., 2013; Sacino et al., 2013; Recasens et al., 2014; Peelaerts et al., 2015). A second paradigm to induce progressive α-syn build-up is based on injections of adeno-associated viral vectors (AAVs) carrying human α-syn DNA into the rat vagus (X) nerve. This treatment resulted in targeted transduction in the medulla oblongata and overexpression of human α-syn within medulla oblongata neurons connected to the rat vagus nerve. Interestingly, enhanced levels of intra-neuronal human α-syn were sufficient to trigger its trans-synaptic passage and caudo-rostral diffusion that, in a time-dependent fashion, reached pontine, midbrain and finally forebrain regions (Ulusoy et al., 2013, 2015). Similarities and differences between models of protein transmission bear important pathogenetic implications as they could elucidate toxic properties of α-syn relevant to human synucleinopathies. Two intriguing features characterize α-syn diffusion in mice receiving intraparenchymal α-syn injections. Following brain inoculations, the ensuing pathology was characterized by accumulation and propagation of insoluble forms of the protein (Luk et al., 2012a, b; Masuda-Suzukake et al., 2013; Sacino et al., 2013; Recasens et al., 2014; Peelaerts et al., 2015). Secondly, α-syn spreading in this model appeared to be contingent upon expression of endogenous α-syn and interactions between endogenous α-syn and deleterious forms of the protein present in the inoculates. A prion-like mechanism of aggregation and propagation has been suggested to underlie such interactions; indeed, similar to observations in prion models, no diffusion of pathology was observed if α-syn-containing preparations were injected into the brain of α-syn-deficient mice (Blättler et al., 1997; Angot et al., 2010; Luk et al., 2012a; Mougenot et al., 2012; Recasens et al., 2014).

The role played by non-fibrillar versus fibrillar α-syn remains unclear in animals in which protein transmission is triggered by its increased intraneuronal expression. Similarly, it is unknown whether the presence of endogenous α-syn affects protein aggregation and propagation under this latter experimental paradigm. The current study was designed to address these important questions. Following up on earlier work in rats (Ulusoy et al., 2013, 2015), a mouse model of AAV-mediated human α-syn transduction and caudo-rostral protein diffusion was developed and used to compare pathology in wild-type versus α-syn-deficient animals. Conformation-specific antibodies were used for immunohistochemical analyses correlating protein transmission with the accumulation of non-fibrillar and/or fibrillar α-syn species. Data revealed intriguing features of this model that apparently differ from observations in paradigms of prion-like α-syn spreading. In particular, we found that propagation of transduced α-syn throughout the mouse brain involved transfer and build-up of monomeric and oligomeric species, and was enhanced rather than counteracted by ablation of the endogenous protein.
Materials and methods

Vectors

Transgene expression of human α-syn or enhanced green fluorescent protein (GFP) was induced using recombinant AAVs (AAV 2/6; Vector Biolabs). Gene expression was under the control of the human SYN1 promoter and enhanced using a woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) and a polyadenylation signal sequence (Loeb et al., 1999; Ulusoy et al., 2013). Stock preparations were diluted to generate injection titres of 0.75 and 1.0 × 10^{13} gc/ml for human α-syn-AAV, and 1.0 × 10^{13} gc/ml for GFP-AAV.

Animals, surgical procedure and tissue preparation

Experiments were carried out in 8-week-old female mice. Mice harbouring a spontaneous Snca deletion (C57BL/J Ola Hsd) and corresponding wild-type controls (C57BL/JRj) were obtained from Harlan and Janvier Labs, respectively. Animals with a targeted Snca deletion on exons 1–2 (B6;129X1- Snca<sup>min1Ros</sup>) and wild-type controls (B6;129F2) were from Jackson Laboratory. Mice were housed with ad libitum access to food and water under a 12-h light/12-h dark cycle. Experimental protocols were approved by the State Agency for Nature, Environment and Consumer Protection in North Rhine Westphalia.

Following anaesthesia, a 1-cm incision was made at the midline of the neck. The left vagus nerve was isolated from the carotid artery, and vector solution (750 nl) was injected at a flow rate of 160 nl/min using a 36-gauge blunt steel needle fitted onto a 10 μl NanoFil syringe. The syringe was connected to an UltraMicroPump with control unit (World Precision Instruments). After injection, the needle was kept in place for two to three additional minutes.

Animals were killed under pentobarbital anaesthesia. Unless differently specified, they were perfused through the ascending aorta first with saline containing heparin and then with ice-cold 4% (w/v) paraformaldehyde. Brains were removed, immersion-fixed in 4% paraformaldehyde and cryopreserved in 30% (w/v) sucrose solution. Coronal sections (35 μm) throughout the brain were cut using a freezing microtome and stored at −20°C in phosphate buffer (pH 7.4) containing 30% glycerol and 30% ethylene glycol.

Western blot analysis and enzyme-linked immunosorbent assay

A list of antibodies used for these measurements is provided in Supplementary Table 1. Fresh tissue was used for these analyses. Brains were removed, and coronal brain blocks encompassing the entire medulla oblongata or pons were cut and used for dissection of the dorsal left (AAV injected side) and right (contralateral side) quadrants. Tissue was first homogenized and then sonicated in phosphate-buffered saline (pH 7.4) containing protease inhibitors (Roche) and 1% Triton<sup>®</sup> X-100 (Roth). Samples were centrifuged at 18 000g for 30 min, protein content was determined, and the supernatants (30 or 50 μg protein) were separated on a 12% acrylamide/bis-acrylamide gel. The gel was blotted on a polyvinylidene difluoride membrane (Millipore). Post-transfer membranes were treated with 0.4% paraformaldehyde as described by Lee et al. (2011) and blocked with 5% fat-free milk. Overnight incubations in mouse anti-human (h) α-syn (4B12, Genetex; 1:500), mouse anti-α-tubulin (DM1A, Sigma-Aldrich; 1:20 000) or rabbit anti-β-synuclein (ab6165, Abcam; 1:500) were followed by 1-h treatment with alkaline phosphate-conjugated anti-mouse or anti-rabbit IgG (Promega; 1:1000 or 1:10 000). Signal was detected by enhanced chemiluminescence (Applied Biosystems) and a ChemiDoc MP imaging system (Bio-Rad). Signal intensity was quantified by densitometry with Fiji software (Schindelin et al., 2012).

For quantification of human α-syn and total (mouse plus human) α-syn by enzyme-linked immunosorbent assay (ELISA), 384-well microplates were coated and incubated overnight at 4°C with 0.1 μg/ml sheep anti-α-syn (Syn-140) in 200 mM NaHCO<sub>3</sub>, pH 9.6 (50 μl/well). After washing and blocking, 50 μl of mouse brain lysate (30 μg/ml of protein concentration) were added to each well in duplicates. Tissue was dissected as described above, and homogenized in lysis buffer (CellLyte<sup>™</sup> M; Sigma) containing protease/phosphatase inhibitors (Pierce) and EDTA. After centrifugation (3000g for 30 min), supernatants were collected and used for analyses. For detection of total α-syn, samples were incubated at 37°C for 2 h with rabbit anti-α-syn (FL-140, Santa Cruz Biotechnology; 1:5000); for measurements of human α-syn, incubations were carried out with mouse anti-hα-syn (11D12; 1:1000). Plates were washed and then incubated for 2 h at 37°C with horseradish peroxidase-conjugated donkey anti-mouse IgG or goat anti-rabbit IgG (Jackson ImmunoResearch). Following a final wash, 50 μl/well of an enhanced chemiluminescent substrate (Pierce Biotechnology) was added. Chemiluminescence was measured with VICTOR<sup>™</sup> X3 plate reader (PerkinElmer). A standard curve was generated using serial dilutions of recombinant human α-syn.

Reverse transcription polymerase chain reaction

The four quadrants of paraformaldehyde-fixed coronal sections of medulla oblongata (Bregma: −7.08 to −7.64 mm) or rostral pons (Bregma: −5.68 to −5.02 mm) were dissected (Paxinos and Franklin, 2001). Total RNA was extracted using Nucleic Acid Isolation Kit (Ambion). cDNA was synthesized from 100 ng template RNA using SuperScript<sup>®</sup> VILO Master Mix (Life Technologies) (20 μl final volume). WPRE, mouse α-syn and hypoxanthine guanine phosphoribosyl transformase (Hprt) were assayed by conventional reverse transcription polymerase chain reaction (RT-PCR). The following primer combinations were used: (i) 5’caattccgtggtgttgtcgg forward and 5’caagggagctgggaatatag reverse (WPRE); (ii) 5’agtgcatccctctcgttct forward and 5’cagaggagtgggaattaatag reverse (mouse α-syn); and (iii) 5’tcctcctcagaccgctttt forward and 5’ctgctgctcctctcgcgtc reverse (Hprt). RT-PCR products obtained from 30 cycles were separated on 1.5% agarose gels. For quantitative RT-PCR, triplicate measurements were made using a StepOnePlus<sup>™</sup> real-time PCR system (Applied Biosystems). The reaction mix (20 μl) contained 1 μl cDNA, Power SYBR® Green (Applied Biosystems) and
overnight with pairs of these antibodies. Immunoreactivity was avoided detection of basal dilutions were adjusted to visualize highly expressed protein (Syn-O1 and Syn-O2; 1:1000 and 1:2000). AB5038P and Axioscope microscope (Carl Zeiss) under a 63× Plan-Apochromat objective. Analyses were performed by investigators blinded to treatment/experimental group. Length and density of human α-syn-containing axons were estimated using the Space Balls stereological probe (Stereo Investigator version 9, MBF Biosciences). A previously described method was adapted (Ulusoy et al., 2015). Measurements were made on three sections of the pons (Bregma: −5.68, −5.51 and −5.34 mm) where an area encompassing the locus coeruleus and the nucleus parabrachialis was delineated. A virtual hemisphere (10-μm radius) was placed randomly within this area, and systematic sampling was done at intervals of 60 μm in both x- and y-axes.

Fluorescence microscopy

To retrieve antigen-binding sites, free-floating medulla oblongata and pontine sections were treated with 2 μg/ml proteinase K (Sigma-Aldrich) for 10 min at room temperature. Six primary antibodies were used (Supplementary Table 1). Two of these antibodies recognize both monomeric and aggregated forms of the protein (AB5038P, Millipore; 1:400, and C-20, Santa Cruz Biotechnology; 1:1000). The other four antibodies, Syn-F1, Syn-F2, Syn-O1 and Syn-O2, are non-commercial and have been previously characterized (Vaikath et al., 2015). They recognize only mature amyloid α-syn fibrils (Syn-F1 and Syn-F2; 1:1000 and 1:500), or both α-syn fibrils and α-syn oligomers (Syn-O1 and Syn-O2; 1:1000 and 1:2000). AB5038P and C-20 dilutions were adjusted to visualize highly expressed protein avoiding detection of basal α-syn. Sections were incubated overnight with pairs of these antibodies. Immunoreactivity was then visualized with secondary antibodies conjugated with DyLight® 488 and DyLight® 594 (Vector Laboratories). Double-stained sections were analysed using a LSM700 Zeiss confocal microscope equipped with 488 and 555 nm excitation lasers. Stack images (1-μm intervals) were collected by sequential scanning using a 63× Plan-Apochromat objective.

A separate set of free-floating medulla oblongata and pontine sections were not subjected to antigen retrieval. They were sequentially incubated with syn211 (1:6000), biotinylated horse anti-mouse secondary and a streptavidine-conjugated fluorophore (Dylight® 594, Vector Laboratories; 1:200). Thioflavin S staining was performed on mounted syn211-immunolabeled sections. Sections were incubated for 8 min in 0.05% Thioflavin S dissolved in water, rinsed and then washed (3 min/wash) in 80, 95 and 95% ethanol. Slides were coverslipped with PVA-DABCO (Sigma), and tissue was analysed under a Zeiss LSM 710 NLO confocal microscope using 488 and 561 nm lasers with sequential acquisition.

Proximity ligation assay

The method originally described by Roberts et al. (2015) was used with modifications. To detect human–human α-syn interactions, syn211 antibody (Millipore) was conjugated with Plus or Minus oligonucleotide probes (Duolink® Probemaker kit, Olink). To detect mouse–human α-syn interactions, a murine-specific α-syn antibody (D37A6, Cell Signaling) was conjugated with Plus and syn211 antibody was conjugated with Minus probes (Supplementary Table 1). Antigen retrieval was achieved by incubation of free-floating sections with citrate buffer (pH 6.0) for 15 min at 95°C. After quenching, a proximity ligation assay (PLA) was performed using Duolink® Brightfield detection kit (Olink). Sections were blocked with Duolink blocking solution and then incubated overnight with PLA® Plus and Minus probes (1:100 dilution for human–human α-syn interactions; 1:40 and 1:200 for mouse–human α-syn interactions). Sections treated without the Minus probe served as negative controls. On the following day, ligation, amplification and detection were carried out according to manufacturer’s specifications, and the signal was developed with novaRED™ substrates. Sections were mounted on coated slides and, in some instances, counterstained with haematoxylin prior to coverslipping with Duolink® in situ brightfield mounting medium.

Statistical analyses

Unpaired t-test was used for comparisons of means between two groups. Analyses were performed using JMP Pro Statistical software (version 10.0.0; SAS Institute). Statistical significance was set at P < 0.05.

Results

Targeted expression of transgenic human α-syn in the mouse medulla oblongata

Experiments were carried out in control mice as well as mice lacking α-syn due to a spontaneous deletion of the α-syn gene (Specht and Schoepfer, 2001). In these mutant mice, absence of α-syn was not compensated by any significant increase in β-syn protein, a close homologue of α-syn (data not shown). AAVs carrying human α-syn DNA (SNCA) were injected into the left vagus nerve in
the mouse neck to induce targeted human $\alpha$-syn overexpression in the medulla oblongata. Successful transduction was already evident at 2 weeks post-AAV injection and could be demonstrated histologically as well as by western blot and PCR analyses. Immunostaining of medulla oblongata sections with a specific anti-$\alpha$-syn antibody (syn211) revealed localization of the exogenous protein that matched the anatomical distribution of efferent and afferent fibres forming the vagus (X) nerve. Efferent fibres are strictly ipsilateral and stem from cell bodies within the dorsal motor nucleus of the X nerve (DMnX) and nucleus ambiguus (Leslie et al., 1982). Viral vectors injected into the vagus nerve reached these two nuclei that contained cell bodies and neuronal projections robustly labelled for human $\alpha$-syn; staining of DMnX and nucleus ambiguous neurons was only seen on the left (ipsilateral to the injection side) side of the brain (Fig. 1). Afferent fibres of the vagus nerve originate from neuronal cell bodies in the inferior and superior vagal ganglia. They convey sensory information mostly to the ipsilateral nucleus of the tractus solitarius, but also innervate the contralateral medulla oblongata (Kalia and Sullivan, 1982; Leslie et al., 1982; Odekunle and Bower, 1985). Consistent with this bilateral pattern of distribution, human $\alpha$-syn-stained axons occupied the left nucleus of the tractus solitarius but also crossed the midline to reach the dorsal medulla oblongata on the right side of the brain (Fig. 1A). The dense arborization of labelled fibres contrasted with the absence of human $\alpha$-syn-containing cell bodies in the nucleus of the tractus solitarius and other terminal fields of vagal afferents. Of note, pattern and intensity of human $\alpha$-syn immunoreactivity were similar in medulla oblongata sections from either control or mutant mice (Fig. 1).

Western blot analyses carried out at 2 weeks post-AAV injection showed robust bands immunoreactive for human $\alpha$-syn in specimens from the dorsal left (injected side) medulla oblongata; human $\alpha$-syn protein was also detected, albeit in the form of weaker bands, in the dorsal right medulla oblongata contralateral to vagal injections (Fig. 2A). Semi-quantitative densitometric measurements revealed no significant differences in levels of the transduced protein between wild-type controls and mice lacking $\alpha$-syn (Fig. 2B). In contrast to findings in the medulla oblongata, samples from the pons were devoid of human $\alpha$-syn at this 2-week time point (Fig. 2A). Human $\alpha$-syn protein was also assayed by ELISA in the dorsal left and right medulla oblongata. Data confirmed that expression of the transduced protein was similar in wild-type and mutant mice; in both groups of animals, values were ~4 times higher in the left as compared to the right medulla oblongata (Fig. 2C). ELISA measurements were then carried out to compare levels of total (mouse plus human) $\alpha$-syn in the dorsal left medulla oblongata of untreated versus AAV-injected wild-type mice. Total $\alpha$-syn was increased by ~50% as a consequence of AAV administration (Fig. 2D). This increase was found in whole tissue specimens that, in injected mice, contained both transduced and non-transduced neurons. It is noteworthy,

therefore, that levels of $\alpha$-syn overexpression in these treated animals are likely to be significantly greater within cells targeted by AAV transduction.

Transgene expression at the mRNA level was assessed by quantitative RT-PCR after amplification with human $\alpha$-syn (SNCA) specific primers. To compare expression in control versus $\alpha$-syn-deficient mice, SNCA mRNA levels were quantified relative to RNA extracted from human brain. At 2 weeks post-AAV treatment, SNCA mRNA was detected in tissue from the dorsal left but not the dorsal right medulla oblongata (Fig. 2E); this finding is consistent with the presence of transduced neuronal cell bodies in the DMnX on the side of the brain ipsilateral but not contralateral to vagal injections. Levels of human $\alpha$-syn expression in left DMnX-containing tissue were comparable between control animals and mice lacking $\alpha$-syn (Fig. 2E).
To further demonstrate confinement of AAVs within targeted areas of the medulla oblongata, measurements of WPRE (an enhancer element incorporated into the AAV genome) mRNA were used as markers of AAV transduction. Assays were initially carried out in mice sacrificed 2 weeks after AAV injection. After amplification using WPRE-hybridizing primers, RT-PCR analysis showed a pattern of tissue-specific expression characterized by strong and faint bands in samples from the dorsal left and ventral left medulla oblongata, respectively (Fig. 2F). This pattern, evident in both control and mutant mice, reflects transduction of DMnX (dorsal medulla oblongata) and nucleus ambiguus (ventral medulla oblongata) neurons ipsilateral (left) to AAV injections. No evidence of AAV-
derived mRNA was found in the right medulla oblongata as well as in the left or right pons (Fig. 2F). Similar measurements were then performed in medulla oblongata, pontine and midbrain specimens collected at 6 and 12 weeks post-AAV injection. Even at these later time points, specific WPRE bands were detected in the medulla oblongata but not in other brain regions rostral to it (Fig. 2G).

Caudo-rostral propagation of human α-syn in wild-type mice

Tissue sections from mice killed at different time points post-AAV injection were immunostained with syn211 antibody. While no immunoreactivity was detected in the pons, midbrain and forebrain at 2 weeks, neuronal projections positive for human α-syn were observed in brain regions rostral to the medulla oblongata at later time points. In wild-type mice injected with \(0.75 \times 10^{13}\) gc/ml AAV, a few human α-syn-containing axons occupied the pons (Bregma: \(-5.40\) mm), caudal midbrain (cMB; Bregma: \(-4.60\) mm), rostral midbrain (rMB, Bregma: \(-3.40\) mm) and forebrain (FB; FB1, Bregma: \(-2.18\) mm; FB2, Bregma: \(-0.94\) mm; FB3, Bregma: \(+0.14\) mm). Findings at 6 weeks were then compared to fibre counts in tissue sections from animals killed at 12 weeks after AAV transduction. The number of stained axons was significantly increased in mice treated with either of the two AAV titres at this later time point (Fig. 3A and Supplementary Fig. 1A).

Figure 3 Counts of human α-syn-immunoreactive axons in AAV-injected mice. Wild-type (WT, A) and α-syn-deficient (−/−, B) mice received a single injection of human α-syn-carrying AAVs (0.75 or \(1.5 \times 10^{13}\) gc/ml) into the left vagus nerve. Analyses were performed at 6 and 12 weeks post-treatment. Tissues were immunostained with an anti-hα-syn antibody, and human α-syn-positive axons were counted in the left (injected side) hemisphere of coronal sections from the: pons (Bregma: \(-5.40\) mm), caudal midbrain (cMB; Bregma: \(-4.60\) mm), rostral midbrain (rMB, Bregma: \(-3.40\) mm) and forebrain (FB; FB1, Bregma: \(-2.18\) mm; FB2, Bregma: \(-0.94\) mm; FB3, Bregma: \(+0.14\) mm). Error bars are + SEM. Statistical comparisons are reported in Supplementary Table 2.
Furthermore, consistent with progressive caudo-rostral propagation, neuritic projections immunoreactive for the exogenous protein were found in sections of the forebrain; most rostral outposts were at Bregma −2.18 and −0.94 mm after injections with the lower and higher AAV titre, respectively.

To further rule out the possibility that neuron-to-neuron translocation of viral vectors rather than inter-neuronal protein transfer was responsible for human α-syn transmission, a group of mice received vagal injections of GFP-rather than human α-syn-carrying AAVs. Results of these experiments showed that, even in the presence of robust transgene expression in the medulla oblongata, GFP-containing neurons were virtually absent in tissue sections from the pons, midbrain and forebrain of mice killed at either 6 or 12 weeks after GFP-AAV treatment (Supplementary Fig. 2).

**Human α-syn propagation in α-syn-deficient mice**

Counts of neuronal projections immunoreactive for human α-syn were carried out in brain tissue from α-syn-deficient mice injected with human α-syn-AAVs. Regardless of whether animals were treated with the lower or higher AAV titre and whether analyses were performed at the 6- or 12-week time point, the number of human α-syn-positive axons was at least twice greater in brain regions rostral to the medulla oblongata of mutant mice as compared to wild-type controls (Fig. 3B, Supplementary Fig. 1B and Supplementary Table 2). In the former group of animals, the advancing protein also reached areas that were considerably more anterior; in particular, when animals lacking α-syn were killed at 12 weeks after treatment with the higher AAV titre, human α-syn-immunoreactive fibres could be seen in forebrain sections as rostral as Bregma + 0.14 mm.

Caudo-rostral brain propagation of human α-syn followed a stereotypical pattern of topographical distribution and affected predilection sites that were alike between control and mutant mice. They included the coeruleus-subcoeruleus complex and parabrachial area in the pons (Fig. 4A and B), the dorsal raphae and periaqueductal grey in the midbrain (Supplementary Fig. 3A and B), the hypothalamus in the diencephalon and the amygdala in the medial temporal lobe (Supplementary Fig. 3C and D). Axons loaded with human α-syn appeared as beaded winding threads, and immunoreactivity was typically more robust within the irregularly spaced varicosities (Fig. 4B and Supplementary Fig. 3B and D).

Differences in human α-syn diffusion between wild-type and α-syn-deficient mice were confirmed by measurements of the length and density of human α-syn-labelled fibres using the Space Balls stereological tool. A region encompassing the coeruleus and parabrachial complexes (CPC) was delineated throughout serial pontine sections from animals injected with 1.5 × 10^{13} gc/ml AAV and killed at 6 and 12 weeks post-treatment. Data obtained by unbiased CPC sampling revealed that both the mean total length and density of human α-syn-immunoreactive axons were significantly greater in mutant as compared to control mice; ~4- and 2-fold differences were seen at 6 and 12 weeks post-treatment, respectively (Fig. 4C and D).

A separate set of experiments was carried out in a different line of α-syn-deficient mice generated by targeted disruption of exons 1 and 2 of the α-syn gene (Abeliovich et al., 2000). Knockout mice and genetically matched controls received vagal human α-syn-AAV injections (1.5 × 10^{13} gc/ml) and were sacrificed at 5 weeks post-treatment. Counts of human α-syn-positive axons showed higher numbers in the pons and caudal midbrain of animals lacking endogenous α-syn (Fig. 5A). Most rostral areas occupied by labelled fibres were in the caudal and rostral midbrain in control and knockout mice, respectively. Enhanced propagation in the absence of endogenous α-syn was confirmed by Space Balls measurements of human α-syn-immunoreactive axons in the CPC; data revealed 2-fold increases in fibre length and density in α-syn-deficient as compared to α-syn-expressing animals (Fig. 5B and C).

**Relationship between protein overexpression, aggregation and propagation**

Immunohistochemical analyses were carried out on tissue sections from the medulla oblongata and pons using a variety of anti-α-syn antibodies. Two of these antibodies (AB5038P and C-20) recognize both monomeric and aggregated forms of the protein. The other antibodies display conformation-specific reactivity towards aggregated forms of the protein. A progressive burden of aggregate pathology is suggested by the observation that, with both α-syn oligomers (Syn-O1, Syn-O2 and 5G4) (Kovacs et al., 2012; Vaikath et al., 2015). All antibodies stained transduced medulla oblongata tissue containing the DMnX, nucleus ambiguus and nucleus of the tractus solitarius and, with all antibodies, no significant differences in labelling distribution and intensity were observed between wild-type and α-syn-deficient mice (Fig. 6 and Supplementary Fig. 4). Representative images in Fig. 6 show α-syn-containing neuronal projections in the DMnX stained with AB5038P, Syn-F1 and Syn-O2. Immunoreactivity for AB5038P revealed a dense fibre pattern that likely reflected the presence of both monomeric and aggregated α-syn. Of the two aggregate-specific antibodies, Syn-O2 revealed a denser network of immunoreactive neuronal processes than Syn-F1 (Fig. 6C and D versus Fig. 6A and B), consistent with its ability to detect oligomeric as well as fibrillar forms of the protein. A progressive burden of aggregate pathology is suggested by the observation that, with both Syn-F1 and Syn-O2, a more pronounced pattern of immunoreactivity was seen at 12 weeks as compared to 6 weeks.
post-vagal injection (Fig. 6). Results obtained with AB5038P, Syn-F1 and Syn-O2 matched the pattern of immunoreactivity seen in medulla oblongata tissue stained with C-20, Syn-F2 and Syn-O1, respectively (Supplementary Fig. 4). Moreover, labelling with 5G4 revealed close similarities with the staining produced by Syn-O1 or Syn-O2 (data not shown).

To further assess the formation of amyloid fibrils, sections of the medulla oblongata were immunolabelled with anti-h\(\alpha\)-syn and then stained with Thioflavin S. In these sections, a number of neurons (mostly neuronal projections) immunoreactive for human \(\alpha\)-syn were also stained with Thioflavin S (Fig. 7A and B). To confirm protein oligomerization, samples were processed by PLA. This technique allows labelling of adjacent \(\alpha\)-syn molecules and has recently been used to detect non-fibrillar, most likely oligomeric \(\alpha\)-syn in histological brain specimens from patients with Parkinson’s disease (Roberts et al., 2015). Two
separate sets of PLA probes were designed to recognize self-interaction between human \(\alpha\)-syn molecules or to label human \(\alpha\)-syn in close proximity to mouse \(\alpha\)-syn. Specific human–human \(\alpha\)-syn signal was present in medulla oblongata tissue from either wild-type or \(\alpha\)-syn-deficient mice injected with AAV (Fig. 7C). Immunoreactivity for human–mouse \(\alpha\)-syn was also detected, but only in sections from AAV-injected wild-type animals; in these mice, in situ amplification revealed a stronger signal for human–human \(\alpha\)-syn than human–mouse \(\alpha\)-syn interactions (Fig. 7C versus Fig. 7D).

A final set of analyses was carried out on pontine tissue from AAV-transduced wild-type and \(\alpha\)-syn-deficient mice. Sections were double-stained with either AB5038P plus Syn-F1 or AB5038P plus Syn-O2. Labelling with the first pair of antibodies showed no evidence of Syn-F1 immunoreactivity within axons positive for total \(\alpha\)-syn at either 6 (Supplementary Fig. 5A and B) or 12 weeks (Fig. 8A and B) post-treatment. In contrast, staining with AB5038P and Syn-O2 revealed co-localization of immunoreactivity at both time points (Supplementary Fig. 5C and D, and Fig. 8C and D). Thioflavin S staining and human–human \(\alpha\)-syn PLA were used to gain further evidence consistent with absence of \(\alpha\)-syn amyloid fibrils and presence of \(\alpha\)-syn oligomers, respectively. No reactivity for Thioflavin S was detected within pontine axons affected by human \(\alpha\)-syn propagation (data not shown). On the other hand, when pontine tissue was processed for human–human \(\alpha\)-syn PLA, specific labelling was observed only in sections from AAV-injected mice; reactivity was particularly intense within axonal swellings, conferring an image of punctate fibre profiles on single-plane microscopy (Fig. 8E).

**Figure 5** Count, length and density of human \(\alpha\)-syn-positive axons in control versus \(\alpha\)-syn-knockout mice. Control and \(\alpha\)-syn-knockout (KO) mice received a single injection of human \(\alpha\)-syn-carrying AAVs (1.5 \(\times\) 10\(^7\) gc/ml) into the left vagus nerve. Analyses were performed at 5 weeks post-treatment when brain tissue sections were immunostained with an anti-h\(\alpha\)-syn antibody. (A) Human \(\alpha\)-syn-positive axons were counted in the left (injected side) hemisphere of coronal sections from the: pons (Bregma: \(5.40\) mm), caudal midbrain (cMB; Bregma: \(6.60\) mm) and rostral midbrain (rMB, Bregma: \(-3.40\) mm). Error bars are + SEM. Unpaired t-test comparing data in knockout mice versus the corresponding values in control animals. \(F(1,8) = 3.569\) in the pons, and \(F(1,8) = 8.143\) in the caudal midbrain. *\(p < 0.05\); **\(p < 0.005\).

Length (B) and density (C) of human \(\alpha\)-syn-immunoreactive axons were estimated in a pontine area encompassing the locus coeruleus and the nucleus parabrachialis. Error bars are + SEM. Unpaired t-test comparing data in knockout mice versus the corresponding values in control animals. \(F(1,8) = 4.830\) for length comparison; \(F(1,8) = 1.175\) for density comparison. **\(p < 0.005\); ***\(p < 0.0005\).

**Discussion**

Inter-neuronal transfer and brain spreading of proteins implicated in human neurodegenerative diseases may be facilitated by interactions between pathological forms of the proteins generated within donor cells and the corresponding normal protein expressed within recipient neurons (Guo and Lee, 2014). Extending earlier work in rats (Ulusoy et al., 2013, 2015), we generated a mouse model in which protein propagation was triggered by overexpression of human \(\alpha\)-syn within donor neurons in the medulla oblongata. Spreading was then compared between wild-type mice and \(\alpha\)-syn-deficient animals, i.e. in the presence and absence of endogenous \(\alpha\)-syn expression within recipient cells. We found that intravagal injections of viral vectors carrying human \(\alpha\)-syn DNA were indeed capable of inducing targeted overexpression of human \(\alpha\)-syn in the mouse medulla oblongata, which was followed by caudo-rostral protein diffusion. The rate and extent of this propagation were dependent upon the transduction titre and progressed over time following a stereotypical pattern of anatomical distribution. Starting at 6 weeks post-vaginal injection, immunoreactivity for human \(\alpha\)-syn could be detected within axons first in the pons, then in the midbrain and finally in the forebrain. Human \(\alpha\)-syn reached areas such as the coeruleus–subcoeruleus complex (pons),
dorsal raphae (midbrain) and amygdala (medial temporal lobe) that have no direct anatomical connections with the vagus (X) nerve; data are therefore consistent with trans-synaptic passage of the protein from donor medulla oblongata neurons to recipient axons projecting into the medulla oblongata from higher brain regions. To assess potential differences in protein transfer due to expression of endogenous /C0-syn, human /C0-syn-positive axons were counted in wild-type versus /C0-syn-deficient mice. This comparative analysis yielded intriguing results. Protein propagation was not enhanced by /C0-syn expression within recipient cells. On the contrary, it was significantly more pronounced in animals lacking endogenous protein. In this group of mice, counts of human /C0-syn-positive axons were consistently greater in the pons, midbrain and forebrain, and human /C0-syn diffusion toward regions rostral to the medulla oblongata was also more advanced.

Extensive efforts were focused on elucidating mechanisms of differential protein transmission in the presence and absence of endogenous /C0-syn. Increased propagation in /C0-syn-deficient mice was not a mere consequence of enhanced human /C0-syn expression as AAV-induced transduction, assessed at both the mRNA and protein levels, was comparable in control mice and mice lacking /C0-syn. Quite importantly, more pronounced diffusion in the absence of endogenous /C0-syn was observed in two separate lines of /C0-syn-deficient animals, one carrying a spontaneous deletion of the /C0-syn (Snca) gene and the other generated through genetic engineering techniques (Abeliovich et al., 2000; Specht and Schoepfer, 2001). This observation makes it unlikely that differences in protein transmission seen in this study may arise from factors (e.g. the genetic background of mice with spontaneous /C0-syn loss) unrelated to /C0-syn deficiency. Lack of /C0-syn may be compensated by enhanced expression of its homologue β-syn (encoded by Snch) that could in turn modify pathological features triggered by human /C0-syn transduction in mutant mice (Hashimoto et al., 2004). A role of β-syn in modulating

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**Figure 6** Immunohistochemical characterization of aggregated /C0-syn in the DMnX. Wild-type (WT, A and C) and /C0-syn-deficient (−/−, B and D) mice received a single injection of human /C0-syn-carrying AAVs (1.5 × 10^{13} gc/ml) into the left vagus nerve. Analyses were performed at 6 and 12 weeks post-treatment. Representative sections of the medulla oblongata (Bregma: –7.32 mm) were double-immunostained with either (i) an antibody against total (monomeric and aggregated) /C0-syn (AB5038P) plus an antibody that specifically recognizes /C0-syn fibrils (Syn-F1) (A and B); or (ii) AB5038P plus an antibody that recognizes oligomeric and fibrillar forms of the protein (Syn-O2) (C and D). Images of the left (injected side) DMnX are shown. Scale bars = 15 μm.
human α-syn propagation was ruled out, however, by findings showing that its levels were similar in the brain of wild-type and α-syn-deficient animals. Changes in protein conformation and aggregation have been suggested to affect α-syn’s tendency to pass from one neuron to another and could therefore explain variations in protein spreading (Lee et al., 2005, 2008; Angot and Brundin, 2009; Freundt et al., 2012). Immunohistochemical analyses carried out as part of this study did not detect overt differences in monomeric, oligomeric and fibrillar α-syn in the medulla oblongata of control versus α-syn-deficient mice. Nonetheless, it is still conceivable that generation and accumulation of
discrete α-syn species (e.g. specific oligomeric forms of the protein) characterize these two groups of animals, contributing to enhancement or suppression of human α-syn diffusion (Danzer et al., 2007). Different aggregate species with greater or lower tendency to propagate could be generated, for example, by distinct interactions of endogenous and/or exogenous α-syn with lipid surfaces in wild-type versus α-syn null animals (Barceló-Coblijn et al., 2007; Galvagnion et al., 2015).

Results of this study support the interpretation that decreased or enhanced protein transmission could be a consequence of human α-syn expression on a normal versus null α-syn background since, depending on this background, important differences in protein–protein interactions were found. Self-interaction of adjacent molecules of human α-syn was detected in mutant mice, whereas both human–human α-syn and human–mouse α-syn interactions were observed in AAV-injected wild-type animals. A variety of mechanisms influenced by α-syn interactions could ultimately modify protein propagation. One of these mechanisms may be the formation of distinct α-syn aggregates (see above), as in vitro evidence indicates that both rates and pathways of protein assembly were dramatically different in incubations of human α-syn alone, mouse α-syn alone or human and mouse α-syn together (Rochet et al., 2000). Furthermore, changes in protein–protein interactions in wild-type versus null α-syn mice, as identified in the present study, could diversely affect neuronal functions relevant to intercellular transfer and brain propagation of the protein, such as synaptic vesicle exo- and endocytosis and axonal transport (Cabin et al., 2005; Nemani et al., 2010; Prasad et al., 2011; Prots et al., 2013; Vargas et al., 2014).

Another important aim of this study was to assess the relationship between α-syn aggregation and propagation and, in particular, to determine if protein transmission was associated with the accumulation of oligomeric and/or fibrillar α-syn. Using several antibodies with distinct affinities toward aggregated forms of the protein (Kovacs et al., 2012; Vaikath et al., 2015), data revealed that both oligomeric and fibrillar aggregates were formed within donor neurons in the mouse medulla oblongata. In contrast, when recipient pontine axons were stained with either antibodies that recognize oligomeric and fibrillar α-syn or antibodies specific for α-syn amyloid fibrils, immunoreactivity was only observed with the former reagents. Taken together, these findings are consistent with the conclusion that, in this model, fibrillar α-syn is not transferred from donor to recipient neurons, nor is it required for caudo-rostral propagation and axonal accumulation of α-syn. On the other hand, the presence of oligomeric α-syn aggregates within these axons is compatible with two non-mutually exclusive interpretations. First, small α-syn aggregates may be capable of passing from one neuron to another and diffusing along axonal projections. The second possibility is that inter-neuronal transfer primarily involves monomeric α-syn that, once crowded within recipient axons, undergoes aggregation into oligomeric species. Axonal build-up of oligomeric α-syn aggregates was confirmed by analysis of tissue sections processed for α-syn PLA and was accompanied by evidence of pathological changes (e.g. thread-like axonal swelling). Experimental data linking α-syn transmission and oligomerization to axonal pathology bears significant implications. They support a primary role of non-fibrillar α-syn in early axonal injury. They also underscore the potential relevance of our experimental model to pathogenetic processes in Parkinson’s disease since, in a recent report, neuritic accumulation of PLA-positive oligomeric α-syn was suggested to be an early pathological feature of Parkinson’s disease brain (Roberts et al., 2015).

The readiness of monomeric and/or oligomeric human α-syn to pass from one neuron to another and propagate within the mouse brain, as indicated by our present findings, is in line with results of an earlier work by Rey et al. (2013). In this previous study, different molecular species of human α-syn, i.e. monomeric, oligomeric or fibrillar, were directly injected into the mouse olfactory bulb; monomeric and oligomeric, but not fibrillar α-syn were shown to rapidly and efficiently diffuse along olfactory bulb-connected neuronal pathways. Important differences can instead be noted between results of this current study and features that characterize other models of α-syn propagation in which pathological forms of the protein are directly inoculated into the brain (Luk et al., 2012a, b; Masuda-Suzukake et al., 2013; Sacino et al., 2013; Recasens et al., 2014). Taking these differences into consideration, one could conclude that long-distance diffusion of α-syn occurs through at least two mechanisms. One of these mechanisms involves inter-neuronal transfer of non-fibrillar protein that diffuses via axonal projections and directly damages these recipient neurons; trans-synaptic passage, axonal propagation and pathology are seemingly lessened when interactions between human and mouse α-syn occur. The second paradigm of protein spreading is triggered by exposure to insoluble α-syn and characterized by pathological accumulation of Thioflavin S-positive fibrillar aggregates. Under these experimental conditions, propagation appears to require expression of endogenous α-syn that may change conformation and become aggregated due to the seeding properties of a pathological form of the protein (Guo and Lee, 2014).

It could be speculated that different mechanisms of α-syn diffusion may play distinct roles under varying pathophysiological conditions. On the other hand, they may not necessarily represent diverging pathological pathways but act in sequence or synergistically during neurodegenerative processes. For example, highly mobile oligomeric forms of the protein, which may be predominantly accumulated at early pathological stages, could later acquire seeding properties that would fuel aggregation reactions, promote fibrillation and give rise to the deposition of intra-neuronal inclusions. Fibril formation may also signify progression toward a more aggressive neurotoxicity.
(Peelaerts et al., 2015). Alternatively or in addition, it is conceivable that initial build-up of oligomeric α-syn may predispose to secondary toxic 'hits' (e.g. oxidative stress and impairment of protein degradation pathways) that would induce seeding-competent conformational changes and trigger more advanced aggregate pathology.

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Supplementary material

Supplementary material is available at Brain online.

References


