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Non-invasive delivery of an α -synuclein gene silencing vector with MR-guided focused ultrasound

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Abstract

Background: The characteristic progression of Lewy pathology in Parkinson's disease likely involves intercellular exchange and accumulation of misfolded α -synuclein amplified by a prion-like self-templating mechanism. Silencing of the α -synuclein gene could provide long-lasting disease modifying benefits by reducing the requisite substrate for the spreading aggregation.

Objectives: Due to poor penetration of viral vectors across the blood-brain barrier, gene therapy for central nervous system disorders requires direct injections into affected brain regions, and invasiveness is further increased by the need for bilateral delivery to multiple brain regions. Here we test a non-invasive approach by combining low intensity MR-guided focused ultrasound and intravenous microbubbles that can transiently increase the access of brain impermeant therapeutic macromolecules to targeted brain regions.

Methods: Transgenic mice expressing human α -synuclein were subjected to MR-guided focused ultrasound targeted to four brain regions (hippocampus, substantia nigra, olfactory bulb, and dorsal motor nucleus) in tandem with intravenous microbubbles and an adeno-associated virus serotype 9 vector bearing a short hairpin RNA sequence targeting the α -synuclein gene.

Results: One month following treatment, α -synuclein immunoreactivity was decreased in targeted brain regions, whereas other neuronal markers such as synaptophysin or tyrosine hydroxylase were unchanged, and cell death and glial activation remained at basal levels.

Conclusions: These results demonstrate that MR-guided focused ultrasound can effectively, non-invasively, and simultaneously deliver viral vectors targeting α -synuclein to multiple brain

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Conflicts of interest:

areas. Importantly, this approach may be useful to alter the progression of Lewy pathology along selected neuronal pathways, particularly as prodromal PD markers improve early diagnoses.

Keywords

Parkinson's disease; Gene therapy; Adeno-associated virus; Inhibitory RNA; Blood-brain barrier

INTRODUCTION

Alpha-synuclein (α -syn) is an abundant presynaptic protein in mammalian brains^{1, 2} and a key pathogenic factor in Parkinson's disease (PD) and related synucleinopathies. Aggregated and phosphorylated α -syn accumulates progressively in affected individuals causing selective neuronal dysfunction and degeneration^{3–6}. Braak staging of PD Lewy pathology suggests that the initial appearance of α -syn aggregates in the central nervous system (CNS) occurs in the dorsal motor nucleus of the vagus (DMN) in the lower brainstem and in the olfactory bulb (OB), followed by a rostral progression of pathology into the midbrain and cerebral cortex^{7–9}. These early manifestations of synucleinopathy correspond to prodromal non-motor features of PD, including constipation, due to impaired vagal innervation of the gastrointestinal tract, and a loss of olfaction¹⁰.

The spreading Lewy pathology is linked to α -syn's ability to self-template in a manner similar to prions^{11–14}. α -Syn can adopt stable conformations with high beta-sheet content and misfolded variants can confer their structure onto additional α -syn molecules. In multiple experimental cell and animal models, α -syn pathology spreads to nearby cells or along neuronal tracts following inoculation with either fibrillar α -syn or synucleinopathy-sourced brain homogenates^{15–22}. The underlying process, initially postulated to explain Lewy pathology within fetal grafts implanted in PD patients^{23, 24}, implicates a general spreading cascade whereby exchange of misfolded α -syn is coupled to its interaction with native α -syn in recipient neurons²⁵.

The inherent requirement for α -syn expression in recipient neurons for permissive templating is consistent with PD genetics showing that elevated α -syn expression by gene multiplication raises the risk for PD^{26–28}. Conversely, therapeutic down-regulation of α -syn expression may be a viable approach to delay or block spreading pathology in PD²⁹. Several studies have reported α -syn knockdown in rodents or primates^{30–38} and as protection against dopaminergic degeneration induced by either rotenone or α -syn fibrils^{39–41}. However, a key challenge to adapting putative therapeutics for clinical use is the low penetrability of the blood-brain barrier (BBB) to molecules larger than ~400 Da and of poor lipid solubility, thereby limiting the effectiveness of systemically delivered immuno- or gene therapies for brain disorders⁴². CNS entry of therapeutic molecules such as immunoglobulin, growth factors, or non-viral gene sequences are enhanced by conjugation to brain-penetrant ligands (molecular Trojan horse) recognized by an endogenous transporter⁴³. For example, encapsulation of α -syn siRNA into exosomes labeled with a brain-targeting viral peptide³⁴ or direct conjugation to a monoamine reuptake inhibitor³⁷ reduced α -syn expression in brain neurons. One limitation of non-viral gene expression is its transient effect, such that treatments must be repeated to maintain gene regulation.

Stable gene expression lasting more than a decade can be safely achieved with viral vectors^{44, 45}, although brain delivery requires intracerebral injections, which are invasive and carry risks of infection, hemorrhage, and neuronal damage around the injection site⁴⁶. Moreover, the practicality of intracerebral injections is diminished by the need for multiple injections to cover an entire brain region or bilateral coverage in multiple brain regions. Alternatively, transcranial magnetic resonance (MR)-guided focused ultrasound (FUS) combined with microbubbles injected into the bloodstream can locally and transiently increase the permeability of the BBB for several hours after sonication⁴⁷. This approach permits brain targeted delivery of systemically administered therapeutics, such as immunoglobulin or viral vectors, efficiently across the BBB without invasive surgery^{48–58}. Here, we use FUS to deliver a virally-expressed short hairpin RNA (shRNA) to silence the α -syn gene in multiple discrete brain regions that are known to develop Lewy pathology in PD. Our study demonstrates the feasibility of simultaneous modulation of α -syn expression in distal brain regions without multiple invasive procedures.

METHODS

Virus

Recombinant adeno-associated virus serotype 9 (AAV9) was utilized to express shRNA targeting human α -syn to knockdown human α -syn gene expression in our transgenic mouse model. Plasmids containing a silencing sequence targeting nucleotides 299–309 of the human α -syn gene (AAV9-hSNCA-shRNA)³³ were generously provided by Dr. Martha C. Bohn (Northwestern University). Control plasmids contain a scrambled sequence of the same nucleotides (AAV9-Scr-shRNA). Both vectors used in this study, and as previously reported⁵⁹, contain short mir-30 microRNA insertions flanking the 5' and 3' ends of the silencing sequence, which can reduce cell death associated with hSNCA-shRNA. Upstream to the silencing cassette included the cytomegalovirus (CMV) promoter, a reporter turbo green fluorescent protein (turboGFP) sequence, and an internal ribosome entry site (IRES) for bicistronic expression. Single-stranded rAAV9 viruses expressing either hSNCA- or the Scr-shRNA were produced by Virovek. For each animal, virus was intravenously delivered via the tail vein as a single dose of 1.25×10^{10} VG/g, or approximately 3.75×10^{11} vector genomes per 30 g animal.

Animals

Transgenic (Tg) mice (3–4 months) lacking endogenous murine α -syn but expressing wild-type human α -syn were used in this study^{60, 61}. Pan-neuronal expression of the human Tg α -syn is driven by the hamster prion promoter^{62–69}. Brain α -syn protein levels are 1–1.5-fold of nonTg wild-type animals and the Tg animals do not manifest behavioural changes or brain abnormalities at the ages studied. 8 Tg mice received FUS targeting the hippocampus (HC) and substantia nigra (SN), and another 8 Tg mice received FUS targeting the OB and DMN. These FUS-targeted groups were subdivided into 2 virus treatment groups (n = 4 per group): AAV9-Scr-shRNA or AAV9-hSNCA-shRNA. Animal procedures were carried out in compliance with the Canadian Council on Animal Care and the Animals for Research Act of Ontario.

MR-guided FUS

Mice were anesthetized with isoflurane and a 26-gauge catheter was inserted into the tail vein. Mice were secured on an MRI-compatible sled and imaged using a 7.0 T MRI (BioSpin 7030, Bruker). Pre-sonication T2-weighted (T2w) scans were used to target ultrasound foci. In one set of animals (n = 8), FUS was targeted to the HC and the ipsilateral SN. In a second set of animals (n = 8), FUS was targeted bilaterally to the OB and DMN. FUS was conducted using the RK100 system (FUS Instruments Inc.). Ultrasound waveforms were generated using a spherically focused transducer (1.68 MHz, 75 mm diameter, 60 mm radius of curvature). A polyvinylidene difluoride hydrophone was positioned in the centre of the transducer, as illustrated in Fig. 1A.

At the start of sonication, Definity microbubbles (0.02 mL/kg, Lantheus Medical Imaging) were injected via the tail vein. Standard BBB disruption parameters were used for sonication (10 ms bursts, 1 Hz pulse repetition frequency, 120 s duration). The applied acoustic pressure was incrementally increased with each pulse. Once subharmonic emissions were detected, the acoustic pressure was dropped by 50% and maintained for the rest of sonication, as described⁷⁰. The peak pressure required for BBB opening was averaged across the focal spots per animal to calculate the mean peak pressure for each animal.

Immediately following FUS, a gadolinium-based MRI contrast agent (0.2 mL/kg Gadovist, Schering AG) was injected via the tail vein and T1w scans were obtained to assess BBB permeability. Mice subsequently received the viral solution in saline via the tail vein catheter and 200 μ L of saline to facilitate transfer of the virus into the bloodstream.

The relative enhancement for each animal was calculated as pixel intensity in a 2 mm by 2 mm region on the post-sonication T1w MR images normalized to the intensity in a reference region of the brain. The enhancement values were averaged over all ultrasound foci per animal using a custom program (Matlab).

Immunohistochemistry

One month post-sonication, mice were deeply anesthetized with a mixture of ketamine (150 mg/kg) and xylazine (10 mg/kg). Animals were transcardially perfused with saline and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer. Whole brains were sectioned axially at 40 μ m using a sliding microtome.

For detection of transgene expression, free floating, brain sections were incubated in 10 mM sodium citrate buffer (pH 6.0) for 30 min at 80°C and then in blocking solution (5% goat serum, 0.25% Triton X-100 in PBS) for 2 h at room temperature (RT). Sections were incubated with anti-turboGFP antibody (PA5–22688, 1:500; Thermofisher Scientific) for 72 h at 4°C and then a secondary antibody for 2 h at RT, followed by 4'6'-diamidino-2-phenylindole (DAPI, D9542, 1:10 000; Sigma-Aldrich) in PBS for 10 min at RT.

To evaluate the extent of α -syn knockdown in the HC and SN, triple labeling for α -syn, synaptophysin and tyrosine hydroxylase (TH) was performed, while α -syn, synaptophysin and choline acetyltransferase (ChAT) was performed for the OB and DMN. Sections were incubated in blocking solution at RT for 2 h, followed by anti- α -syn (32–8100, 1:1000;

Invitrogen), anti-synaptophysin (ab52636, 1:250; Abcam) and anti-TH (ab76442, 1:1000; Abcam) primary antibodies for 72 h at 4°C. Sections were incubated in secondary antibodies for 2 h, followed by DAPI for 10 min at RT.

To detect neuronal cell death associated with shRNA delivery, terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick-end labeling (TUNEL), cleaved caspase-3 and neuronal nuclei (NeuN) immunofluorescent colabeling was evaluated. Briefly, TUNEL-positive cells were detected with an *in situ* cell death detection kit (DeadEnd Fluorometric TUNEL system, Promega), according to the supplier's instructions. Sections were incubated in blocking solution (10% goat serum, 0.3% Triton X-100) for 2 h at RT, and then for 24 h at 4°C with primary antibodies against cleaved caspase-3 (9661S, 1:300; Cell Signaling Technology) and NeuN (ABN90, 1:500; Millipore), followed by secondary antibodies for 24 h at 4°C.

To characterize the immune response to viral gene therapy, including the activation of glia, we performed double immunolabeling with ionized calcium-binding adapter molecule 1 (Iba-1) and glial fibrillary acidic protein (GFAP), for microglia and astrocytes, respectively. Sections were incubated in 10 mM sodium citrate buffer (pH 6.0) for 30 min at 80°C and then in blocking solution (5% donkey serum, 0.25% Triton X-100, PBS) for 2 h at RT. Sections were incubated with anti-Iba-1 antibody (019–19741, 1:1000; Wako Chemicals) and anti-GFAP (NB100–53809, 1:1000; Novus Biologicals) for 24 h at 4°C followed by secondary antibodies for 2 h and DAPI for 10 min at RT.

Imaging and quantification

Serial sections were processed in parallel and the regions of interest were applied to four serial sections throughout the HC, SN, OB and DMN, as defined using mouse stereotaxic coordinates⁷¹. All images shown were acquired using a Nikon A1 laser scanning confocal microscope (20X and 60X magnification objectives) coupled to NIS-Elements software (Nikon Instruments). Z-series of 0.7 µm optical section thickness were merged. All quantification of α-syn, synaptophysin, and TH immunoreactivity was done using images acquired at 20X magnification on a Zeiss spinning disk microscope coupled to the PV camera (Zeiss Axio Observer.Z1) and Zen software (Carl Zeiss). Tiled Z-stack images were obtained with 0.7 µm optical section thickness and projected to generate maximum intensity images. Signal intensity was examined in regions of interest at sonicated and corresponding non-sonicated structures in the brain. Pixel densitometry was analyzed with ImageJ software (NIH).

Statistical analysis

Prism software (GraphPad Software Inc.) was used for statistical analysis and graph generation. All graphs are presented as mean ± SEM. Statistical significance was set at $p < 0.05$. Power analyses were performed using G*Power software⁷².

RESULTS

MRI enhancement confirms BBB permeability in sonicated brain regions

FUS was targeted using T2w MRI scans either unilaterally to the HC and SN in one cohort of animals or bilaterally to the OB and DMN in a second cohort (Fig. 1B,C). Post-sonication, gadolinium enhancement was measured from T1w images of targeted brain areas (Fig. 1D-G), reflecting the relative amount of FUS-induced BBB opening⁷³. We found no significant difference in enhancement level between AAV9-hSNCA-shRNA and AAV9-Scr-shRNA treated animals, indicating comparable FUS-mediated increase in BBB permeability between the treatment groups (Fig. 1H,J). Normalized to the reference region, the mean increase in voxel intensity in sonicated regions was $43\% \pm 17\%$ and $47\% \pm 13\%$ in the AAV9-hSNCA-shRNA and AAV9-Scr-shRNA injected groups, respectively ($p = 0.62$).

Similarly, there was no difference in the mean acoustic energy delivered to the FUS-targeted regions between treatment groups (Fig. 1I,K). For the AAV9-hSNCA-shRNA and AAV9-Scr-shRNA groups, respectively, the mean peak pressure during sonication was 1.0 ± 0.2 and 1.1 ± 0.2 ($p = 0.52$). These data suggest that consistent BBB opening was achieved by using similar acoustic pressures in both treatment groups.

Reduction in α -syn expression after FUS-mediated delivery of silencing vector

The AAV9 vectors contained a reporter turboGFP sequence to verify transgene expression and location to FUS-targeted brain regions. All sonicated areas showed immunoreactivity to turboGFP (Fig. 1L-W). In contrast, there was no turboGFP expression in corresponding contralateral regions of brains which were not targeted by FUS. These results confirm the poor penetration of the virus across an intact BBB at an intravenous dose of 1.25×10^{10} VG/g, and that AAV9 delivery to the brain is selectively increased by FUS application. Varying levels of turboGFP expression were evident between brain areas, possibly reflecting regional neuronal versus non-neuronal populations and differences in afferent projections containing nerve terminals enriched in α -syn and presynaptic marker synaptophysin. In adult mice, AAV9 displays neuronal tropism, particularly when delivered to the brain and spinal cord using intraparenchymal injections or systemic administration combined with FUS^{48, 51, 74–77}. Here, the amount and localization of turboGFP-positive cells found in the hippocampal formation are comparable to previously reported using scAAV9-GFP⁵¹. The targeting of the OB, SN and DMN also results in a subpopulation of neurons expressing turboGFP and suggest that α -syn is suppressed within that subpopulation of neurons transduced.

One month following FUS-mediated delivery of the AAV9 vectors, α -syn immunoreactivity was observed in the FUS-targeted regions receiving the control Scr-shRNA (Fig. 2,3; red). The α -syn expression was a diffuse, punctate pattern matching that of synaptophysin, consistent with its preferentially presynaptic localization as observed in previous studies^{78–85}. In comparison, FUS-targeted regions of animals treated with hSNCA-shRNA had lower α -syn immunoreactivity. Synaptophysin expression was similar between the two treatments (Fig. 2E,3E), suggesting that synaptic integrity remains unaltered following FUS gene delivery. Therefore, to account for anatomical variations in the regions of interest, we

normalized the intensity of α -syn immunofluorescence to that of synaptophysin (Fig. 2F-G, 3F-G). Regions of interest in the SN and DMN were identified by the presence of dopaminergic (TH-positive) and cholinergic (ChAT-positive) soma (Supplemental Fig. 1). Relative to FUS-mediated delivery of AAV9-Scr-shRNA, we found a significant decrease in the α -syn/synaptophysin ratio following AAV9-hSNCA-shRNA treatment in the FUS-targeted HC ($p = 0.021$), SN ($p = 0.012$), and OB ($p = 0.018$). Similarly, we noted that α -syn levels in the DMN were 1.6 ± 0.3 and 2.6 ± 0.4 , when treated with AAV9-hSNCA-shRNA and AAV9-Scr-shRNA, respectively ($p = 0.081$, t-test, $n = 4$). Based on these results, a power analysis reveals that sample sizes of 6 animals per treatment group have an 80% power to detect differences with a significance level of 0.05 (two-tailed).

There are reports of a decline in nigral TH expression following suppression of α -syn in the SN^{32, 36, 38}, raising the possibility of neurotoxicity associated with low α -syn levels. However, despite the ~60% reduction in α -syn, there was no change in TH immunoreactivity in the SN (Fig. 4), whether in comparison to the control shRNA treatment ($p = 0.181$), or to the contralateral hemisphere ($p = 0.773$).

Absence of cell death or glial activation one month following FUS treatment

As measures of potential neuronal damage, we evaluated markers of cell death and glial activation in brain areas targeted with FUS and the AAV9 vectors. There was no discernible increase in TUNEL or cleaved caspase-3 staining due to FUS or α -syn knockdown in any brain region evaluated (Fig. 5A-L). The OB displayed some neuronal apoptosis in all groups (Fig. 5G-I), which is related to normal granule cell turnover^{86–88}. Moreover, the distribution and intensity of glial markers including astrocytes (GFAP) and microglia/macrophages (Iba-1)^{89–91} were comparable in FUS-targeted areas between mice treated with Scr-shRNA or hSNCA-shRNA, and hSNCA-shRNA without FUS (Fig. 5M-X; Supplemental Fig. 2–5). These results are consistent with previous studies that reported a transient increase in glial activation and inflammatory markers after FUS treatment, using parameters that minimized cell death, and a subsequent return to baseline levels^{50, 55, 92–95}. In accord with the unchanged TH staining in Fig. 4, the absence of cell death markers after one month of transgene expression further indicates a lack of neurotoxicity associated with α -syn gene silencing.

DISCUSSION

There are multiple difficulties associated with pharmacological treatment of CNS disorders. Primary among these is the inability of many drugs to cross the BBB at therapeutic levels. This limitation can be circumvented with transcranial MRI-guided FUS, which increases the permeability of the BBB locally and transiently for up to 6 hours when combined with intravenous microbubbles⁹⁶. We and others have shown that FUS is a safe and practical technology for the therapeutic delivery of macromolecules, genes and cells into the CNS^{50, 52–55, 57}. It is effective in a broad spectrum of species, from rodents to humans, to target selectively brain regions as small as 1–2 mm to an entire hemisphere.

In this study, we show that a single intravenous injection of AAV9 encoding α -syn inhibitory RNA, delivered to the brain using FUS in combination with microbubbles, is

sufficient to decrease α -syn expression in FUS-targeted brain regions. Based on the Braak histopathological staging of PD, we sonicated the OB, DMN, SN, and HC, which represent a spectrum of brain regions affected from early to late PD^{8, 97, 98}. The FUS-mediated increase in BBB permeability was confirmed by the diffusion of MRI contrast agent gadolinium around target areas and by the robust expression of the reporter turboGFP at one-month post-treatment, neither of which was detectable in non-sonicated hemispheres. Each FUS focal spot demarcated by gadolinium enhancement (Fig. 1D,F) is approximately 1 mm in diameter laterally and 3–4 mm along the beam, which is challenging to dissect without contamination from surrounding tissue expressing normal levels of α -syn. Therefore, to validate the FUS-mediated knockdown of α -syn in multiple discrete brain areas, we relied on immunofluorescence which offers superior spatial resolution compared to western blotting to detect changes in protein levels. α -Syn immunoreactivity was decreased in FUS-targeted areas of animals treated with hSNCA-shRNA relative to control Scr-shRNA. The overall extent of the α -syn knockdown varied depending on the brain region, as transgene expression is influenced by local differences in transduction efficiency, cell-specific tropism, and the ratio of cell soma to axons⁹⁹. Transduction efficiency was consistent with our previous reports^{48, 51} and the graded expression of turboGFP in cells within each brain region implies a corresponding range of α -syn knockdown in those cells. In the current study, FUS targeting was intentionally limited to small brain volumes to establish proof of concept. FUS exposure to broader brain areas⁵¹, coupled with improvements in AAV vectors, including capsid, promoter and transgene, could further enhance therapeutic efficacy. Nevertheless, the decrease was clearly selective for α -syn expression because synaptophysin, which is also highly expressed in nerve terminals, was unchanged by the shRNA treatment and was therefore used to normalize α -syn within each region of interest. The α -syn shRNA reduced α -syn expression by at least 50% ($p < 0.05$) in the HC, SN, and OB, and by 40% ($p = 0.08$) in the DMN. A power analysis indicates that a total of 6 animals per treatment group would be required to reach the recommended power level of 0.80 with alpha set to 0.05 (one-tailed). Previous reports using stereotaxic injections to deliver α -syn interfering RNA or antisense oligonucleotides also effectively reduced transgenic and endogenous brain α -syn in rodents and primates^{30, 31, 33, 34, 39–41}. Although the 35–60% knockdown in α -syn expression in those studies had minimal adverse effects, higher levels of α -syn gene silencing appeared to induce some nigral toxicity causing a loss of TH expression^{32, 36, 38}. In contrast, we did not detect any loss of TH immunoreactivity in the SN following sonication and α -syn shRNA, either in comparison to the sonicated SN with control shRNA or to the contralateral non-sonicated SN in hSNCA-shRNA injected animals. Moreover, markers of apoptotic cell death or glial activation were not notably changed one month after the virus or FUS treatments, in accord with our previous studies^{50, 94, 95}.

Idiopathic PD is an ideal neurodegenerative disorder for gene therapy: α -syn pathology, neurodegeneration, and dopamine loss are confined to discrete brain regions, and clinical motor symptoms are quantifiable. As the identification of prodromal motor and non-motor symptoms increasingly refine early diagnoses of PD^{10, 100–104}, drug delivery to appropriate brain regions will be relevant for initiating disease-modifying treatments as early as possible. The ability to target specific brain regions for gene therapy, as shown here, may prevent spreading Lewy pathology following α -syn knockdown, and also rescue failing neurons

when combined with regeneration strategies using growth factors. Previous Phase I-II clinical trials used viral vectors to express growth factors (neurturin, GDNF) or increase dopamine biosynthesis (TH, aromatic L-amino-acid decarboxylase, and GTP cyclohydrolase-1)^{105–107}. These trials involved small numbers of patients with fairly advanced disease and revealed only transient or modest benefits despite long-lasting and well-tolerated transgene expression. Several current Phase I-II trials are targeting α -syn (NPT200–11, Neuropore Therapeutics; PRX002, Biotech Prothena; AFFITOPE-PD01A, AFFiRiS AG; BIIB-054, Biogen; anti-aggregant EGCG, Ludwig Maximillians University) ([ClinicalTrials.gov](https://clinicaltrials.gov)). Whether these treatments, in single or combination therapies, will provide sufficient neuroprotection and regeneration for clinical improvement at early disease stages remains to be determined. Nevertheless, it is likely that enhanced delivery across the BBB will be beneficial therapeutically. For example, recent FUS-mediated GDNF expression in rat brain was shown to protect against 6-hydroxydopamine toxicity^{56, 58}, and a Phase I clinical trial to evaluate low intensity FUS in Alzheimer patients has recently been completed (NCT02986932, [ClinicalTrials.gov](https://clinicaltrials.gov)).

Our results represent an opportunity to alter the infiltration of α -syn pathology into the CNS prior to its effects on the SN. To our knowledge, not only is this the first report targeting the OB and the DMN regions for α -syn gene silencing, but also that multiple distal and discrete brain regions were targeted simultaneously. One limitation of our study is that we did not assess therapeutic benefits of α -syn knockdown. Studies are underway to determine the therapeutic window for neuroprotection and the rescue of motor symptoms after initiation of Lewy pathology in α -syn fibril seeding models. We anticipate that the choice of regimen and brain region will be aided by ongoing advances in prodromal biomarkers and imaging diagnostics. The use of FUS to deliver therapeutics in a targeted manner could open a range of opportunities for the treatment of synucleinopathies, particularly with complementary approaches including gene silencing, immunotherapy, anti-aggregants, and growth factors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

AAV9	adeno-associated virus serotype 9
BBB	blood-brain barrier
CMV	cytomegalovirus
DMN	dorsal motor nucleus of the vagus
FUS	focused ultrasound
GFAP	glial fibrillary acidic protein
HC	hippocampus
hSNCA	human α -syn gene
Iba-1	ionized calcium-binding adapter molecule 1
IRES	internal ribosome entry site
OB	olfactory bulb
SN	substantia nigra
shRNA	short hairpin RNA
Scr	scrambled
TH	Tyrosine hydroxylase
turboGFP	turbo green fluorescent protein
T1w	T1 weighted
T2w	T2 weighted
VG	vector genomes

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Fig. 1: MRI-guided FUS-induced BBB opening and transgene expression following FUS-mediated delivery of AAV9-hSNCA-shRNA and AAV9-Scr-shRNA to targeted brain regions. (A) Schematic of the FUS experimental setup. (B,C) Selected focal spots in the HC, SN, OB and DMN (indicated by purple, green, blue and red dots, respectively) for FUS-mediated BBB disruption. (D,E) Following FUS, T1-weighted MR images were taken to confirm increased BBB permeability at selected focal spots in the HC and SN in one cohort of animals, and (F,G) in the OB and DMN in a second cohort. There was no significant difference in (H,J) enhancement level and (I,K) mean peak pressure required to induce BBB

opening between AAV9-hSNCA-shRNA (black bar) and AAV9-Scr-shRNA treated animals (white bar) for both targeting schemes. **(L-W)** Representative confocal images (60X magnification) of brain sections prepared from AAV9-hSNCA-shRNA and AAV9-Scr-shRNA injected animals to detect turboGFP immunofluorescence. For both experimental groups, viral-mediated turboGFP was restricted to the FUS-treated side in the **(L,M)** HC and **(O,P)** SN, and bilateral **(R,S)** OB and **(U,V)** DMN. In contrast, turboGFP was not detectable on the contralateral hemisphere in the **(N)** HC and **(Q)** SN, nor in the untreated **(T)** OB and **(W)** DMN. Statistics: Paired, two-tailed, student's *t*-test. Data represent the mean $\pm$ SEM. *n* = 4 per group. TurboGFP, green; DAPI, blue. Scale bars: **(D-G)** 5 mm; and **(L-W)** 20 μ m.

Fig. 2: α -Syn knockdown following FUS delivery of AAV9-hSNCA-shRNA to the hippocampus and substantia nigra.

(A-D) Representative low (20X) and high (60X) magnification confocal images of brain sections immunolabeled for α -syn, synaptophysin and DAPI following FUS delivery of either AAV9-hSNCA-shRNA or AAV9-Scr-shRNA. Mean α -syn immunoreactivity was significantly decreased in animals that received AAV9-hSNCA-shRNA (black bar) compared to AAV9-Scr-shRNA (white bar) in the (A, B, F) HC and (C, D, G) SN. Dashed outline in C,D denotes area with TH-positive cells. Measurements of the α -syn (red) immunofluorescence signal are expressed as a percentage of the synaptophysin signal in the same region of interest. There was no significant difference in synaptophysin (green) immunoreactivity between AAV9-hSNCA-shRNA (black bar) and AAV9-Scr-shRNA injected animals (white bar) with FUS targeted to the (A, B, E) HC and (C, D, E) SN. Statistics: Paired, two-tailed, student's *t*-test. Data represent the mean $\pm$ SEM. *n* = 4 per group. Synaptophysin, green; α -syn, red; DAPI, blue. Scale bars: (A-D) 20x magnification, 100 μ m; and 60x magnification, 20 μ m.

Fig. 3: α -Syn knockdown following FUS delivery of AAV9-hSNCA-shRNA to the olfactory bulbs and dorsal motor nucleus.

(A-D) Representative low (20X) and high (60X) magnification confocal images of brain sections immunolabeled for α -syn, synaptophysin and DAPI following FUS delivery of either AAV9-hSNCA-shRNA or AAV9-Scr-shRNA. Mean α -syn immunoreactivity was significantly decreased in animals that received AAV9-hSNCA-shRNA (black bar) compared to AAV9-Scr-shRNA (white bar) in the (A, B, F) OB and (C, D, G) DMX. Dashed outline in C,D denotes area with ChAT-positive cells. Measurements of the α -syn (red) immunofluorescence signal are expressed as a percentage of the synaptophysin signal in the same region of interest. There was no significant difference in synaptophysin (green) immunoreactivity between AAV9-hSNCA-shRNA (black bar) and AAV9-Scr-shRNA injected animals (white bar) with FUS targeted to the (A, B, E) OB and (C, D, E) DMX. Statistics: Paired, two-tailed, student's *t*-test. Data represent the mean $\pm$ SEM. n = 4 per

group. Synaptophysin, green; α -syn, red; DAPI, blue. Scale bars: (**A-D**) 20x magnification, 100 μ m; and 60x magnification, 20 μ m.

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Fig. 4: Tyrosine hydroxylase expression is not reduced in the substantia nigra following FUS delivery of AAV9-hSNCA-shRNA.

(A,B) TH expression was measured in nigral dopaminergic neurons following sonication to the SN. (C,D) There was no significant difference between AAV9-hSNCA-shRNA (black bar) and AAV9-Scr-shRNA injected animals (white bar), and comparing the FUS-treated side to the contralateral non-FUS hemisphere. Statistics: Paired, two-tailed, student's *t*-test. Data represent the mean $\pm$ SEM. *n* = 4 per group. Tyrosine Hydroxylase, green. Scale bars: (A-B): 250 μ m.

Fig. 5: MRI-guided FUS gene delivery does not induce neuronal apoptosis or inflammation. (A-L) Representative confocal images of brain sections immunolabeled for TUNEL (green), cleaved caspase-3 (Cas3, red) and NeuN (blue). TUNEL- and cleaved caspase-3 positive cells were not detected in sonicated AAV9-Scr-shRNA or AAV9-hSNCA-shRNA-transduced areas of the (A, B) HC, (D, E) SN, (G, H) OB (relative to basal expression levels), and (J, K) DMN. (C, F, I, L) Immunostaining in non-FUS areas showed a similar pattern of apoptotic labeling. (M-X) Confocal images of brain sections depicting Iba-1 (green), GFAP (red), and nuclei (DAPI, blue) immunolabeled cells. There was no

difference in expression between the sonicated AAV9-hSNCA-shRNA and AAV9-Scr-shRNA treatment in the (**M, N**) HC, (**P, Q**) SN, (**S, T**) OB, and (**V, W**) DMN, as compared to immunolabeling in corresponding non-FUS regions (**O, R, U, X**). Scale bar: 100µm.

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