FOCUSED ULTRASOUND SURGERY FOUNDATION

RESEARCH AWARDS PROGRAM

FINAL REPORT

I. PROJECT TITLE:

Focused Ultrasound for Increased Delivery of Intranasal DNA Nanoparticles to Rat Brain

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IV. TOTAL PROJECT BUDGET (IN US DOLLARS): **\$100,000**

V. TOTAL FUNDING AWARDED FROM FUSF (IN US DOLLARS): **\$100,000**

VI. LENGTH OF PROPOSED FUNDING PERIOD (E.G., 1 YEAR): 1 year (February 1, 2015 - January 31, 2016) with no cost extension requested until January 31, 2017

VII. HYPOTHESIS AND RATIONALE

We hypothesized that focused ultrasound (FUS) would increase delivery to the brain of a non-viral gene vector encoding glial cell line-derived neurotrophic factor (GDNF) when given by the intranasal route of administration. We proposed that an increase in total DNA delivery should result in increased transfection and transgene expression in the sonicated regions of the rat brain. GDNF is a trophic factor for substantia nigra (SN) dopamine neurons which die in Parkinson's disease (PD). The SN and striatum were chosen as the FUS-targeted regions because they are the sites of neuropathology in PD.

All of the studies required pUGG nanoparticles (NPs) formulated and produced by our collaborators at Copernicus Therapeutics, Inc. pUGG is a plasmid that encodes GDNF linked to green fluorescent protein (eGFP). eGFP served as a "reporter" for the protein expressed by transfected cells, allowing detection of that GDNF in the rat brain resulting from transfection by pUGG plasmid DNA. pUGG was given either as naked plasmid or compacted into NPs. If FUS increased pUGG delivery and transgene expression in the FUS-targeted regions, this would provide proof-of-principle for our hypothesis and support development of a combined "FUS plus intranasal administration" option as a non-invasive means of gene therapy for PD, and possibly other neurodegenerative disorders of the brain.

VIII. SUMMARY OF WORK ACCOMPLISHED

At the beginning of the grant, we requested a new batch of pUGG NPs because the remainder of our original supply was insufficient to carry out all of the proposed studies. We purchased the pUGG plasmid from Aldevron and Copernicus compacted it into NPs. Copernicus shipped us 8.2 mg of pUGG NPs in late April 2015. We refer to these NPs as the "new batch", and the original batch as the "old batch" of pUGG NPs.

1. TIME-COURSE AND DOSE-RESPONSE STUDIES

During the summer and fall of 2015, we completed both a dose-response study and a time-course experiment to assess how much eGFP expression was generated throughout the rat brain after different intranasal doses, and for how long it persists. These studies were part of Aim 1. For the time course study we used a single dose of the old batch of pUGG NPs. All rats received 90 µg of pUGG DNA, a dose that we knew from previous work yields significant eGFP expression throughout the rat brain 1 week after intranasal administration. One group of 8 rats was sacrificed at 1 week, 3 months and 6 months after intranasal dosing. Other groups received intranasal saline and were sacrificed at the same time points. Analysis of eGFP expression at the 3 time points revealed that eGFP expression (above background in saline-treated controls) was highest at one week, and persisted at ~30% of maximal expression at both the 3 and 6 month time points ($p < 0.05$ for 1 week versus 3 and 6 months). These results provided evidence that intranasal delivery of Copernicus' pGDNF NPs results in significant and prolonged transgene expression in rat brain, with highest levels at 1 week and continued expression over at least 6 months.

For the dose-response study we used three doses of the new batch of pUGG NPs. Groups of 6 rats received either intranasal saline (control), 45 µg, 90 µg, or 180 µg pUGG DNA NPs. All were sacrificed 1 week later, at the time of maximal transgene expression. Since the middle dose (90 µg pUGG DNA) induced significant increases in eGFP expression throughout the rat brain, we expected the lower dose to yield less and the higher dose to yield more eGFP. However, very little expression of eGFP was observed in the whole brain or in individual brain regions of rats given any of the three doses of the new NPs (left graph below). The slight increases over baseline (saline) were not significant at any of the three pUGG doses, and they were much lower than previously seen with the old NPs. This suggested the new batch of pUGG NPs was not performing like the old batch. Further studies were done to directly compare the two batches in vivo. A 90 µg dose of each batch was given intranasally to 2 rats and all were sacrificed one week later. The old NPs yielded significantly more eGFP expression in whole brain than the new NPs (about twice as much; $p < 0.05$), as shown below in the graph on the right.

Copernicus ran quality control tests on samples of both batches and confirmed that all of the NPs were still intact, i.e. not degraded. We next sent samples of the two batches to Copernicus' collaborator, Dr. David Yurek at the University of Kentucky, so that he could test them by direct injection into the rat brain using methods he has published. We analyzed the samples by eGFP-ELISA and found that the old pUGG NPs generated significantly more eGFP than the new NPs at the injection site in left (L) striatum compared to the uninjected, control right (R) striatum, as shown below.

These results again suggested differences in the integrity of the pUGG NPs between the two batches. Our lack of confidence in the “new” NPs, and our lack of sufficient “old” pUGG NPs prevented us from re-doing the dose-response study that was part of Aim 1. We would like to return to this study, after completion of the grant, if we can purchase more pUGG NPs that reliably transfect cells in vivo.

2. TROUBLE-SHOOTING PROBLEMS WITH PUGG NPs

We couldn’t proceed to the FUS studies of Aim 2 until we had pUGG NPs capable of robust in vivo transfection in brain, equivalent to our original batch of NPs. We first carried out a DNA restriction digest to confirm that the naked pUGG plasmid was intact and that issues with the last batch of NPs was not due to compromised integrity of the DNA itself. For the DNA digests, we used NHE-I, STU-I and PAC-I to digest samples of the three different batches of naked pUGG in our stock. As shown in the gels below, we saw identical bands for digests from all three naked plasmids. This confirmed that all three batches of our naked pUGG were identical.

We next carried out in vitro studies to confirm that the naked pUGG plasmid was capable of transfection and resulted in detectable transgene expression. We transfected SHEP1 cells (a human neuroblastoma cell line) with the same three batches of naked pUGG. All transfections were done using the transfection reagent, Lipofectamine LTX with Plus reagent. All three batches transfected well and gave very high and similar levels of eGFP expression in cell lysates. Lower levels of eGFP were found in the media of these cultures, as shown below.

Having confirmed that the DNA was intact, Copernicus prepared a new batch of pUGG NPs using plasmid from our remaining stock of the naked plasmid DNA and did testing to ensure that the new NPs met standard quality control criteria. We then sent a sample of the new NPs to David Yurek at the University of Kentucky for testing by direct injection into the rat brain, as above. We analyzed the samples by eGFP-ELISA and found that the newest batch of pUGG NPs generated significantly more eGFP at the injection site in left (L) striatum versus the control right (R) striatum, as shown below (left graph). We next tested them ourselves intranasally in rats (n=4), and one week later we measured eGFP expression in the brain. We found that intranasal administration of the newest batch of pUGG NPs resulted in eGFP expression comparable to the first batch of NPs (used in the time course study), with no significant difference in whole brain levels, as shown below (right graph).

This trouble-shooting and making new pUGG NPS took months, but it ultimately yielded a new batch of pUGG NPs that could be used for the FUS studies of the second Aim. The new NPs were sufficient to complete the FUS studies, but not enough to repeat the dose-response curve of Aim 1.

3. FUS ENHANCEMENT OF INTRANASALLY ADMINISTERED NPS

The goal of Aim 2 was to evaluate whether FUS combined with intranasal administration of pUGG NPs or the naked plasmid would increase overall delivery to the brain, improve tissue penetration, and enrich transgene expression in the desired locations in brain. Before beginning these studies, a number of preliminary studies were performed to establish sonication protocols.

Preliminary studies: We first worked to establish FUS treatment conditions that reliably increase delivery of intranasally-administered fluorescent tracers to the brain of rats without inducing tissue damage. We constructed a new MRI-compatible transducer and passive cavitation detector system for use in these experiments. This system consisted of a 690 kHz transducer and 1.5 MHz cavitation detector which were mounted on a holder so that their focal areas overlapped. The transducer was calibrated using a needle hydrophone and a radiation force balance. The sensitivity profile of the detector was mapped using a hydrophone to verify that it was correctly mounted. Finally, we performed a series of experiments using a feedback control system developed in our laboratory to drive the transducer so that a pre-defined amount of harmonic activity was produced when microbubbles were injected. These experiments were performed in mice (n=20). The controller also automatically reduced the exposure level if broadband emission was detected. Such emission is a signature for inertial cavitation, which can produce vascular damage. The new controller system allowed us to maximize sonications while minimizing tissue damage.

In parallel, we conducted tests in rats in preparation for the experiments with the nanoparticles. We performed a series of experiments with different fluorescent dyes (Trypan blue, fluorescent dextrans) and with an MRI contrast agent to optimize the FUS parameters for enhancing nasal delivery. We initially tried intranasal administration of Trypan blue. We disrupted the BBB in the putamen and thalamus in two rats approximately 40 min after intranasal delivery. Fluorescent imaging of the brain after the experiments suggested that there might have been increased delivery in regions where vascular damage was observed. However, these findings were not definitive, and we were not convinced that delivery was enhanced by FUS. Therefore, we changed to the same fluorescent dextran that was used in the study by Chen et al. (PLoS One 9: 10 e108880, 2014), who found that FUS can enhance delivery to brain after intranasal administration in mice. To date, we have evaluated six rats with these agents. Again, the findings were not definitive, and we were not convinced that we were able to enhance the nasal delivery.

Finally, in parallel with these experiments, we performed several experiments where an MRI contrast agent Gadavist was delivered intranasally. In serial T1-weighted imaging, we were able to observe the agent being transported in the nasal mucosa, but we did not observe evidence of transport through the brain parenchyma up to an hour after intranasal administration. This led us to question whether we had waited long enough for the agent to reach the sonicated area in our previous FUS-treated animals (above). For this reason, we are now performing tests where we sonicate multiple times over two hours. This will ensure that the ultrasound is applied when the agent is passing through the targeted brain area. In our most recent rat experiment, we targeted a location in the putamen for sonication with microbubbles, and we used multiple sonications. Approximately 50 min before the first sonication we administered 40 μ l of a fluorescent dextran intranasally. We then sonicated at the putamen target repeatedly every 15 min for two hours in conjunction with administration of Optison (100 μ l/kg). Cavitation monitoring was used to ensure that similar microbubble activity occurred during each sonication. The brain was extracted and examined by fluorescent microscopy. While some promising examples were observed, we did not see consistent delivery of the fluorescent tracer to the rat brain. At this point, we concluded that if we were indeed enhancing nasal delivery, the enhancement was below our detection threshold for the fluorescent tracer. We do not know why we were unable to replicate the findings of the Columbia group. In that study, they administered the same nasal dose to a mouse that we used in rats. Given the difference in size of the animals, it is perhaps not surprising that we were unable to reliably detect the fluorescent tracer at the levels seen previously. We decided at this point to go forward with the transfection experiments, where we will have higher sensitivity.

FUS increased whole brain transgene expression by intranasal naked pUGG: Our previous work showed that when given intranasally to rats, naked pUGG results in some transgene expression in the whole brain, albeit minimal. We reasoned that FUS with circulating microbubbles might increase that delivery and transgene expression at the sonicated sites. We used the real-time, closed-loop, acoustic emission-based controller developed in the McDannold lab, which sustains stable cavitation (harmonic emission, HE) while suppressing inertial cavitation (broadband emission, BE). HE and BE were analyzed in real time during 32 msec bursts and fed back for control of the next pulse. To avoid inertial cavitation, the pressure was reduced if BE was detected and terminated if it crossed a set threshold (30 dB), as shown in the controller profile below..

For these studies, FUS bursts (32 msec bursts at 4 Hz for up to 100 sec) were applied during infusion of Optison microbubbles. This was done twice, once before and once after intranasal naked pUGG. The first FUS treatment was applied to a dorsal brain region in the right hemisphere beginning 10 min before intranasal delivery of naked pUGG (172 μ g DNA). The same FUS sequence was repeated 1 hour later at a caudal location on the same side. Contrast-enhanced MRI following the FUS treatment confirmed the BBB disruption (as shown below). No statistical difference in contrast enhancement between forebrain and midbrain sites was found, indicating comparable BBB disruptions in the two targeted areas.

All rats were sacrificed one week later, and 4 rostral-to-caudal coronal slabs of the brain were cut, separating left and right sides of each slab. eGFP-ELISA showed that FUS with circulating microbubbles increased total expression of eGFP in the whole brain relative to rats that received intranasal naked pUGG without FUS, or saline-treated controls, as shown below. However, separate analysis of sections from each hemisphere revealed that the increase in expression was not confined to the sonicated side, but occurred in parallel on both sides. It is unclear why the effect was bilateral. It may mean that FUS increased transfection efficiency of the naked plasmid via sonoporation or some other biophysical means, or by increasing overall perivascular flow and thus pUGG delivery throughout the brain. Alternatively, our stereotaxic placement of the FUS bursts in this study may have resulted in sonication at more medial sites than intended in some rats, thereby increasing transgene expression on what appeared to be both sides. We are looking into whether this may have occurred by comparing MRI evidence of BBB disruption after FUS treatments with eGFP-ELISA data for each rat. Collectively, these results indicate that FUS with circulating microbubbles did significantly increase total transgene expression in the brains of rats given intranasal naked pUGG.

FUS increases transgene expression by intranasal pUGG NPs in the sonicated regions: To next determine whether FUS could increase delivery and transfection by pUGG NPs, we used the same study design. Again, rats (n=7) were subjected to FUS with circulating microbubbles applied both before and after intranasal administration of the same dose of pUGG NPs. Rats were sacrificed one week later, and transgene expression throughout the brain was assessed by eGFP-ELISA. In contrast to the results of the naked pUGG study, FUS with circulating microbubbles did significantly increase eGFP expression in the three most rostral brain regions (A, B, and C) of the sonicated right hemisphere, compared to the unsonicated left brain regions (left graph below; one-way ANOVA: $p=0.0004$; paired t-test, L vs R for A: $p=0.023$, B: $p=0.0056$, C: $p=0.0655$). This corresponded to a significant increase in the weighted average of eGFP expression in the sonicated right hemisphere of rats given intranasal pUGG NPs with FUS compared to the unsonicated left hemisphere (right graph below; paired t-test: $p=0.0457$).

Despite the increase in transgene expression on the sonicated side, FUS did not significantly increase the whole brain weighted average of eGFP expression in rats given intranasal pUGG NPs compared to rats given intranasal pUGG NPs without FUS (one-way ANOVA: $p=0.0134$; t-test, IN pUGG vs IN pUGG NP + FUS: $p=0.4573$, n.s.). Thus, FUS with circulating microbubbles resulted in higher transgene expression in the sonicated brain regions and increased total transgene expression on the sonicated side of the brain, but it did not increase the total brain-wide amount of eGFP expression, as shown below. This suggests that FUS increased delivery and enhanced cellular transfection at the sonicated sites at the relative expense of unsonicated sites, i.e. FUS improved targeting of transgene expression to sonicated regions.

We next tried to assess whether application of FUS before or after intranasal administration was more effective. By calculating the difference between the eGFP expression levels in the left (control) and right

(sonicated) sides of each brain region, it was determined that there was no significant difference between the changes in eGFP expression between the forebrain (which was sonicated before intranasal administration) and the midbrain (which was sonicated after intranasal administration); data not shown. Thus, FUS with circulating microbubbles had a similar effect in enhancing transgene expression regardless of whether it was applied before or after intranasal administration.

FUS alters the number and type of transfected cells at the sonication site after intranasal pUGG NPs:

Aim 2 was also to assess whether FUS increases penetration of pUGG NPs into brain parenchyma and/or changed the pattern of cellular transfection in the sonicated regions. We previously used double-label immunohistochemistry (IHC) to show that the cells transfected after intranasal pUGG NPs were almost always abluminal to and aligned along vascular endothelial cells, which were stained for rat endothelial cell antigen (RECA-1). None of the eGFP-labeled cells co-expressed the astrocyte marker GFAP or the neuronal marker NeuN, although they were very close (within 15 μ m) to these cell types. We concluded that the cells normally transfected by intranasal pUGG NPs were likely to be pericytes, the cells which first encounter the NPs as they are transported by perivascular flow outside microvessels.

In the FUS studies with pUGG NPs, we used the same treatment protocol to determine if FUS with microbubbles could increase cellular transfection at the sonication site, increase tissue penetration and/or change the types of cells that were transfected. Rats (n=4) were subjected to FUS with circulating microbubbles, applied both before and after intranasal administration of the same dose of pUGG DNA as above (but now in NPs). The rats were sacrificed one week later by perfusion with 4% paraformaldehyde. Coronal sections were cut from the sonicated areas (forebrain at the level of the striatum, and midbrain at the level of the substantia nigra) and were subjected to double-label IHC for eGFP and different cellular markers, i.e. RECA-1, GFAP, NeuN, and tyrosine hydroxylase (TH), a marker for SN dopamine neurons. In animals that received intranasal pUGG NPs, the pattern of transfection in the non-sonicated areas was similar to that seen after intranasal delivery alone, e.g. the majority of transfected cells were located immediately adjacent and abluminal to blood vessels, presumably pericytes. However, at the sonication sites in both the midbrain and forebrain, there were large numbers of eGFP-positive cells in irregular clusters amidst areas of bright eGFP background staining (see images below). This pattern suggests that FUS increased delivery of the pUGG NPs in localized areas, and this in turn increased cellular transfection and transgene expression at the sonicated site. Interestingly, the eGFP-positive cells did not co-localize with RECA-1, GFAP, NeuN, or TH, and also did not align along microvessels like pericytes. They were small, round cells, often in clumps, sometimes next to a blood vessel but not always. Based on their cellular morphology, we suspect these cells may be microglia. If so, they may indicate several processes at work. For instance, they may have been recruited to the sonication site by localized release of microglial-activating tissue factors (cytokines, albumin etc.), and once there became transfected by pUGG NPs that had penetrated the parenchyma at those sites. Alternatively, microglia may have been attracted to the site by the pUGG NPs themselves after they penetrated the parenchyma. In this case, microglial phagocytosis of the NPs may have led to their transfection and expression of eGFP. Deciphering the means of recruitment and the meaning of these clusters of transfected cells will require double-label IHC to establish whether they are indeed microglia. We hope to do this study after the grant ends using brain sections remaining from these rats. We will determine if the eGFP-positive cells co-express an immunohistochemical marker for microglia, Iba-1.

These clusters of eGFP-positive cell at the sonication sites may account for the increases in eGFP detected by ELISA in the previous study. Taken together, these findings support one part of our hypothesis – FUS does increase transgene expression at the sonication site 1 week after intranasal administration of pUGG NPs. However, FUS did not shift the distribution of cellular transfection from pericytes to neurons or astrocytes. Instead, FUS appeared to increase transfection of another cell type, possibly microglia, at the sonication sites. The pros and cons of this shift are not yet clear.

Another part of Aim 2 was to determine if FUS increased tissue penetration of the NPs and increased the proximity of the transfected cells to the targets, dopamine neurons in the SN and their terminals in the striatum. Outside the sonication sites, in the forebrain and midbrain of rats that received intranasal pUGG NPs + FUS, we found that eGFP-positive cells remained in close proximity to RECA-1-positive

capillary endothelial cells, again resembling pericytes. Correspondingly, they were also found within 15 μm of GFAP-positive astrocytes, similar to previous results in rats that received the same intranasal dose of pUGG NPs without FUS. However, in the FUS-treated rats, a higher percentage of eGFP-positive cells were located within 15 μm of a NeuN-positive neuron in both the forebrain and the midbrain, and a much higher percentage of transfected cells in the substantia nigra were in close proximity to a TH-positive dopamine neuron than in rats not treated with FUS. These results support another part of our hypothesis – FUS with microbubbles does improve tissue penetration in the target brain regions of intranasally-administered plasmid DNA NPs encoding GDNF.

This work completed the Aims of the funded proposal, and provided new insights and additional questions regarding the feasibility of FUS for increasing intranasal delivery to the brain. We hope to continue this research to refine FUS protocols, address the new questions, and further explore the feasibility of the approach for treatment of PD and other CNS disorders.