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Focused ultrasound–mediated brain genome editing

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Gene editing in the brain has been challenging because of the restricted transport imposed by the blood–brain barrier (BBB). Current approaches mainly rely on local injection to bypass the BBB. However, such administration is highly invasive and not amenable to treating certain delicate regions of the brain. We demonstrate a safe and effective gene editing technique by using focused ultrasound (FUS) to transiently open the BBB for the transport of intravenously delivered CRISPR/Cas9 machinery to the brain.

genome editing | CRISPR/Cas9 | blood–brain barrier | focused ultrasound | gene delivery

CRISPR gene editing technologies provide exciting opportunities to advance gene therapy and treat many intractable genetic diseases, including neurodegenerative disorders (1, 2). However, effective in vivo delivery of CRISPR components remains a significant barrier. Except for the liver, CRISPR delivery mainly relies on local administration. Given its highly invasive nature, this can be particularly problematic when targeting the brain. A safer yet effective method of delivery would help empower the use of somatic gene editing in this critical organ. We and other groups have demonstrated focused ultrasound (FUS) delivery of drugs and biologics to the brain through systemic routes (3). Our efforts have led to a clinical trial (NCT04804709) for delivering Panobinostat to children with diffuse midline glioma in the brainstem, a difficult and sensitive region for direct administration. Here, we report the feasibility of applying FUS to achieve gene editing in targeted brain regions following the intravenous injection of adeno-associated virus (AAV) vectors encoding CRISPR/Cas9 machinery.

FUS-mediated BBB opening is accomplished through the cavitation of systemically administered microbubbles in the FUS focus, temporarily permeabilizing the BBB at the FUS-targeted site for the delivery of various payloads including AAV (Fig. 1*A*). Based on the therapeutic goal, the target region, dose, and FUS parameters can be modified to maximize the transport of AAV into the brain. We previously developed two different FUS systems (Fig. 1*B*). Spherical single-element FUS could transiently open the BBB in a confined manner through its geometrically focused transducer (3), while the FUS array, a theranostic system built by reprogramming the commercially available P4-1 transducer to generate rapid sequences of short focused pulses, enabled a transient BBB opening in a widespread region with simultaneous real-time cavitation imaging (4). We first started with the spherical system to test whether FUS could improve the delivery of AAV9/*Staphylococcus aureus* Cas9 (SaCas9) vector into the mouse brain. Although AAV9 was reported to have CNS-tropism when given intravenously (5), its brain disposition was still significantly lower than the level seen in other organs on our hands. In contrast, FUS enhanced the disposition of SaCas9-encoding AAV9 by ~13 times at the target hemisphere, thus allowing targeting of the brain at levels similar to other organs apart from the liver (Fig. 1*C*).

We next optimized the SaCas9 vector by swapping the promoter and modifying the guide RNA (gRNA) scaffold as certain viral promoters (e.g., CMV) could be transcriptionally silenced in brain cells (6), while the poly-T motif in the wild-type gRNA scaffold may lead to gRNA early termination (7, 8). As expected, the constitutive mammalian promoter EF1α enhanced in vivo SaCas9 expression by 7.4-fold when compared with its parental vector containing the CMV promoter (Fig. 1*D*). After further optimizing the gRNA scaffold, we tested our optimized vector using a well-characterized *Pcsk9* guide (9) to determine the efficacy of our system when packaged into AAV and delivered systemically. At a dose of 2×10^{11} genome copies (GC)/mouse, we observed results consistent with the previous report (9), indicating a significant reduction in total cholesterol, a downstream marker of PCSK9-mediated lipid metabolism (104 ± 4 to <50 mg/dL, $P < 0.001$). However, we only detected a minimal indel rate in the target locus from the brain samples (0.71 ± 0.11%).

Next, we increased the dose to 10^{12} GC/mouse and included the FUS array system to see if gene editing in the brain could be significantly improved. At the FUS-targeted regions, we could detect significant SaCas9 transcripts from both FUS groups, and as aforementioned, the spherical FUS produced gene editing in a confined volume, while the FUS array led to

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The authors declare no competing interest.

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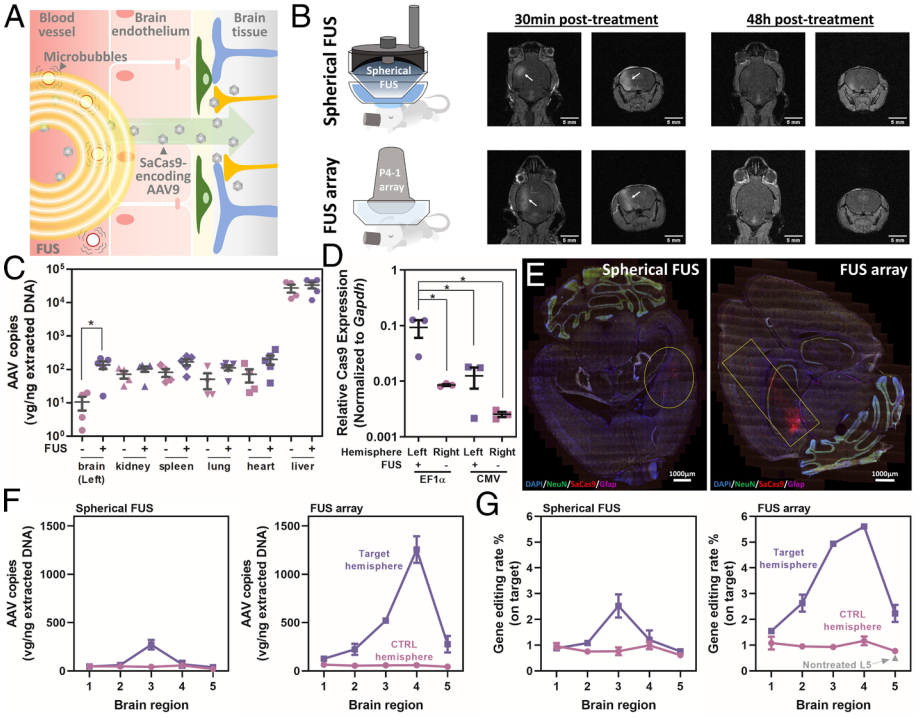

Fig. 1. FUS to enhance systemic AAV/CRISPR vector delivery to the brain. (*A*) Schematic overview of FUS-mediated BBB opening. (*B*) FUS systems used in this study and the MRI images showing the transient opening induced by FUS (arrowed). (*C*) Biodistribution of AAV9/SaCas9 vector (N = 5 for the FUS group and N = 4 for control, adult male C57BL/6). Mice received the vector in a dose of 2×10^{11} GC/mouse, and the disposition was quantified at week 2 post-administration. (*D*) SaCas9 expression in the brain when mice given with different AAV9/SaCas9 vectors. Adult male C57BL/6 were intravenously given the vector in a dose of 2×10^{11} GC/mouse (N = 3). (*E*) Representative RNA in situ hybridization images to confirm the SaCas9 expression in the FUS-targeted region. (*F*) Disposition of AAV9/SaCas9 vector and (*G*) Gene editing efficiency in different brain regions (two biological repeats). For *E*–*G*, adult male C57BL/6 were received an intravenous AAV9/SaCas9 dose of 10^{12} GC/mouse, and the brain was dissected at week 3 post-administration.

Cas9 expression that was more widespread (Fig. 1*E*). For analysis, we further divided the brain tissues into five regions, and the qPCR and amplicon sequencing results matched the results from RNA in situ hybridization. When comparing these two systems, we did see higher Cas9 vector disposition (>3×) in the FUS array group (Fig. 1*F*), leading to an enhanced editing efficiency (Fig. 1*G*). Overall, using the FUS array system with a systemic dose of 10^{12} GC/mouse, we could reach >5% gene editing from this unbiased, bulk tissue analyses.

To further validate the gene editing efficacy, we tested our approach in the Ai9 mouse reporter line carrying a CAG-loxP-STOP-loxP-TdTomato cassette (10). Deletion of the loxP-flanked transcriptional terminator (STOP) by dual guide-mediated Cas9 editing can activate the expression of TdTomato (7). Given the need of two gRNAs to completely remove the STOP signal, an additional U6-gRNA expression cassette was added to our vector. In light of the use of a high AAV dose (10^{13} GC) for Cas9 editing via intracranial administration setting in other work (11) and potentially lower efficiency by requiring Cas9 to cut two targets, we chose 2×10^{12} GC/mouse as our systemic administration dose for these follow-up validations. Adult Ai9 mice received our Ai9-targeting vector intravenously under the FUS array system. At the end point (week 3 post-administration), we observed significant TdTomato activation at the target hemisphere when tissue was examined using a Lightsheet microscope (Fig. 2*A*). Normalizing the TdTomato+ volume to the whole hemisphere, the overall editing efficiencies were determined to be 12.3% and 1.21% for the FUS-targeted and contralateral hemispheres, respectively (Fig. 2*B*). In parallel, we serially sectioned the mouse brains and carried out histological analysis. Aligned with the Lightsheet results, we saw significant gene editing in the FUS-targeted hemisphere with an efficiency of 15.7% (Fig. 2*C*). We grouped the sections based on their locations and quantified the average gene editing efficiency for each region (Fig. 2*D*). The editing performance profile in Ai9 correlated with the trend we saw from the sequencing result in C57BL/6 (Fig. 1*G*), and two sets of serial sections gave consistent results (Fig. 2*D*). The use of FUS significantly improved the brain gene editing efficacy by enhancing the brain disposition of the AAV vectors. We then analyzed the editing efficiency in neurons in the hippocampus, where the center of our FUS array was positioned. In the selected regions-of-interest (ROIs: 1,200 × 1,200 μm^2; the focal size of our FUS probe), we found 25.6% of the neurons edited in this particular region, while <1% observed in the contralateral control side (Fig. 2*E*).

Through participation in the NIH Somatic Cell Genome Editing (SCGE) Program (12), we worked with the SCGE Small Animal Testing Center (SATC) at the Jackson Laboratory (JAX Lab) to verify the effectiveness and reproducibility of our technology. The same analysis pipeline performed at the SATC showed a consistent result, 25.5% of the neurons edited in the ROIs in the Ai9 model (Fig. 2*F*). The SATC validated our approach using an independent Traffic Light Reporter model (TLR2) generated for the SCGE program. This reporter strain carries a mutated Venus-P2A-TagRFP cassette, where a double-strand break (DSB) in the reporter followed by a nonhomologous end-joining DNA repair event can activate TagRFP, and if a donor is provided, a homology-directed repair event can restore Venus expression (13). In our in vivo TLR2 validation with the optimized gRNA, 15.8% of the neurons were TagRFP-positive in the ROIs at the FUS array–targeted side (Fig. 2*G*). Because only indels in the +1/−2 frame could activate the TagRFP expression, we established a correction factor (2.07) based on the amplicon sequencing results obtained from whole FUS-targeted hemisphere to estimate the actual overall editing efficiency (32.6%, Fig. 2*H*). Better performance seen in TLR2 may be because only one DSB is needed, which could be more efficient versus the two DSBs and deletion of the STOP cassette required for activation of Ai9. Altogether, the results in two mouse models across two different laboratories confirmed the robustness of the FUS technology for enhancing brain gene editing.

In summary, we demonstrate that FUS is a reproducible CRISPR delivery approach for effective gene editing in specific brain regions through systemic administration of CRISPR-encoding vectors. By combining FUS with AAV-mediated gene delivery, we can achieve >25% editing efficiency of particular cell types. It is noteworthy that our approach is still dependent upon the tropism of the AAV capsid used, which may not transduce all the neurons equally, and liver is still the main tissue target of the serotype (AAV9) we used. Furthermore, the efficiency of the gRNAs and the ability of the

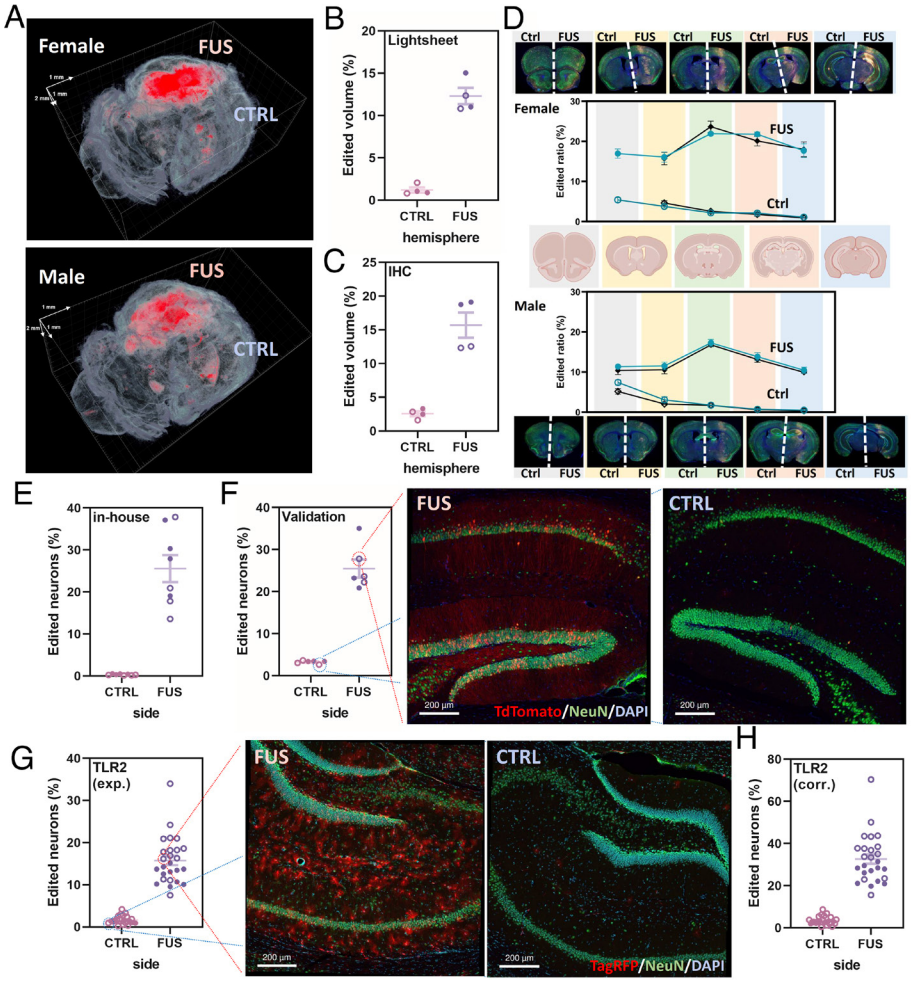

Fig. 2. Validation of FUS-mediated brain gene editing in reporter mouse models. (*A*) Representative 3D Lightsheet images showing the Cas9-activated TdTomato signals (red) in the brain. (*B*) Gene editing efficiency quantified by Lightsheet microscope. (*C*) Efficiency quantification by immunostaining. (*D*) Gene editing efficiency in different brain regions. Two sets of serial sections were used for immunostaining and quantification for each sex. (*E*) In-house quantification of edited neurons in ROIs in FUS-AAV9/SaCas9–treated Ai9 mice by immunostaining. (*F*) Independent quantification of neuron editing performed by the SCGE SATC and representative confocal images. (*G*) Quantification of TagRFP+ neurons in ROIs in FUS-AAV9/SaCas9–treated TLR2 reporter mice by immunostaining and the representative confocal images. (*H*) Neuron editing performance determined with the experimental correction. For *B* and *C* and *E*–*H*, data are presented in dots and circles for the results from female and male mice, respectively.

promoters to drive robust expression of the CRISPR components also play key roles in driving editing rates. In future studies, the efficiency and specificity of this FUS-based approach is likely to be enhanced with the use of a different carrier (e.g., different AAV serotypes or nonviral systems) and by further engineering the CRISPR components. Our previous studies in larger animals (14–16) and human trials (NCT04804709 and NCT04118764) have proven the safety and applicability of FUS. The method established here has the potential to expand the toolkit options for CRISPR delivery and open opportunities for treating diseases of the brain, such as neurodegenerative disorders, with somatic genome editing.

Materials and Methods

AAV/SaCas9 vectors used in this study were built on the vector from Takara. The AAVs were produced either in-house or by PackGene. All the in vivo FUS experiments were approved by the IACUC at Columbia; briefly, adult mice (C57BL/6, Ai9, or TLR2) were given intravenously with AAV9/SaCas9 vectors and microbubbles in saline solution followed by FUS sonication. Information regarding the vectors, guides, and the experimental details are provided in *SI Appendix*.

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*.

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