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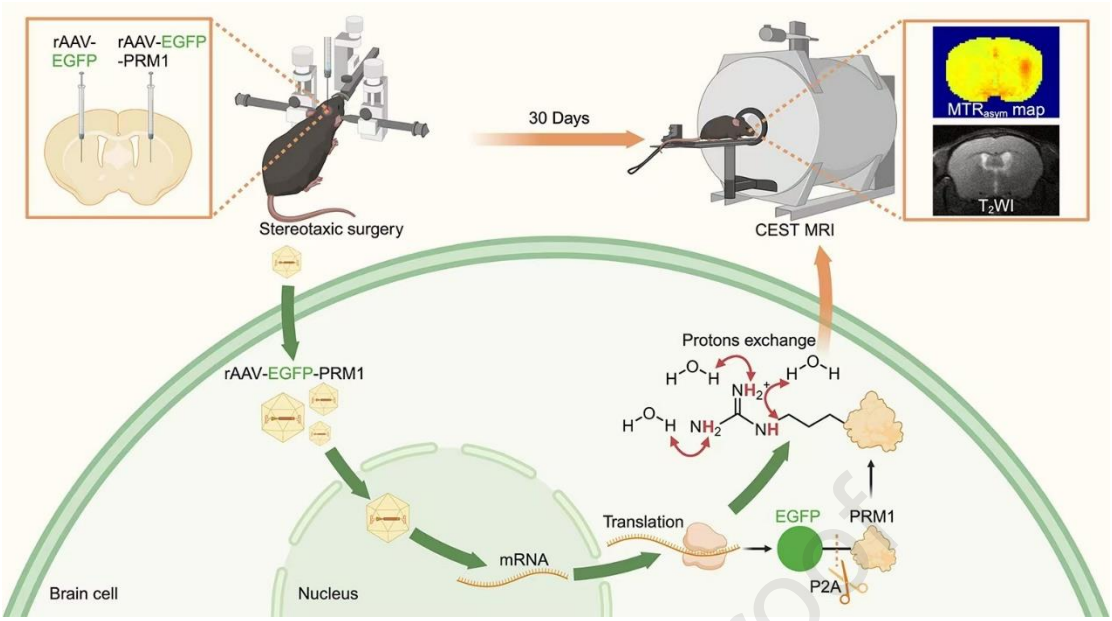


**Research Article**

# Protamine-1 Encoded Recombinant Adeno-associated Virus for Enhanced Brain Magnetic Resonance Imaging

Kairu Xie [a], Yaping Yuan [a,b], Mou Jiang [a], Daiqin Chen [a,b], Shizhen Chen [a,b*],Xin Zhou [a,b,c*]

[a] State Key Laboratory of Magnetic Resonance Spectroscopy and Imaging, National Center for Magnetic Resonance in Wuhan, Wuhan Institute of Physics and Mathematics, Innovation Academy for Precision Measurement Science and Technology, Chinese Academy of Sciences-Wuhan National Laboratory for Optoelectronics, Huazhong University of Science and Technology, Wuhan, 430071, China

[b] University of Chinese Academy of Sciences, Beijing, 100049, China

[c] Key Laboratory of Biomedical Engineering of Hainan Province, School of Biomedical Engineering, Hainan University, Haikou, 570228, China

Corresponding author: chenshizhen@wipm.ac.cn; xinzhou@wipm.ac.cn.

**Abstract**

Magnetic resonance imaging (MRI) is a powerful tool for diagnosing and monitoring brain diseases, but its low sensitivity can hinder early detection. To address this challenge, we utilized chemical exchange saturation transfer (CEST) MRI, which greatly enhances sensitivity for detecting low-concentration compounds. In this study, we developed a CEST contrast agent based on a recombinant adeno-associated viruses (rAAVs) encoding the protamine-1(PRM1) MRI reporter gene. CEST MRI revealed that PRM1 contrast agent effectively highlighted caudate putamen region after injection of the rAAVs into the mouse brain, clearly distinguishing it from the surrounding tissue, with no observable damage. This method provides a sensitive, metal-free CEST contrast agent for *in vivo* brain cell detection, demonstrating potential for both diagnostic and therapeutic applications in brain diseases.

**Keywords:**

Magnetic resonance imaging (MRI)

Chemical exchange saturation transfer (CEST)

Protamine 1 (PRM1)

Recombinant adeno-associated virus (rAAVs)

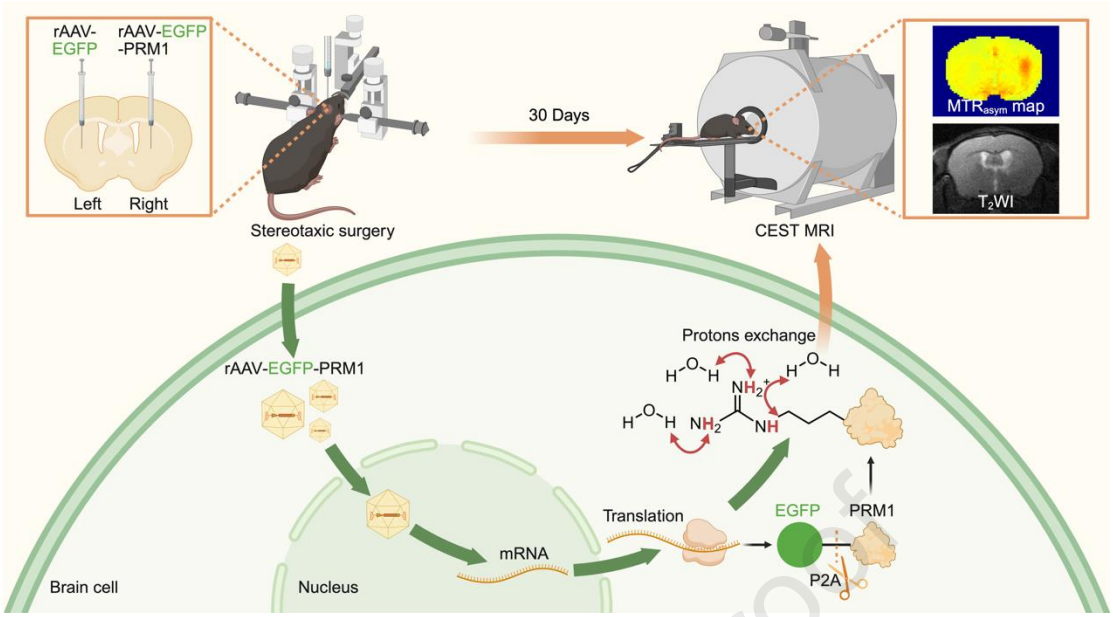
## 1. Introduction

Magnetic resonance imaging (MRI) is a highly practical technique for soft tissue imaging with superior spatial and temporal resolution, making it particularly valuable for diagnosing and monitoring brain diseases [1, 2]. However, the relatively low sensitivity of MRI can pose challenges for early diagnosis and precise monitoring in clinical settings. This limitation can be addressed by increasing magnetic field strength, utilizing contrast agents, or adopting novel imaging techniques [3-5]. The widely utilized gadolinium-based contrast agents may enhance MRI signals by shortening the longitudinal relaxation time ($T_1$) of hydrogen nuclei ($^1$H) in lesion areas [6, 7]. However, they are diffused uniformly throughout the extracellular space when intravenously administered due to their poor selectivity, posing high risks of toxicity and deposition in the brain [8, 9].

Chemical exchange saturation transfer (CEST) MRI indirectly detects native molecules without labelling by manipulating the water proton signal through selective saturation of exchangeable protons. CEST method provides an effective sensitivity enhancement mechanism, allowing the visualization of low-concentration solutes via the water signal [10, 11]. Recent researches have identified several effective CEST agents, including glucose [12, 13], myo-inositol [14], glutamate [15], and creatine [16, 17]. However, imaging endogenous substances in living organisms often suffers from poor specificity due to background signal interference. To overcome this limitation, exogenous agents not naturally present in the brain can be introduced. For example, protamine-1 (PRM1), primarily found in male germ cells, has been proposed as a potential CEST contrast agent due to its high concentration of guanidine-based protons, which can exchange with water protons [18].

Recombinant adeno-associated viruses (rAAVs) vectors are intensively used for delivering exogenous genes in brain research due to their excellent transfection efficacy and low cytotoxicity [19]. Utilizing MRI contrast agents encoded by rAAVs can further enhance the utility of MRI in the brain imaging [20, 21]. For instance, rAAVs can encode aquaporins, which selectively enhance water diffusivity in neurons, thus enabling diffusion-weighted MRI [22].

Herein, we proposed a novel methodology for *in vivo* brain-enhanced MRI. After demonstrating the CEST properties of PRM1 in solution, we transfected 293T cells with a plasmid expressing PRM1 and subjected them to CEST MRI analysis. Additionally, we established a recombinant adeno-associated virus 2 (rAAV2) delivery system encoding PRM1 and injected it into the caudate putamen (CPU) region of the mouse brain. Thirty days post-injection, CEST MRI was performed to assess the targeted CPU region (See Graphical abstract). This approach offers a promising approach for detecting specific brain cell types in live animals, providing valuable insights into dynamic brain changes and various pathological conditions.




**Graphical abstract.** Schematic illustration of the protamin-1 encoded rAAVs used for CEST MRI of the mouse brain.

## 2. Materials and methods

### *2.1 Peptide synthesis*

The amino acid sequence of PRM1 is MARYRCCRSQSRSRYYRQRQRSRRRRRRSCQTRRRA MRCCRPRYRPRCRRH-$NH_2$.

PRM1 peptides were synthesized by KS-V PEPTIDE (Hefei, China) with a purity of 95%. The purity and identity of the synthesized peptides were assessed using analytical high-performance liquid chromatography (HPLC) (Fig. S1) and mass spectrometry (Fig. S2).

### *2.2 In vitro CEST MRI of PRM1 solutions*

All peptide solutions were prepared at a concentration of 0.733 mM using phosphate-buffered saline (PBS) or distilled water ($ddH_2O$). The pH of the solutions was adjusted to specific values of 6.5, 6.8, 7.2, and 7.5 by titration with 3 M HCl or NaOH. The prepared samples were then placed in 5 mm nuclear magnetic resonance (NMR) tubes for CEST MRI analysis. MRI measurements were performed using a Bruker 400 MHz wide-bore NMR spectrometer with imaging accessories, equipped with a 25 mm birdcage transmit/receive coil. For CEST MRI, the pulse sequence includes a saturation module followed by a rapid acquisition with relaxation enhancement (RARE) readout, with the following parameters: repetition time/echo time (TR/TE) = 8,000/5 ms, RARE factor = 8, slice thickness = 3 mm, matrix size = 128 × 96, $B_1$ = 0.6-7.2 μT, saturation time ($t_{sat}$) = 4 s, with saturation offset frequencies ($\Delta\omega$, where $\omega$ is the saturation pulse frequency) ranging from −15 to 15 ppm in 0.2-ppm increments, with the water resonance set at 0 ppm. $B_0$ inhomogeneity was corrected using the water saturation shift referencing (WASSR) method. The CEST signal was quantified using the metric $MTR_{asym} = [S_{sat}(-\Delta\omega)/ S_0] - [S_{sat}(\Delta\omega)/S_0]$, where $S_{sat}(-\Delta\omega)$, $S_{sat}(\Delta\omega)$ and $S_0$ represent the water signal with a saturation frequency offset at $-\Delta\omega$, $\Delta\omega$ and without saturation, respectively [23].

### *2.3 Plasmid construction*

The plasmids pAAV-CAG-EGFP-WPRE-polyA (pAAV-EGFP) and pAAV-CAG-EGFP-P2A-PRM1-

WPRE-polyA (pAAV-EGFP-PRM1) were purchased from Brain Case (Shenzhen, China). To facilitate the co-translational proteins PRM1 and EGFP, a P2A self-cleavage sequence was inserted between the two genes.

*2.4 In vitro cell studies*

293T cells were seeded into T75 flasks and incubated at 37 °C in a 5% $CO_2$ atmosphere for 24 hours to reach approximately 80% confluence. Cells were then transfected with either pAAV-EGFP or pAAV-EGFP-PRM1 plasmids using Lipo8000 transfection reagent (Beyotime, China), following the manufacturer's instructions. After transfection, the cells were incubated for an additional 48 hours for MRI analysis. Briefly, cells were treated with trypsin, resuspended in 200 μL of PBS, and centrifuged at 12,000 g for 10 min in 0.2 ml PCR tubes. The supernatant was then prepared for MRI experiments. The MRI sequence used was similar to the one previously described, with the following adjustments: TR/TE = 6,000/4.75 ms, number of averages= 1, slice thickness = 2 mm, FOV = 17 × 17 $mm^2$, matrix size = 96 × 96, $B_1$ = 3.6 μT, and $t_{sat}$ = 3 s, with saturation offset frequencies ranging from −4 to 4 ppm in 0.4-ppm increments, with the water resonance set at 0 ppm. $B_0$ inhomogeneity was corrected using the WASSR method to ensure accurate frequency alignment in the CEST spectra. The cell studies comprised seven replicates and all raw data were processed using MATLAB (R2022a, MathWorks, Natick, MA).

*2.5 Production of the rAAVs*

The rAAVs were produced in HEK293 cells using the traditional triple-plasmid transfection method [24]. The rAAV-EGFP and rAAV-EGFP-PRM1 vectors were bought from Brain Case (Shenzhen, China).

*2.6 Animals*

All surgical and experimental procedures were conducted in accordance with the guidelines set by the Animal Care and Use Committee of Innovation Academy for Precision Measurement Science and Technology, Chinese Academy of Sciences (APM21013T). Eight-week-old male C57BL/6 mice were obtained from Beijing Vital River Laboratory Animal Technology Co. Ltd. The mice were housed in a controlled environment with a 12-hour light/dark cycle, stable temperature, and humidity, with unrestricted access to food and water.

*2.7 Stereotaxic surgery*

Five mice were intraperitoneally injected with pentobarbital sodium (50 mg/kg), and positioned in a stereotaxic frame (RWD, China). After exposing the skull, a small hole was drilled to allow the insertion of a glass micropipette (World Precision Instruments, USA). One microliter of purified rAAV solution ($5 \times 10^{12}$ viral genomes/ml in PBS) was stereotaxically injected into the target region, the CPU, using coordinates based on the mouse brain atlas. The coordinates for the CPU were as follows: 0.51 mm anterior to Bregma, ± 2 mm lateral from midline, and 3.3 mm in depth relative to Bregma. The infusion rate was set at 50 nL/min. Upon completion of the injection, the pipette was kept in place for 20 min before being slowly withdrawn. The mice were placed on a warm pad to fully recover from anesthesia before being returned to cages for further observation.

*2.8 In vivo CEST MRI*

Thirty days after the virus injection, all the five mice were under MRI observation. Anesthesia was induced with 3.5–4.0% isoflurane (RWD, China) then maintained at 1.0–1.5% isoflurane. The respiratory

rate was monitored and maintained at approximately 60 breaths per minute. A warm water pad was used to keep the body temperature at approximately 36.5 °C. The mouse brain was scanned using a transmit birdcage coil and a receive-only surface coil (20 mm in diameter). For the CEST MRI, a pulse sequence was employed with the following parameters: TR/TE = 6,000/4.75 ms, number of averages = 1, slice thickness = 2 mm, FOV = 16 × 14 $mm^2$, matrix size = 96 × 72, $B_1$ = 2.4 μT, and $t_{sat}$ = 3 s. Saturation offset frequencies from −4 to 4 ppm in 0.4-ppm increments, with the water resonance set at 0 ppm. $B_0$ inhomogeneity was corrected using the WASSR method, with a total acquisition time of 5 min. The total scan time for CEST MRI was 12 minutes and 48 seconds.

*2.9 Statistical analysis*

Statistical differences between groups were assessed using Student's t-test, with significance levels set at $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. The number of independent replicates for each experiment is indicated in the figure captions. All statistical analyses were performed using GraphPad Prism 10 software.

## 3. Results and discussion

To evaluate the influence of pH levels and saturation field strengths on the CEST contrast of PRM1 solutions, we analyzed a 0.733 mM PRM1 solution. PRM1 exhibited two distinct peaks in the CEST spectra: one corresponding to the amide protons of the protein backbone at a 3.6 ppm offset, the other to the guanidyl protons of the arginine side chain at a 1.5 ppm offset. The CEST signals varied with different pH values due to changes in the exchange rate [25]. Optimal CEST performance was observed within a pH range of 7.2–7.5, which aligns with the physiological range of human brain [26, 27]. At pH 7.2, the optimal CEST contrast was noted at 1.5 ppm (Fig. 1a and Fig. S3). The $MTR_{asym}$ spectrum of 0.733 mM PRM1 solution reached a peak of 40%. To identify the optimal saturation power for detecting PRM1, we tested a range of $B_1$ values. A saturation field of 3.6 μT provided the clearest CEST signal, producing the two characteristic peaks at 3.6 and 1.5 ppm (Fig. 1b), consistent with previously reported data [18]. Based on these findings, we selected 3.6 μT as the saturation power for subsequent *in vitro* experiments evaluating PRM1 as a CEST contrast agent.

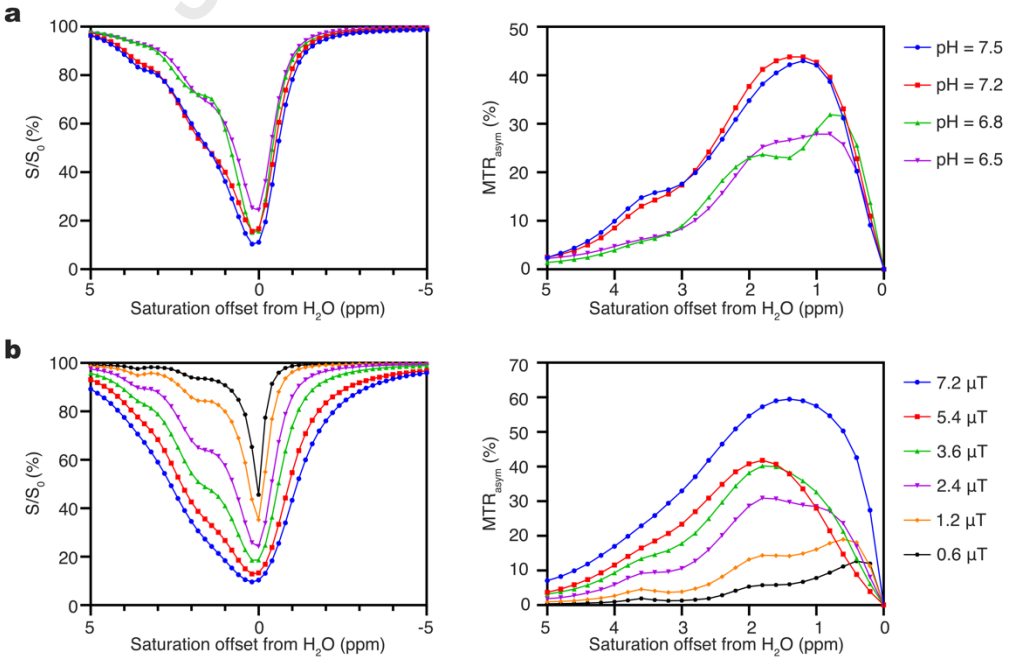


**Fig. 1.** Z-spectra and $MTR_{asym}$ spectra of PRM1 solutions under different conditions. (a) pH dependence of PRM1 CEST contrast at $B_1$ = 3.6 μT, $T_{sat}$ = 4 s; (b) Effect of saturation field strength on PRM1 CEST contrast at pH = 7.2, $T_{sat}$ = 4 s.

To investigate the potential of PRM1 as a CEST contrast agent in cells, we conducted an *in vitro* study using 293T cells. These cells were transfected with a plasmid encoding both EGFP and PRM1, while those transfected with pEGFP alone served as the control group (Fig. 2a). Forty-eight hours post-transfection, the cells were collected for CEST MRI analysis. The Z-spectrum and $MTR_{asym}$ spectrum of the transfected cells are shown in Fig. 2b. The $MTR_{asym}$ spectrum around the 1.5 ppm frequency offset for PRM1-expressing cells was remarkably higher than that for the control group. Additionally, a representative $MTR_{asym}$ map at the 1.5 ppm frequency showed a significantly stronger signal in cells with expressed PRM1 compared to those without PRM1 encoding (Fig. 2c-d). These findings suggest that PRM1 is an effective CEST contrast agent in cellular contexts. However, a reduction in CEST contrast was observed *in vivo* compared to the phantom studies, likely due to interference from the nuclear overhauser enhancement (NOE) and magnetization transfer (MT) effects [28, 29]. The complex cellular environment, such as phosphorylation or interactions with negatively charged metabolites, may alter the proton exchange behaviors and peptide structure of PRM1, thus affecting its contrast performance [30].

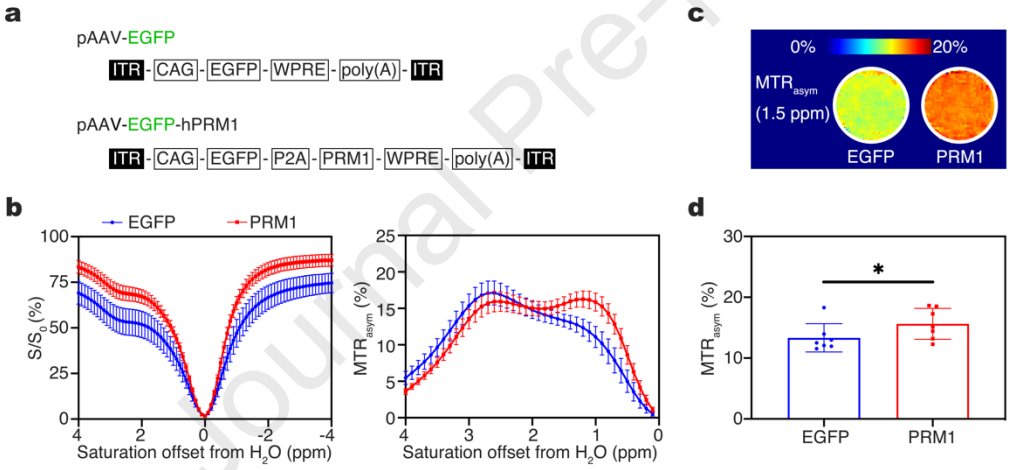



**Fig. 2.** CEST-MRI of PRM1-expressing 293T cells. (a) Schematic representation of recombinant AAV transfer plasmids encoding the gene of interest (GOI). The GOI, driven by the CAG promoter, is inserted between inverted terminal repeats (ITRs) for ubiquitous protein expression in mammalian cells and gene packaging into the AAV particles. The woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) is included to enhance transgene expression. Plasmid names: pAAV-EGFP and pAAV-EGFP-PRM1. (b) Z-spectra and $MTR_{asym}$ spectra of 293T cells expressing EGFP or EGFP-PRM1. (c) Representative $MTR_{asym}$ maps of 293T cells expressing EGFP or EGFP-PRM1 at a frequency offset of 1.5 ppm. Cells were transfected with AAV transfer plasmids and analyzed using *in vitro* CEST MRI. (d) Mean $MTR_{asym}$ (± SD) at a frequency offset of 1.5 ppm ($n$ = 7). CEST data were acquired at 9.4 T, $B_1$ = 3.6 μT, and $T_{sat}$ = 3 s.

Prior to the *in vivo* studies, we performed optimization experiments using a healthy mouse and a range of $B_1$ saturation powers (0.5 to 5.0 μT). Higher saturation powers led to reduced image detail and spectral resolution, particularly affecting brain region differentiation in $MTR_{asym}$ maps (Fig. S4). To optimize both contrast and clarity, 2.4 μT was selected as the optimal saturation power for subsequent *in vivo* studies.

To investigate whether CEST MRI could successfully detect PRM1 encoded by rAAV *in vivo*, we stereotactically injected rAAV-EGFP-PRM1 or rAAV-EGFP into the bilateral CPU regions of the mouse brain (Fig. 3a). The mice were observed under a 9.4 T MRI scanner 30 days post-injection. The brain region injected with rAAV-EGFP-PRM1 exhibited enhanced CEST contrast compared to that of those received rAAV-EGFP treatment, while their $T_2$-weighted images showed no significant differences (Fig. 3b-c and Fig. S5). The Z-spectrum and $MTR_{asym}$ spectrum of the infected brain demonstrated an enhanced CEST signal at a 1.5 ppm frequency shift in the right CPU ($MTR_{asym}$ = 4.91 ± 0.52%), confirming the efficacy of PRM1 for *in vivo* CEST imaging in mouse brains (Fig. 3d-e). In the study that applied amide proton transfer (APT) to assess the CEST value in the human brain, the APT-weighted value was reported as 1.35 ± 0.15% [31]. In contrast, our data showed that the $MTR_{asym}$ value in the right CPU is 3.6 times higher than the APT value, suggesting that PRM1 is a more promising contrast agent compared to endogenous molecules.

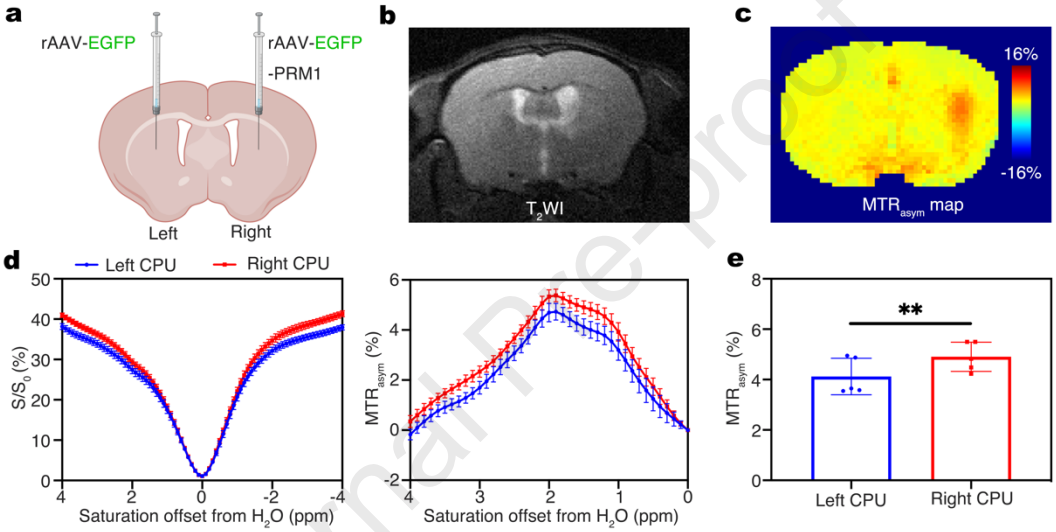



**Fig. 3.** CEST-MRI of PRM1 in mouse brain. (a) Scheme illustrating a horizontal brain slice with rAAV-EGFP and rAAV-EGFP-PRM1 injections into the bilateral CPU regions. Representative $T_2$-weighted image (b) and $MTR_{asym}$ map (c) of the mouse brain 30 days post-injection. (d) Z-spectra and $MTR_{asym}$ spectra of left and right CPU regions 30 days post-injection. (e) Mean $MTR_{asym}$ (± SD) at the 1.5 ppm frequency offset for the left and right CPU regions ($n$ = 5). CEST data were acquired at 9.4 T, $B_1$ = 2.4 μT, and $T_{sat}$ = 3 s.

This study integrates two promising techniques: MRI reporter-encoding AAV2 and *in vivo* CEST MRI. By using a Cre-dependent expression cassette in combination with a Cre-transgenic mouse model, this approach promises to enhance the specificity of *in vivo* cell type investigations. Such developments are especially relevant for studying neurodegenerative disorders, such as Parkinson's disease. For example, recent studies have demonstrated a reduction in APT and CEST contrast in the substantia nigra of Parkinson's patients compared to healthy controls [31, 32]. However, because this contrast depends on endogenous cellular proteins and peptides, it often encounters sensitivity limitations due to background signal interference. The selective expression of PRM1 in the substantia nigra may provide more precise insights into dopaminergic neurons, offering potential for improved monitoring of Parkinson's disease progression.

The study has some limitations. In complex biological environments, factors such as MT, NOE, and

direct water saturation (DS) can affect the accurate quantification of $MTR_{asym}$ signal intensity. To mitigate these effects, multi-pool Lorentz fitting provides an alternative approach for future experiments[33, 34]. Additionally, the study utilized stereotactic injection of rAAV vectors into specific brain regions, which is relatively invasive. Non-invasive delivery techniques, such as focused ultrasound blood-brain barrier opening (FUS-BBBO), offer an alternative method for delivering rAAV directly to targeted brain areas [35, 36]. FUS-BBBO has been successfully applied for rAAVs delivery in various animal models. Integrating non-invasive gene delivery with MRI imaging could present a promising strategy for future studies, especially in non-human primates [37].

## 4. Conclusion

This study presents a novel approach for the *in vivo* detection of brain cells using a CEST contrast agent. By incorporating the PRM1 gene into an rAAV2 vector and employing CEST MRI, we successfully detected transfected brain cells in the CPU region. This method provides valuable insights into dynamic brain changes under both physiological and pathological conditions, with promising applications for advanced analysis in larger animals, including non-human primates.

## CRediT author contribution statement

**Kairu Xie:** Investigation, Methodology, Data analysis, Writing-Original draft preparation. **Yaping Yuan:** Methodology, Data analysis. **Mou Jiang:** Methodology, Data analysis. **Daiqin Chen:** Methodology, Data analysis, Supervision, Writing-Review and Editing. **Shizhen Chen:** Conceptualization, Supervision, Writing-Review and Editing. **Xin Zhou:** Conceptualization, Supervision, Writing-Review and Editing.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Xin Zhou is an editorial board member of *Magnetic Resonance Letters* but was not involved in the editorial review or the decision to publish this article.

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**Xin Zhou** received Ph.D. degree from Wuhan Institute of Physics and Mathematics, Chinese Academy of Sciences in 2004. After his postdoctoral research periods in Harvard University (2005-2007) and research fellow experience in University of California Berkeley (2007-2009), he joined Wuhan Institute of Physics and Mathematics, Chinese Academy of Sciences. The research interests of his group are mainly focused on the development of novel methodology and techniques of NMR. Group homepage: http://zhou.apm.ac.cn/.

**Shizhen Chen** received her Ph.D. degree in Analytical Chemistry from Central China Normal University in 2011. From 2011 to 2013, she completed postdoctoral training at the Wuhan Institute of Physics and Mathematics, Chinese Academy of Sciences, where she now serves as a professor. Her work focuses on multi-nuclear magnetic resonance molecular imaging.

**Kairu Xie** received her M.D. from the Tongji Medical College at Huazhong University of Science and Technology (HUST), Wuhan, in 2018. She is currently pursuing a Ph.D. at the Wuhan National Laboratory for Optoelectronics, HUST. Her research focuses on nucleic acid delivery vectors and magnetic resonance imaging.