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Supplementary Information for

A Novel Positron Emission Tomography Reporter Gene Strategy for use in the Central Nervous System

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Supplementary text (Animal imaging Methods section)
Captions for movies S1 to S2
Figs. S3 to S6

Other supplementary materials for this manuscript include the following:

Movies S1 to S2

Supplementary Information Text

In Vitro quantitative Polymerase Chain Reaction (qPCR) Analysis:

Transfected cells were formed into pellets by centrifugation and mRNA extracted using Qiagen RNeasy Plus Mini Kit. cDNA was synthesized from the mRNA by heating mRNA in water (50 ng/µL, 10 µL), Oligo(dT)12-18 Primer 1 µL, dNTP Mix (10 mM, 1µL), at 65 ^{o}C for 5 minutes. Following heating, 5x first strand buffer (4 µL), 0.1M DTT (2 µL), M-MLV reverse transcriptase (1 µL) and RNaseOUT™ Recombinant Ribonuclease Inhibitor (1 µL) were added to the mixture. The reaction mixture was heated at 37 ^{o}C for one hour followed by 70 ^{o}C for 15 min to generate the complimentary cDNA strand.

For qPCR, to each well was added Power SYBR® Green PCR Master Mix (12.5 µL), water (8.5 µL), primer designed and synthesized in house, forward (TCGGAGGTTTGATGAAAT) (1 µL) and reverse (TCTCCAGCATCTGAGTAG) (1 µL) and cDNA. Triplicates of each sample were plated. β-actin was used as a housekeeping gene in order to assess the fold-changes in PKM2 mRNA expression. Negative controls were generated by omission of cDNA to ensure no cross-contamination was present. The

qPCR protocol involved a 15 min at 95ºC initialization, 15 s at 95ºC denaturation, 30 s at 56ºC with an optical reading, 30 s at 72ºC extension. Steps 2-4 were cycled 40 times followed by a melting curve of 75ºC to 95ºC over 10 s with an optical reading. Analysis was carried out using Bio-Rad CFX Manager employing a ΔΔCq analysis method.

Animal imaging:

Small animal PET/CT imaging was repeated periodically over 8 weeks. Tail vein catheters (12 cm polyurethane tubing and 27 g butterfly needle) were inserted into the tail vein of the mice and the catheter was glued onto the tail using Vet Bond (tissue glue). PET imaging scans were carried out on a docked Siemens Inveon PET/CT scanner (Siemens Healthcare, Erlangen, Germany) (matrix size, 128 × 128 × 159; CT attenuation-corrected; non-scatter-corrected) after a bolus intravenous injection of 7.4-11.1 MBq of [^{18}F]DASA-23 into mice. Dynamic scans were acquired in list mode format over 30 mins. The acquired data were then sorted into 0.5-mm sinogram bins and 19 time frames for image reconstruction (5 × 15 s, 4 × 60 s, and 11 × 300 s), which was done by iterative reconstruction with the following parameters: 3D ordered-subset expectation maximization (3D-OSEM) followed by fast maximum a posteriori (fastMAP); MAPOSEM iterations, 2; MAP subsets, 16; MAP iterations, 18. Region of interest (ROI) analyses were performed using IRW software (Inveon Research Workplace, Siemens, Germany), ROIs were drawn around the injection site and a corresponding ROI of similar size and location drawn on the contralateral side. All analyses were carried out using mean %ID/g. Due to time constraints and the limited half-life of the radiotracer not all mice could be imaged at each time point. Data from all imaged mice has been reported unless otherwise stated.

MR images were acquired 30 days post injection. MRI was performed at the Stanford Clark Center Small Animal Imaging Facility in an actively shielded Discovery MR901 General Electric 7T horizontal bore scanner (GE Healthcare, Chicago, IL) including Integrated Electronics Company gradient drivers, an Agilent 120–mm–inner diameter shielded gradient insert (600 mT/m, 1000 T/m/s), EXCITE2 electronics, the supporting LX11 platform, and a 3–cm–inner diameter Millipede quadrature transmit/receive volume RF coil. Animals were anesthetized with 2% isoflurane in oxygen, and physiological monitoring included respiration and temperature feedback for maintaining surface body temperature by warm airflow over the animal. A fast spoiled gradient echo sequence (repetition time, 9.7 ms; echo time, 2.1 ms; flip angle, 5°; number of excitations, 13; field of view, 2 cm; image matrix, 160 × 160; slice thickness, 1 mm) was used to acquire 8 orthogonal T2-weighted images in the coronal planes through the region of transduction. PET/MR registration was performed in IRW (Siemens Healthcare, Erlangen, Germany) using the CT image for alignment of the skull.

The expression of Firefly luciferase in mice transfected with AAV-Luc 2 were imaged using bioluminescent imaging 14, 33 and 56 days post AAV injection. Mice were anesthetized as described above; images were acquired 15 min (as determined by sequential imaging), post intra-peritoneal injection of D-luciferin (100 μL, 30mg/mL)(Biosynth, Itasca, IL). Imaging was carried out on an IVIS Spectrum (Perkin Elmer, Waltham MA). Images produced and analyzed using Living Image® software and data expressed as average radiance ($p/s/cm^2/sr$).

Ex Vivo Autoradiography:

Sagittal brain sections of PKM2 transduced mice (n=2), 57 days after injection were obtained 50 min after intravenous injection of 7.4 MBq [^{18}F]DASA-23. Anesthetized mice were perfused with saline (10 mL) to remove intravascular [^{18}F]DASA-23, and after cervical dislocation, the brain was removed and embedded in optimal cutting temperature (OCT) compound (VWR, Radnor, PA) before being frozen on dry ice. Subsequently, 14-µm-thick sagittal brain sections were cut with a cryostat microtome HM 500 (Microm, Thermo Fisher Scientific Inc. Waltham, MA). The sections were mounted on microscope slides (Fisherbrand Superfrost Plus microscope slides, Thermo Fisher Scientific Inc. Waltham, MA), air-dried for a minimum of 5 min, and then exposed to MultiSensitive storage phosphor screens (PerkinElmer) for 18 hours at −20°C. The image plates were analyzed with a Typhoon 9410 variable mode imager (Amersham Biosciences, Amersham, UK). Image data were visualized and processed using ImageJ. Images we converted to 8-bit, regions of interest were drawn around brain sections and histograms produced for each sample to show the distribution of grey values.

Histopathology:

After imaging, anesthetized mice transduced with AAV and control groups were transcardially perfused with 0.9% saline, followed by the removal of the brain. Formalin-fixed brains (10%, v/v) were embedded in paraffin, sectioned into 14-µm-thick slices and mounted on microscope slides according to standard procedures. Sections were taken at regular intervals across the entire brain. Immunofluorescence against PKM2 protein was performed on paraffin-embedded sections with rabbit monoclonal antibodies reactive to

both mouse and human isozymes (1:100, Cell Signaling Technology, Danvers, MA) using standard techniques and according to the manufacturer's instructions, the secondary antibody was conjugated to a fluorophore (594 nm)(Cell Signaling Technology, Danvers, MA). Image analysis was performed using ImageJ. Images were spilt into color channels, Red(R), Green (G), Blue (B) and the red channel used for analysis. ROIs were drawn over areas of varying intensities, and their area, mean grey value and integrated density were measured. Form these values a corrected total cell fluorescence (CTCF) was calculated for each ROI.

Ex Vivo qPCR Analysis of PKM2 mRNA expression in mouse brain:

In a new cohort of mice (n=8), PKM2 AAV9 was stereotactically delivered identically to the previous cohort. They were imaged with [^{18}F]DASA-23 57 days post AAV delivery and their brains excised immediately after imaging. A portion of brain tissue (100-150 mg) corresponding the injection site was used for mRNA extraction, using Zymo Research Direct-zol^{TM} RNA MiniPrep Plus. Following extraction of the mRNA, cDNA synthesis and qPCR was performed as described above.

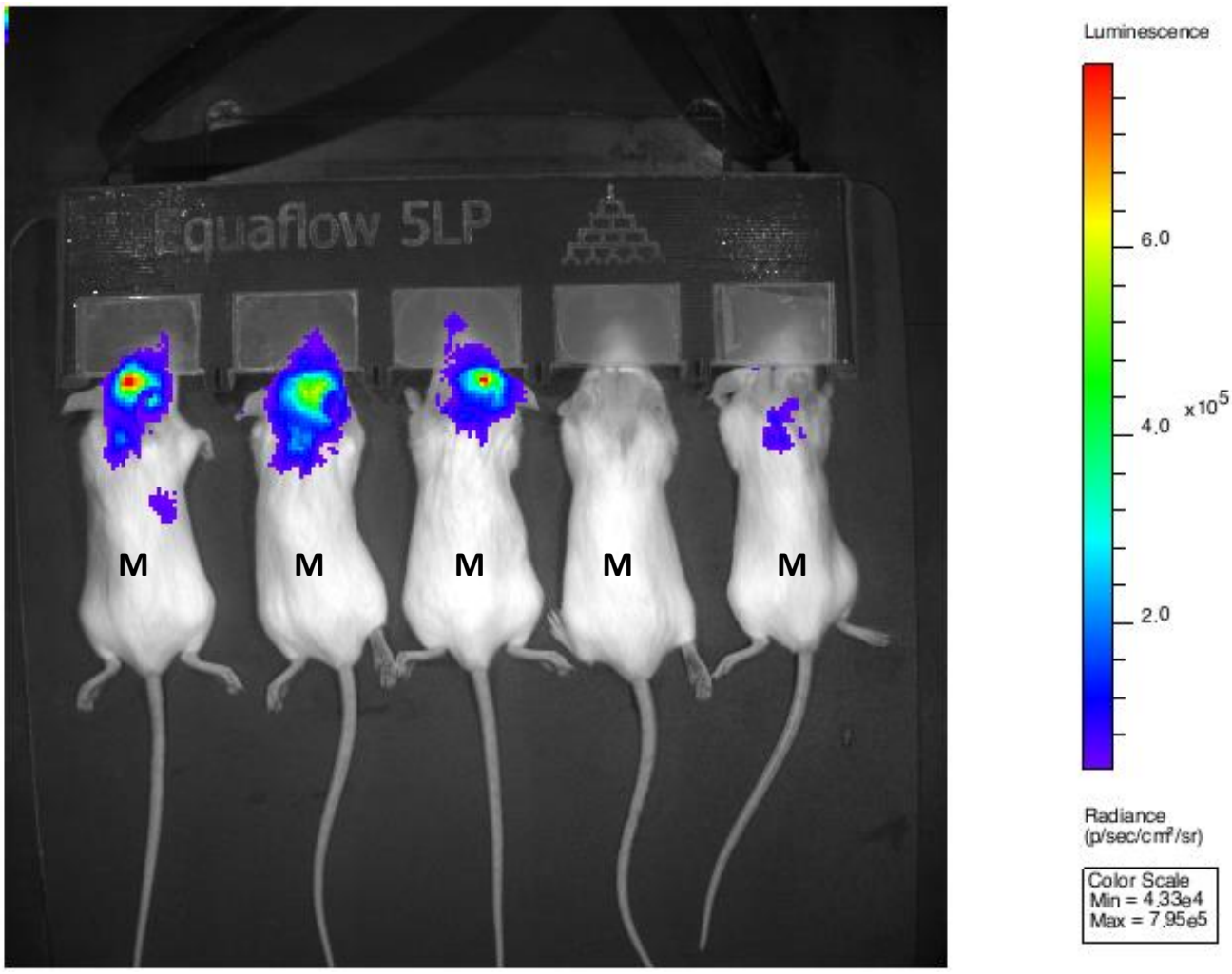

Fig. S1. Bioluminescent images of mice transfected with AAV-Luc2 33 days post injection of AAV (M1, M2, M3, M5) and AAV-PKM2 (M4) acting as a negative control.

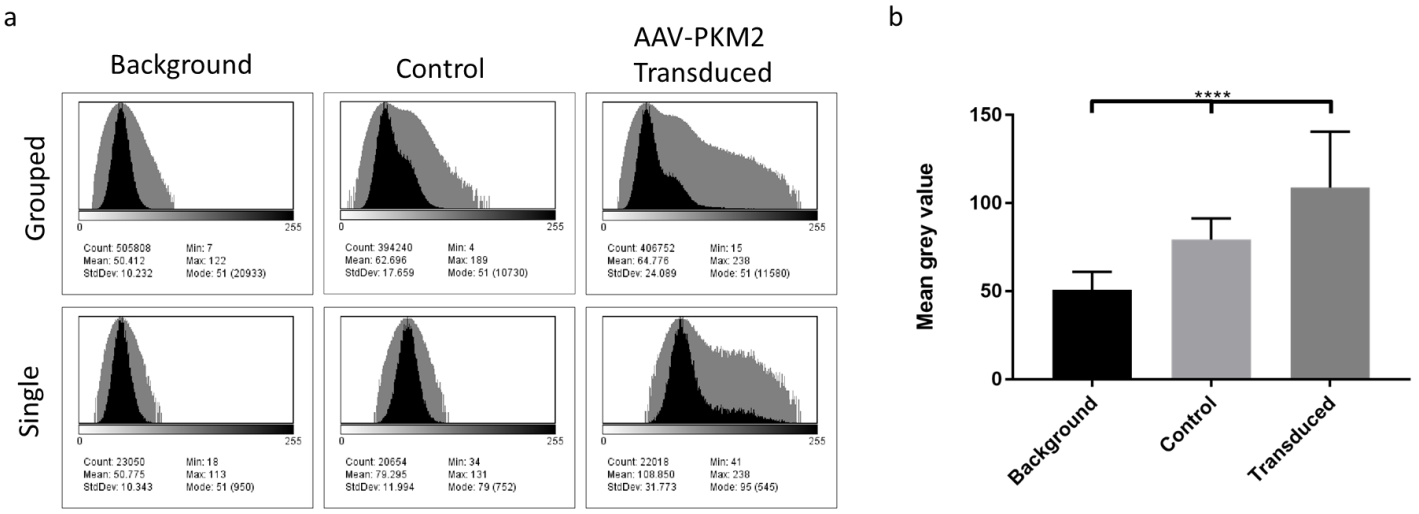

Fig. S2. (A) Histograms showing the distribution of pixels in 8-bit autoradiography images. 0 indicates white (low activity), 255 indicated black (high activity). Black curves are normal distribution while grey curves indicate Log distribution. The top row show a group of sections (1 slide, 5 sections) of control and transduced sections with an area of the background of similar area while the bottom row show examples of individual sections. Control regions show a shift towards the black region compared to background, indicating higher radioactivity in control brains than the background reading. AAV-PKM2 transduced regions show a further shift towards the black, again indicating higher levels of activity. (B) Chart depicting the mean grey value of individual brain sections. Showing that transduced regions have a higher mean grey value than control, indicating that more radioactivity was present in transduced sections. The SD of transduced region is also greater than control, showing that the increased radioactivity is not evenly distributed but in distinct regions of the section. Statistical significances were observed between all comparisons ($P<0.0001$).

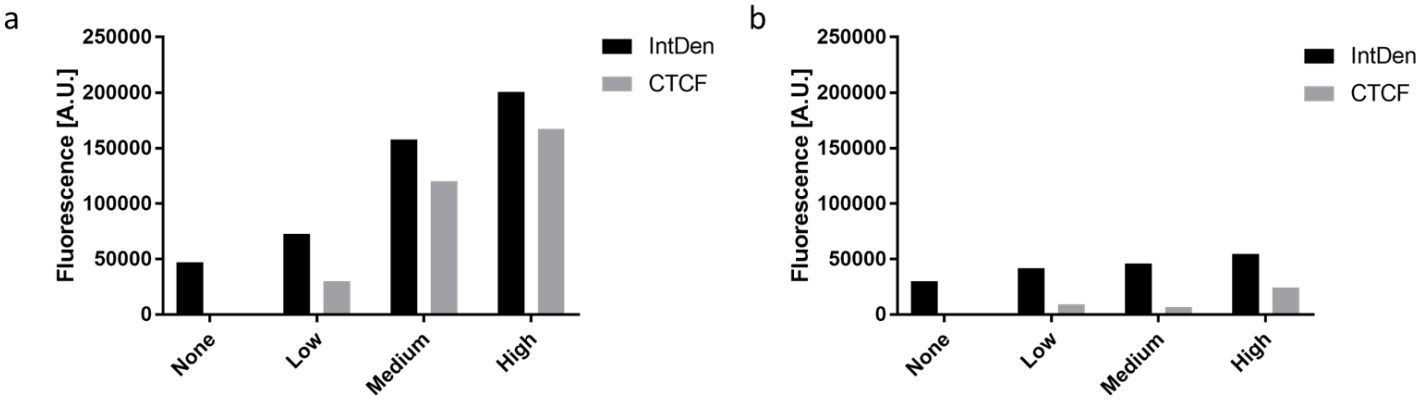

Fig. S3. Charts showing the integrated density (IntDen) and corrected total cell fluorescence (CTCF) of two regions of immunofluorescence images transduced with AAV-PKM2 (A) and control (B). ROIs were drawn over areas of increasing signal (determined visually) and their area, mean pixel value and IntDen, from these values and the mean of the region with the no fluorescent signal (None) CTCF was calculated, representing the fluorescence of each region. There was statistical significance between lowest and highest region of the transduced region ($P<0.0001$), but no statistical significance between regions in the control section.

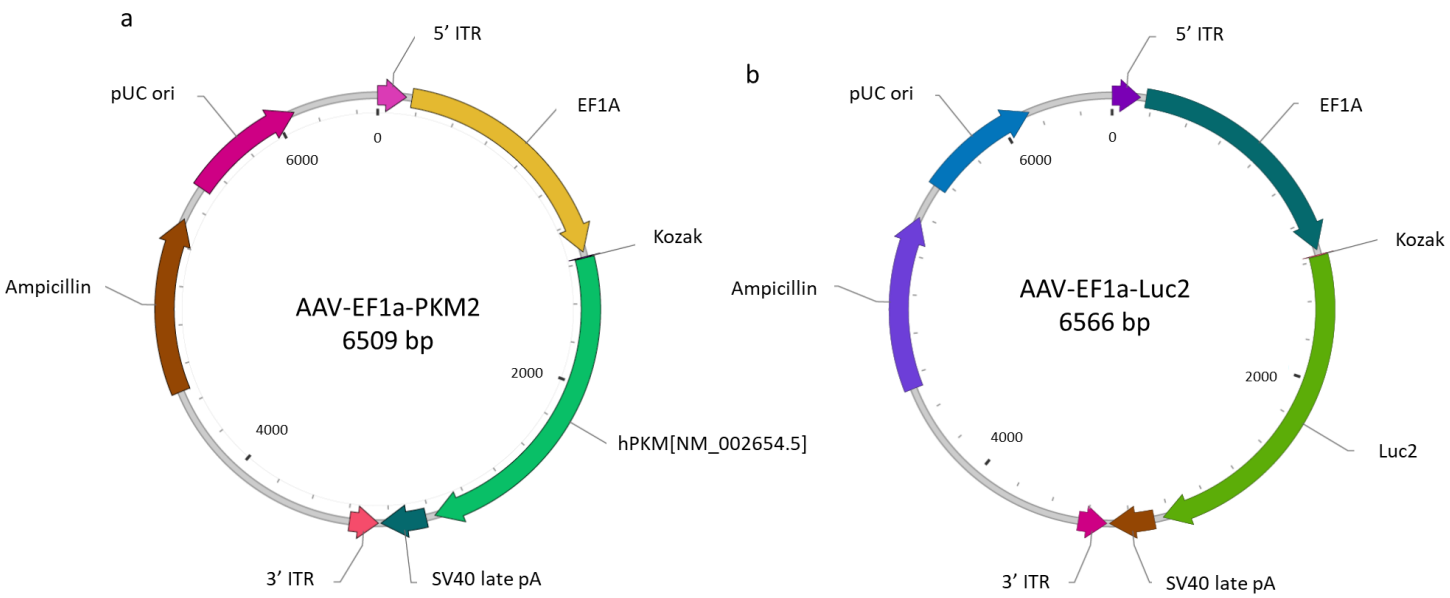

Fig. S4. Graphical depictions of the AAV vectors used in the transduction of mice. A) PKM2 vector B) Luc2 vector**.**

Figure attached separately

Movie. S1. Time-lapse video of a 30 minute dynamic PET scan at 57 days after AAV-PKM2 injection. Coronal view showing the tracer accumulation in the right hemisphere of the brain.

Figure attached separately

Movie. S2. Masked maximum intensity projection (MIP) at 30 minutes of transduced mouse brain 57 days after AAV-PKM2 injection. The image consists of a CT with a MIP color overlay.