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In vivo imaging of astrocytes in the whole brain with engineered AAVs and diffusion-weighted magnetic resonance imaging

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1 ***In vivo* Imaging of Astrocytes in the Whole Brain with Engineered AAVs and Diffusion**
2 **Weighted Magnetic Resonance Imaging**

3
4 **Running title:** *In vivo* Imaging of Astrocytes in the whole brain

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37
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40

41 **Abstract**

42 Astrocytes constitute a major part of the central nervous system, and the delineation of their
43 activity patterns is conducive to a better understanding of brain network dynamics. This study
44 aimed to develop a magnetic resonance imaging (MRI)-based method in order to monitor the
45 brain-wide or region-specific astrocytes in live animals. Adeno-associated virus (AAVs) vectors
46 carrying the human glial fibrillary acidic protein (GFAP) promoter driving the EGFP-AQP1
47 (Aquaporin-1, an MRI reporter) fusion gene were employed. The following steps were included:
48 constructing recombinant AAV vectors for astrocyte-specific expression, detecting MRI
49 reporters in cell culture, brain regions or whole brain following cell transduction, stereotactic
50 injection, or tail vein injection. The astrocytes were detected by both fluorescent imaging and
51 Diffusion Weighted MRI. The novel AAV mutation (Site-directed mutagenesis of surface-
52 exposed tyrosine (Y) residues on the AAV5 capsid) significantly increased fluorescence intensity
53 ($p < 0.01$) compared with the AAV5 wild type. Transduction of the rAAV2/5 carrying AQP1
54 induced the titer-dependent changes in MRI contrast in cell cultures ($p < 0.05$) and caudate
55 putamen (CPu) in the brain ($p < 0.05$). Furthermore, the MRI revealed a good brain-wide
56 alignment between AQP1 levels and ADC signals, which increased over time in most of the
57 transduced brain regions. In addition, the AAV-PHP.eB serotype efficiently introduced AQP1
58 expression in the whole brain via tail vein injection. This study provides an MRI-based approach
59 to detect dynamic changes in astrocytes in live animals. The novel in vivo tool could help us to
60 understand the complexity of neuronal and glial networks in different pathophysiological
61 conditions.

62

63 **Introduction**

64 As the most abundant and most widely distributed cells in the brain of mammals, astrocytes
65 are estimated to represent 19-40% of brain cells with variations in brain regions or species. [1]
66 They have critical physiological functions such as providing structural and metabolic support for
67 neurons, participating in the blood-brain barrier formation, regulating synaptic formation and
68 transmission, as well as exerting neuroprotection. [2-6] A better understanding of how astrocytes
69 mediate modulation of neural circuits and the spatiotemporal dynamics of astrocytes is necessary
70 in order to decipher the roles played by astrocytes in different pathophysiological conditions and
71 eventually develop astrocyte-based strategies for disease therapy. However, reliable and selective
72 tools for detecting astrocytes in living animals are still inadequate. [7]

73 The techniques of molecular biology, genetics, morphology, and physiology have greatly
74 improved and are widely used to study astrocytes systematically. [7] Neuroscience techniques
75 have also provided preliminary observation and manipulation methods for the study of astrocytes,
76 but each has its own advantages and disadvantages. The transcriptome and proteome of astrocytes
77 have been extensively used to explore astrocyte specific molecules and pathways involved in the
78 development of various disease models. These findings were derived from studies in cultured
79 astrocytes or freshly isolated astrocytes. [8-11] The purification process has been shown to
80 markedly change gene expression profiling, which can introduce variations of astrocytes in living
81 animals. [12] For the *in vivo* targeted astrocytes, several genetically encoded reporters are
82 available, including fluorescent and luminescent proteins, but they have limited utility due to
83 poor penetration of light into deep tissues. [13] There are still many problems to be resolved such
84 as detecting morphological and functional changes in astrocytes of the whole brain *in vivo*. [2,
85 14] Therefore, to establish the multi-modal whole-brain synchronous detection of astrocytes, it
86 is necessary to introduce new technologies that upgrade the existing viral tools, organically
87 integrate the existing neuroscience technologies and finally realize the synchronous study of
88 whole-brain astrocytes *in vivo*.

89 Among various imaging approaches, magnetic resonance imaging (MRI) is a unique
90 imaging method that combines the advantages of non-invasion, non-ionizing radiation, high
91 penetration, and compatibility with soft tissues as well as a living system. With a proper reporter,
92 MRI could be used to detect astrocytes in living animals. One example is the aquaporin 1 (AQP1),
93 which is a highly conserved transmembrane transporter enriched in the cell membrane. AQP1 is
94 expressed in a variety of cell types and has a highly selective permeability to water. [15] A
95 previous study reported that AQP1 protein could induce changes in diffusion-weighted imaging
96 (DWI) signals without causing any significant damage to cells or tissues. [16] AQP1 represents
97 a class of metal ions and chelates free MRI reporter genes. To our knowledge, there is no study
98 which has explored the direct use of the viral vector carrying AQP1 gene to produce MRI imaging
99 of astrocytes.

100 Among assorted viral vectors for *in vivo* studies, the recombinant adeno-associated virus
101 (rAAV) has the lowest toxicity, and has been widely utilized in neuroscience. [17] rAAV is known
102 to efficiently cause astrocyte-specific gene transfer by utilizing human glial fibrillary acidic
103 protein (GFAP) and cap5 capsid. [18] AAV-PHP.eB, one of the serotypes of AAVs, has also been
104 shown to efficiently cross the blood-brain barrier (BBB) and deliver exogenous genes to brain
105 cells. [19] Thus, it should be the best candidate for *in vivo* imaging of astrocytes in the whole
106 brain.

107 Here, AAV vectors carrying AQP1 under the control of the GFAP promoter were developed.
108 With the combination of multi-model methods, astrocytes were successfully detected in cell
109 cultures, injected sites and the whole brain. This study provides an MRI-based approach to detect
110 the dynamic changes of astrocytes in live animals, and this novel *in vivo* tool could help us to
111 understand the complexity of neuronal and glial networks in different pathophysiological
112 conditions.

113

114 **Materials and methods**

115 The current study includes steps that optimize and construct the recombinant AAV vectors
116 for astrocyte-specific expression, and detect MRI reporters in the cell culture, brain regions or
117 whole brain following cell transduction, stereotactic injection, or tail vein injection. The
118 methodologies are described as follows.

119 **Cell culture**

120 The human embryonic kidney 293 cells (ATCC) and the U87 human astrocytoma cell line
121 (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone) containing
122 10% fetal bovine serum (FBS; Gibco) and 100 U/ml of penicillin/streptomycin. All cell lines
123 were grown at 37 °C in 5% CO₂.

124 **Animal studies**

125 All procedures were approved by the Animal Care and Use Committee at the Shenzhen
126 Institute of Advance Technology Chinese Academy of Science. The wild-type C57BL/6 mice (6
127 w) were purchased from Hunan SJA Laboratory Animal Co., Ltd (Hunan, China). All animals
128 were kept in a temperature-controlled environment with a constant 12 h/12 h light-dark cycle.
129 Food and water were available *ad libitum*.

130 **Plasmids**

131 This study generated two different AAV plasmids pAAV-GFAP-EGFP-WPRE-polyA and
132 pAAV-GFAP-AQP1-2A-EGFP-WPRE-polyA (Available from Brain Case, Shenzhen, China).
133 The PHP.eB plasmids were bought from Addgene (Plasmid 103005, Addgene, Watertown, USA).

134 To further improve transduction efficiency *in vivo*, mutagenesis was adopted to convert
135 surface-exposed tyrosine (Y436-Y693-Y719) residues to phenylalanine (F) on AAV5 capsid (Fig.
136 S1). Site-directed mutagenesis was performed using primers with point mutations (TAT to TTC)
137 in PCR following the manufacture's instruction. [20] The sequence of primers is provided in the
138 supplemental materials (Table S1).

139 **Construction of recombinant AAV vectors and virus titration**

140 The rAAV virions were produced in HEK293 cells using a traditional triple-plasmid
141 transfection method. HEK293 cells at 80% confluence were co-transfected with pAAV-Cap,
142 pAAV-GFP, and pAAV-helper using PEI reagent. Cells were harvested after 72 h post-infection
143 and the viruses were purified by an iodixanol step density gradient centrifugation as described
144 previously. [21] The titers of the purified AAV virions were determined by qPCR with SYBR
145 Green PCR Master Mix (Bio-Rad) using the following primer-pairs specific for the WPRE: F5'-
146 TCCCATAGTAACGCCAATAGG-3', R5'-CTTGGCATATGATACACTTGATG-3'. The
147 standard curves were generated with 10-fold serial dilutions of standard plasmids.

148 The detailed experimental steps for *in vitro* transduction assays, western blotting, virus
149 stereotaxic injection/tail vein injection, diffusion-weighted MRI, GFP fluorescence imaging, and
150 immunohistochemistry were provided in the supplemental materials.

151

152 **Data analysis and statistics**

153 All the MRI data was transformed to NIFTI format using the Bru2Anz (Bruker, Germany)
154 and converted on the ADC map using the custom-made software developed in MATLAB. Then
155 they were normalized to a homemade mouse brain template with statistical packages (SPM12,
156 www.fil.ion.ucl.ac.uk/spm). Furthermore, a publicly available mouse brain template TMBTA
157 (www.nitrc.org/projects/tmbta 2019) was utilized to segment the whole brain into different
158 partitions for calculating ADC values in different brain regions. The GraphPad Prism (version
159 8.0.1 for Windows, GraphPad Software, San Diego, California USA) was used to perform two-
160 tailed *t*-tests for the *in-situ* injection data and repeated measures ANOVA analyses and multiple
161 comparison corrections were utilized for multiple brain regions of the MRI data from different
162 infected periods. All the *p* values are displayed with: ****P*<0.001, ***P*<0.01 and **P*<0.05. All
163 data are expressed as mean ± SEM unless otherwise specified.

164

165 **Results**

166 **Triple tyrosine mutant rAAV2/5 improves transgene expression *in vivo***

167 To construct the astrocyte-specific viral vector, the reporter cassette was flanked by a human
168 GFAP promoter as illustrated in Fig. 1A and the triple mutant sites in AAV2/5 are shown in Fig.
169 S1. Former studies have reported that mutations of surface-exposed Y residues in AAV2 or AAV6
170 capsids can facilitate the viral nuclear transport by limiting proteasome-mediated degradation,
171 which increases the transduction efficiency. [22, 23] AAV5-GFAP-EGFP-WPRE was found to be
172 selective for transduction of astrocytes *in vivo*. [18] For the capsid crystal structure of AAV5, data
173 from point-mutagenesis on the capsid genes have outlined the critical role of several specific Y
174 residues involved in transduction efficiency of cell culture *in vitro*. [24]

175 To compare the *in vivo* transduction efficiencies of wild type (wt) and Y436F-Y693F-Y719F
176 mutant (triple mutant) AAV2/5, two vectors were produced and respectively injected into the left
177 and right sides of the HIP (Fig. 1B) or CPu (Fig. S2A). Compared with the wt side, the green
178 fluorescence intensity was significantly higher in the mutant side (Fig. 1B-1C) on day 14 after
179 the injection, although there was no significant difference in EGFP positive cell numbers between
180 the two sides (Fig. 1D). Similar results were also found in the CPu (Fig. S2B-S2C). These results
181 confirm that the site-directed mutagenesis at Y436, Y693, and Y719 of *cap5* gene could increase
182 the transduction efficiency of the AAV2/5. To evaluate the cell type specificity of the rAAV2/5
183 mutant, the co-localization of EGFP and astrocyte marker GFAP as well as neuron marker NeuN
184 were measured. The results demonstrate that the rAAV2/5 mutant did not change the tropisms of
185 rAAV2/5 in astrocytes (Fig. 1E). Mutant AAV mediated EGFP expression was distributed
186 exclusively in the neurons. Thus, these results show that the triple mutant rAAV2/5 led to the
187 development of the AAV vector with higher transduction efficiency, while retaining high cell-
188 specificity of the GFAP promoter in animal models.

189 ***In vitro* analysis of MRI contrast induced by AAV mutant mediated AQP1 transfer.**

190 To evaluate the efficiency of AQP1 as a reporter for diffusion-weighted MRI, the mutant
191 rAAV carrying AQP1 was used to infect the U87 cell line *in vitro*. The backbone of the AAV
192 vector is shown in Fig. 2A. The chimeric AQP1 and EGFP gene were spaced by a 2A self-
193 cleavable sequence. The expression of AQP1 and EGFP were driven by the GFAP promoter. As
194 expected, the EGFP intensity gradually increased with MOI in the infected cells. AQP1

195 expression did not cause any changes in cell morphology under the fluorescence microscope (Fig.
196 2B). Meanwhile, western blot showed that the AQP1 protein levels were significantly increased
197 at 48 h after infection (Fig. 2C).

198 Next, the infected U87 cells were detected with the diffusion-weighted MRI. The ADC
199 values, which are sensitive to changes in cell membrane permeability modified by AQP1
200 overexpression, were also measured. AQP1 expression was dose-dependently increased in cells
201 treated with varying amounts of the rAAV mutant. Accordingly, the MRI contrast gradually
202 decreased as the MOI increased (Fig. 2D). Moreover, the ADC values were significantly
203 increased in cells infected with high doses of AAV ($MOI=1\times 10^5$ and 1×10^6) compared with non-
204 infected cells (Fig. 2E). These results demonstrate that the mutant rAAV carrying AQP1 could
205 change the MRI contrast *in vitro* in a dose-dependent fashion.

206 **Assessment of astrocyte specific rAAV-AQP1 in living animals**

207 To evaluate the feasibility of detecting astrocytes with the GFAP promoter controlled AQP1
208 *in vivo*, rAAV-GFAP-AQP1-EGFP and the control virus (rAAV-GFAP-EGFP) were
209 stereotactically microinjected into the right and left sides of the CPu, respectively. After infection,
210 the MRI was implemented in living animals on day 21, followed by the fluorescence analysis of
211 fixed brain slices on day 24 (Fig. 3A). There was a significant difference in DWI signal intensity
212 between the two sides of the CPu (Fig. 3B). AAV mediated gene transfer to the bilateral CPu was
213 confirmed by the fluorescence imaging of fixed brain slices (Fig. 3C). However, ADC values
214 were only significantly increased on the side infected with rAAV-AQP1. Compared with the
215 control side, the average intensity of diffusion-weighted signals was significantly decreased by
216 $21.5\pm 0.04\%$ (Fig. 3D), while ADC values were significantly increased by $15.0\pm 3.8\%$ in AQP1-
217 expressing side ($P=0.001$, $n=8$). This result suggests that rAAV-GFAP-AQP1-EGFP are suitable
218 for tracing astrocytes *in vivo*.

219 Previous studies have reported that AQP1 can increase water diffusion even at low
220 expression levels. [16] The titer-dependent changes in the MRI contrast and the potential
221 cytotoxicity were further investigated. Different volumes (*i.e.*, 300, 750, 1500 nl) of rAAV-
222 GFAP-AQP1-EGFP and rAAV-GFAP-EGFP were stereotaxically injected into the right and left
223 sides of the CPu, respectively. The changes in ADC values were observed in brain regions infused
224 with rAAV as low as 300nl (3.3×10^8 vg/mouse) and gradually increased with injection volumes
225 (Fig. S3A). Immunofluorescence staining of the microglia marker Iba1 was then carried out to
226 assess cytotoxicity. For the lowest volume (300 nl), there was no difference in Iba1-
227 immunoreactivity (ir) between the two sides of the CPu. However, at higher dosages of the virus,
228 the Iba1-ir was significantly increased compared with the control sections ($P<0.05$, Fig. S3B),
229 indicating cytotoxicity. These data suggest that alterations in ADC values were proportional to
230 AQP1 expression levels and that AQP1 expression could be toxic to tissues. Nonetheless, by
231 virtue of the low detecting threshold of AQP1 levels, it is still possible to define a “safe” working
232 dose, while retaining the capability of lighting up astrocytes with DWI.

233 **Longitudinal detection of whole brain astrocytes *in vivo***

234 In this section, we attempted to selectively image brain wide astrocytes using the PHP.eB
235 capsid, which had been applied to infect neurons of the entire brain. As illustrated in Fig. 4A,
236 AAV-PHP.eB carrying a GFAP promoter-controlled AQP1 and EGFP cassette was intravenously
237 injected into an 8-weekold C57BL/6 mouse. The AQP1 expression was detected with *in vivo* MRI
238 on post-injection day 0, 14 and 21. The animals were then sacrificed. The EGFP distributions in

239 the whole brain were directly imaged (Fig. 4B-4C) and aligned to the MRI results. Furthermore,
240 the animal behavioral study was used to evaluate the effects of exogenous AQP1 overexpression
241 on the animals, and there were no significant differences observed between these two groups (Fig.
242 S), which indicates the decreased influence of the exogenous AQP1 overexpression on the
243 animals' behavior.

244 In the longitudinal detection, we found that different brain regions gradually gained an
245 increment in ADC values from 2 weeks (W) to 3W after injection (Fig. 5A-5B). On day 21 after
246 infection, more areas manifested ADC increase with *in vivo* MRI. The highest ADC alterations
247 occurred in cortical areas. The cortex was further divided into 30 partitions (Right cortex: 15;
248 Left cortex: 15), and the ADC values of each partition were calculated at different infected dates.
249 The statistical analysis of ADC values was performed and is illustrated in Fig. 5C-5D, and the
250 significant difference is shown with a different lowercase (a, b and c).

251 To assess the whole-brain ADC alterations, the brain was divided into 34 partitions. Based
252 on patterns of ADC alteration over time, brain regions were assigned to four different groups
253 (Fig. 5E-5F). Group 1 consisting of five brain regions had significant increases in ADC values
254 over time ($P<0.05$). The ADC values of group 2 that comprised of 11 regions reached a plateau
255 two weeks after infection. For group 3, the significant changes ($P<0.05$) in ADC values of eight
256 brain regions were not observed until the third week. The other 10 regions that constituted group
257 4 did not show significant changes during the whole infection period. After three weeks' infection,
258 the fluorescence imaging was collected (Fig. S4), which was almost consistent with MRI results
259 in the third week, and the regions in group 1 had significantly higher EGFP than the other groups.

260 To determine sources of the MRI contrast, the co-localization of EGFP and AQP1 were
261 further analyzed. The EGFP was observed with direct fluorescence imaging, and AQP1 was
262 viewed by immunohistochemistry. The expressions of EGFP and AQP1 in three representative
263 regions (*i.e.*, Cortex, HIP and Cerebellum) are illustrated in Fig. S5. The results indicate that the
264 EGFP and AQP1 were co-expressed in astrocytes in these regions, and that AQP1 induced
265 changes in MRI signals during different infection periods.

266

267 **Astrocytes in the whole brain labelled with rAAV-PHPe.B carrying AQP1**

268 After three weeks' infection, the mouse was sacrificed and the native EGFP fluorescence in
269 brain slices was captured using a fluorescence stereo microscope. Astrocytes were visualized by
270 GFAP immunohistochemistry (Fig. 4B). To examine whether AAV.PHP.eB-GFAP mediated gene
271 expression was restricted to astrocytes, EGFP and GFAP signals were imaged. The EGFP signals
272 were overlapped with GFAP signals in sagittal and coronal slices of the whole brain (Fig. 4B-
273 4C). This was also verified with another astrocytic marker S100B (Fig. S6). Given the high
274 overlap between EGFP and AQP1 (Fig. S5), we concluded that brain-wide astrocytes could
275 efficiently be labelled with the MRI reporter using intravenous injection of AAV-PHP.eB.

276

277 **Discussion**

278 Astrocytes control multiple pathophysiological processes in the central nervous system.
279 However, non-invasive methods for directly observing astrocytes *in vivo* are limited. Currently,
280 fluorescence (*e.g.*, multiphoton microscopy) or radiation (PET) based techniques are the most
281 popular methods for monitoring the activity of astrocytes in live animals. Both techniques have
282 provided us with groundbreaking findings on the biology of astrocytes. Nonetheless, many

283 problems, such as lack of proper viral vectors and imaging reporters, as well as limitations of
284 imaging technologies (*e.g.*, depth of visualized tissues for the multiphoton microscopy and cell
285 specificity for PET), have hampered investigations of brain-wide astrocytes in live animals. This
286 study provides a novel method for investigating spatiotemporal alterations in astrocytes under
287 different pathophysiological conditions.

288

289 **Selection of virus tool**

290 Compared with other viral vectors, rAAV has the lowest toxicity in a living system. For
291 example, lenti-virus has been used to label astrocytes, [25] but it has higher toxicity. [26]
292 Furthermore, using lenti-virus through BBB for the astrocyte detection in the whole brain is not
293 effective. [27, 28] In comparison with AAV, the recombinant VSV was used to label the neuronal
294 network in the whole brain just after 4 days' infection. However, it cannot be used for detection
295 in living animals due to higher toxicity. [29] Furthermore, it is difficult to adopt it to infect
296 astrocytes due to the nature of the RNA virus. [30]

297 Mutagenesis in viral capsid protein is one of the simplest methods that increase the
298 transduction efficiency of the rAAV. [31] The replacement of specific amino acids might modify
299 the phosphorylation and ubiquitination of capsid protein to reduce proteasome degradation,
300 leading to increased transduction efficiency. It is reported that specific tyrosine, serine, threonine
301 and lysine point mutations of serotypes 2, 6, and 8 capsids can significantly increase the
302 transduction efficiency of the virus. [32] However, the tyrosine mutation of cap5 has only been
303 studied *in vitro*. In this study, the triple mutant (Y436F-Y693F-Y719F) rAAV cap5 mediated the
304 enhancement of transgene expression *in vivo*. In this case, the mutant AAV was selected as the
305 proper vector for targeting and detecting astrocytes with diffusion-weighted MRI.

306 **Selection of the MRI reporter**

307 Compared to other MRI reporters, AQP1 has several advantages. First, as a metal-free
308 reporter molecule, aquaporin is not limited by the bioavailability of metals, neither does it require
309 the application of metal ions or chelates. For example, ferritin has been widely utilized as an MRI
310 reporter [33-35] due to the paramagnetic effect after bonding with ferric (Fe^{3+}) and the marked
311 effect on solvent NMR relaxation rates. However, the dependence of metal ions limited the
312 application of ferritin and the change in MRI contrast making it very difficult to completely
313 observe in a short period of virus infection. [35] Second, as a human protein, AQP1 can work
314 without any sequence modification, and can be used as a completely autologous reporter gene to
315 overcome the potential immunogenicity problems faced by the other heterogeneous and
316 engineered reporter genes.

317 Another type of established MRI reporter comprises of the exogenous chemical exchange
318 saturation transfer (CEST) contrast agents. Yet, on account of the background CEST contrast
319 from endogenous biomolecules, it was difficult to detect the CEST agents at low concentrations.
320 On one hand, a successful CEST experiment needs complex pulse sequences for acquisition.
321 AQP1, on the other hand, could be detected at low expression levels using a standard diffusion-
322 weighted imaging, which makes it a more sensitive and easily operable MRI reporter. The
323 labelled astrocytes could be detected as early as two weeks after virus infection.

324 **Astroglia labeling in the whole brain with AAV-PHP.eB encoded AQP1**

325 AAV-PHP.eB capsid enabled the transgenic expression of AQP1 in the brain wide astrocytes.
326 However, different infected patterns of brain regions were observed in both MRI and fluorescence

327 imaging. It is also likely that the varied distribution of astrocytes could cause the differential
328 AQP1 signals across the whole brain. [36] But, since the virus must pass through the BBB prior
329 to cell transduction, the uneven distribution of blood vessels might be attributed to the varied
330 transduction efficiencies in distinct brain regions.

331 **Perspective**

332 This study took the advantages of two tools for neural network research, namely neurotropic
333 virus tracing and MRI, and developed a novel method for synchronous multi-modal imaging of
334 whole brain astrocytes in living animals. The ADC values of AQP1 labelling regions were highly
335 associated with the density of astrocytes, indicative of a good spatial resolution. This feature
336 offers a capacity for determining brain regions with abnormal astrocytes under pathological
337 conditions. Nonetheless, given that the ADC values could increase with time in some brain
338 regions, our method holds potential for monitoring temporal changes in astrocytes of disease
339 models. Future studies aiming to counteract the effects of time on AAV transduction efficiency
340 or generate AQP1 transgenic mice will improve the current method of investigating the temporal
341 dynamic of astrocytes in the neural network.

342 AQP1 is a sensitive, low cytotoxic, and quantifiable MRI reporter that can be detected *in*
343 *vitro* and *in vivo* using standard diffusion-weighted imaging. The MRI scan combined with rAAV
344 mediated astrocyte specific AQP1 expression can be adopted to assess the spatiotemporal
345 alterations in astrocytes of the neural network in living animals. Due to low toxicity of the tools,
346 this novel technology has superior prospects for future application to non-human primates and
347 provides an appropriate way to investigate astrocytes in living animals under pathological
348 conditions.

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361

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457 **Figure Legends:**

458 **Figure 1.** Site-directed AAV5 capsid mutation improved vector-mediated transgene expression
459 *in vivo*. (A) Diagram of the rAAV targeting astrocytes expressing fluorescent reporter genes under
460 the control of the GFAP promoter; (B) Representative images of hippocampus astrocytes using
461 rAAV encoding a GFP reporter with wild (B_L) or mutant cap (B_R). Scale bar: 1 mm. Scale bar of
462 enlarged image: 200 μ m. (C) and (D) Statistical analysis of fluorescence intensity and cell counts
463 in the infected areas for the bilateral hippocampus; (E) Immunohistochemistry of GFAP (purple)
464 and NeuN (red) in the hippocampus of the brain slice. Results indicate that the EGFP expressed
465 cells were co-expression GFAP (purple) or no co-expression with NeuN (red). Scale bar: 100 μ m
466 *Note: Results are expressed as means \pm SEM, ***P < 0.001, n=3.*

467 **Figure 2.** Validation of AQP1 expression for astrocyte targeting rAAV in the cell culture study.
468 (A) Scheme of the genome elements of the astrocyte targeting the AAV vector; (B) Expression of
469 fluorescent reporter protein EGFP in U87 cell lines with the rAAV mutant vector under different
470 MOI, Scale bar: 50 μ m; (C) Western blot analysis of transduction of U87 cell lines with different
471 MOIs of rAAV mutant; (D) Diffusion-weighted MRI analysis of U87 cells infected with different
472 MOIs of rAAV mutant; (E) Statistical analysis of ADC values of different MOIs rAAV mutant
473 infected groups. *Note: Data are expressed as means \pm SEM, *P < 0.05; **P < 0.01 (two-tailed*
474 *t-tests, n=3 per group).*

475 **Figure 3.** Validation of the MRI contrast for rAAV2/5-GFAP-AQP1-EGFP in living animals. (A)
476 Schedule of the experimental procedure. (B) Axial plane view of DWI images. The virus infected
477 area is labeled with dashed lines, and the ADC values are illustrated with pseudo-color image.
478 (C) Comparison of the fluorescent image (Right) and the statistical analysis of DWI imaging (left)
479 in the local injected area (CPu) for two different virus tools. Scale bar: 1 mm. (D) Statistical
480 results of DWI signal intensities and ADC values after three weeks' virus infection on both sides
481 of the CPu. *Note: Data are expressed as means \pm SEM, *p < 0.05; **P < 0.01 (two-tailed t-tests,*
482 *n=4/group).*

483 **Figure 4.** Detection of astrocytes in the whole brain marked by AAV-PHP.eB-GFAP-AQP1-
484 EGFP. (A) Schematic of the experimental design. (B) View of astrocytes in sagittal slices of the
485 mouse brain under different imaging methods. Scale bar: 2 mm. (C) Coronal views of astrocytes
486 in the whole brain. Scale bar: 2 mm. *Note: Green: EGFP; Red: GFAP; Blue: DAPI.*

487 **Figure 5.** Longitudinal detection of astrocytes of the whole brain in living animals with diffusion
488 weighted MRI. (A) and (B): Significant changes in ADC values after 2 W or 3 W's virus infection
489 compared with 0 W. (C) and (D): Statistical analysis of DWI signals in the whole brain (C-Right
490 areas; D-Left areas) under different periods of virus infection (0W, 2W, and 3W). The significant
491 difference was labeled with different lowercase letters a, b, c (Different letters represented *p <
492 0.05 and the same letters represented p > 0.05). (E) and (F): Four different changed patterns of
493 ADC values in the whole brain (E-Average values in different regions of the whole brain; F-
494 Average values and the statistical analysis for every pattern; Multiple t-test, n=7). *Note: Sensory-*
495 *motor cortex related (cortex related1), Poly modal association cortex (cortex related2),*
496 *Hippocampal (HP), Periaqueductal gray (PAG), Medial hypothalamic area (MHA), Striatum*
497 *dorsal region (Striatum2), Striatum-like amygdalar nuclei (Striatum3), Superior colliculus,*
498 *sensory related (SC1), Superior colliculus, motor related (SC2), Lateral hypothalamic area*
499 *(LHA), Striatum ventral region (Striatum1), Lateral septal complex (Striatum4), Endopiriform*

500 *nucleus(EPN), Retrohippocampal region (RHR). Data are expressed as means \pm SEM, * $p < 0.05$,*
501 *(two-tailed t-tests, n=4/group).*

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