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

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

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Abstract

Astrocytes constitute a major part of the central nervous system, and the delineation of their activity patterns is conducive to a better understanding of brain network dynamics. This study aimed to develop a magnetic resonance imaging (MRI)-based method in order to monitor the brain-wide or region-specific astrocytes in live animals. Adeno-associated virus (AAVs) vectors carrying the human glial fibrillary acidic protein (GFAP) promoter driving the EGFP-AQP1 (Aquaporin-1, an MRI reporter) fusion gene were employed. The following steps were included: constructing recombinant AAV vectors for astrocyte-specific expression, detecting MRI reporters in cell culture, brain regions or whole brain following cell transduction, stereotactic injection, or tail vein injection. The astrocytes were detected by both fluorescent imaging and Diffusion Weighted MRI. The novel AAV mutation (Site-directed mutagenesis of surface-exposed tyrosine (Y) residues on the AAV5 capsid) significantly increased fluorescence intensity ($p<0.01$) compared with the AAV5 wild type. Transduction of the rAAV2/5 carrying AQP1 induced the titer-dependent changes in MRI contrast in cell cultures ($p<0.05$) and caudate putamen (CPu) in the brain ($p<0.05$). Furthermore, the MRI revealed a good brain-wide alignment between AQP1 levels and ADC signals, which increased over time in most of the transduced brain regions. In addition, the AAV-PHP.eB serotype efficiently introduced AOP1 expression in the whole brain via tail vein injection. This study provides an MRI-based approach to detect dynamic changes in astrocytes in live animals. The novel in vivo tool could help us to understand the complexity of neuronal and glial networks in different pathophysiological conditions.

Introduction

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

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

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

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

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

Materials and methods

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

Cell culture

The human embryonic kidney 293 cells (ATCC) and the U87 human astrocytoma cell line (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone) containing 10% fetal bovine serum (FBS; Gibco) and 100 U/ml of penicillin/streptomycin. All cell lines were grown at 37 °C in 5% CO_2.

Animal studies

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

Plasmids

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

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

Construction of recombinant AAV vectors and virus titration

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

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

Data analysis and statistics

All the MRI data was transformed to NIFTI format using the Bru2Anz (Bruker, Germany) and converted on the ADC map using the custom-made software developed in MATLAB. Then they were normalized to a homemade mouse brain template with statistical packages (SPM12, www.fil.ion.ucl.ac.uk/spm). Furthermore, a publicly available mouse brain template TMBTA (www.nitrc.org/projects/tmbta_2019) was utilized to segment the whole brain into different partitions for calculating ADC values in different brain regions. The GraphPad Prism (version 8.0.1 for Windows, GraphPad Software, San Diego, California USA) was used to perform two-tailed *t*-tests for the *in-situ* injection data and repeated measures ANOVA analyses and multiple comparison corrections were utilized for multiple brain regions of the MRI data from different infected periods. All the *p* values are displayed with: ***$P<0.001$, **$P<0.01$ and *$P<0.05$. All data are expressed as mean ± SEM unless otherwise specified.

Results

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

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

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

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

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

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

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

Assessment of astrocyte specific rAAV-AQP1 in living animals

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

Previous studies have reported that AQP1 can increase water diffusion even at low expression levels. [16] The titer-dependent changes in the MRI contrast and the potential cytotoxicity were further investigated. Different volumes (*i.e.*, 300, 750, 1500 nl) of rAAV-GFAP-AQP1-EGFP and rAAV-GFAP-EGFP were stereotaxically injected into the right and left sides of the CPu, respectively. The changes in ADC values were observed in brain regions infused with rAAV as low as 300nl (3.3×10^8 vg/mouse) and gradually increased with injection volumes (Fig. S3A). Immunofluorescence staining of the microglia marker Iba1 was then carried out to assess cytotoxicity. For the lowest volume (300 nl), there was no difference in Iba1-immunoreactivity (ir) between the two sides of the CPu. However, at higher dosages of the virus, the Iba1-ir was significantly increased compared with the control sections (P<0.05, Fig. S3B), indicating cytotoxicity. These data suggest that alterations in ADC values were proportional to AQP1 expression levels and that AQP1expression could be toxic to tissues. Nonetheless, by virtue of the low detecting threshold of AQP1 levels, it is still possible to define a “safe” working dose, while retaining the capability of lighting up astrocytes with DWI.

Longitudinal detection of whole brain astrocytes *in vivo*

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

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

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

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

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

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

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

Discussion

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

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

Selection of virus tool

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

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

Selection of the MRI reporter

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

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

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

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

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

Perspective

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

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

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Figure Legends:

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

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

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

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

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

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