Longitudinal neural connection detection using a Ferritin-encoding Adeno-associated virus vector and *in vivo* MRI method

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Abstract

The investigation of neural circuits is important for interpreting both healthy brain function and psychiatric disorders. Currently, the architecture of neural circuits is always investigated with fluorescent protein encoding neurotropic virus and *ex vivo* fluorescent imaging technology. However, it is difficult to obtain a whole-brain neural circuit connection in living animals, due to the limited fluorescent imaging depth. Herein, the non-invasive, whole-brain imaging technique of MRI and the hypotoxicity virus vector AAV (adeno-associated virus) were combined to investigate the whole-brain neural circuits *in vivo*. AAV2-retro are an artificially-evolved virus vector that permits access to the terminal of neurons and retrograde transport to their cell bodies. By expressing the ferritin protein which could accumulate iron ions and influence the MRI contrast, the neurotropic virus can cause MRI signal changes in the infected regions. For mice injected with the ferritin-encoding virus vector (rAAV2-retro-CAG-Ferritin) in the caudate putamen (CPu), several regions showed significant changes in MRI contrasts, such as PFC (prefrontal cortex), HIP (hippocampus), Ins (insular cortex) and BLA (basolateral amygdala). The expression of ferritin in those regions were also verified with *ex vivo* fluorescence imaging. In addition, we demonstrated that changes in T2 relaxation time could be used to identify the spread area of the virus in the brain over time. Thus the neural connections could be longitudinally detected with the *in vivo* MRI method. This novel technique could be utilized to observe the viral infection long-term and detect the neural circuits in a living animal.

Keywords: Neural circuit; Ferritin; *In vivo* MRI; rAAV2-retro; Immunohistochemistry.

Introduction

The brain is the most complex organ in the body, and more than 70 billion neurons exist in the human brain to form a complicated network (Grandjean, et al., 2020). More and more preclinical studies have shown that neural networks play an essential role in instinctive behaviors such as fear (Wei, et al., 2015), reward (Zhang, et al., 2017) and mating (Wei, et al., 2018). In addition, the alteration of neural networks may lead to abnormal animal behaviors, such as epilepsy-like (Citraro, et al., 2013) or depression-like behaviors (Rozov, et al., 2001), suggesting that human brain diseases may emerge from neural network dysfunction. Dissecting neural networks is important for understanding brain function in a physiological or pathological state.

Currently, neurotropic viral vectors have been widely used to investigate the neural networks. As a series of artificially modified neurotropic viruses, neurotropic viral vectors can transport exogenous genes along the synapses-connected neural networks. Numerous virus vectors were constructed to dissect the structure of neural networks after genetic modifications, such as herpes simplex virus (HSV), pseudorabies virus (PRV), rabies virus (RV), *etc.* (Nassi, et al., 2015; Rao and Wang, 2020; Ugolini, 2010). However, most of them can only be used for *ex vivo* imaging due to their virulence. Recombinant adeno-associated virus (rAAV) vectors are effective tools for exogenous gene delivery for living animal studies due to their advantages of high-level transgene expression and low cell toxicity (Kaplitt, et al., 2007). Recently an artificially-evolved AAV series vector (rAAV2-retro) was introduced to mediate retrograde access to neurons (Tervo, et al., 2016). It is a powerful tool to image neural circuits when combined with the fluorescent protein gene (Zheng, et al., 2020). However, due to limitation of the fluorescent imaging depth, it is hard to observe the whole-brain neural circuit in a living animal, which also impedes our understanding of

80 the virus infection procedures. Thus, it was valuable to develop a novel method for *in vivo* neuronal
81 network detection.

82 A vast amount of impressive work has been done for living animal imaging. Near-infrared
83 (NIR) fluorescence imaging methods have been used to increase the imaging depth of fluorescent
84 protein (Frangioni, 2003) and allow for *in vivo* imaging (Hong, et al., 2017). However, the NIR
85 signal is only reliable within the depth of 3 mm (Hong, et al., 2014). Luciferase has also been used
86 as an *in vivo* imaging strategy because, with a highly sensitive detector, the imaging depth can be
87 up to 40 mm. While this has been valuable for labeling cancer cells and gene expression (Li, et al.,
88 2017), the spatial resolution of luciferase imaging is not sufficient enough for tracing neural circuits
89 (Cook and Griffin, 2003). Magnetic resonance imaging (MRI) is a commonly used clinical image
90 technique, which has the advantages of non-invasive and large-scale imaging (Van Leemput, et al.,
91 2009; Wu, et al., 2003). The MRI also provides a good compromise of moderately high spatial
92 resolution (~100 μm) while covering the entire brain (Pagani, et al., 2016; Ullmann, et al., 2013).
93 Thus, with a proper MRI contrast agent encoded by the virus vector, MRI could be an excellent tool
94 to trace the whole-brain neural networks in living animals.

95 Ferritin is a ubiquitous iron storage protein found in most organisms. In general, it protects the
96 cell from damaging active oxide Fe^{2+} and stores the iron ion in the shell of ferritin as Fe^{3+}. As Fe^{3+}
97 is a paramagnetic MRI contrast agent, the overexpression of ferritin is able to change the transverse
98 magnetic relaxation rate (1/T2) of the surrounding tissue. Thus, at sufficiently high concentration
99 and with sufficient access to biological iron, ferritin can change the contrast of the MRI signal and
100 show its presence with hypointensity on T2-weighted MRI images (Iordanova and Ahrens, 2012;
101 Wu, et al., 2018). In our previous work, the ferritin gene was loaded onto the VSV (Vesicular

stomatitis virus) and a multi-synaptic neural network connected to sensory cortex was illustrated (Zheng, et al., 2019). However, the result was obtained using *ex vivo* MRI due to the virulence of the VSV. Here, we tried to display the whole-brain neural network in a living animal with hypotoxicity virus vector AAV.

Herein, a novel tool for *in vivo* whole-brain neural network imaging was developed. We loaded the ferritin gene onto a retrograde transporting AAV vector, delivered it to the caudate-putamen (CPu) of mice and imaged these mice with *in vivo* MRI. In doing so, we were able to visualize a CPu-connected network that includes the upstream brain regions sending projection to the CPu. The ferritin-encoding retrograde transporting AAV vector enabled the investigation of neural network in living animals and long-term observation of the virus infection.

Results

MRI signal changes of the regions with ferritin transduction

Firstly, the function of rAAV2-retro-CAG-Ferritin to label the neural networks and express ferritin was investigated. To this end, a control virus vector rAAV2-retro-CAG-EGFP was constructed for comparison (Fig. 1A). These two virus vectors were injected into the CPu region of mice with similar titers ($5*10^{12}$ vg/mL) and volumes (2.8 μL). Sixty days after receiving the injection the animals were firstly scanned with MRI. Then all animals were sacrificed and the brain slices were performed with immunohistochemical staining and fluorescence imaging. The brain slices of these two groups with same stereotaxic coordinates were chosen for comparison (Fig. 1B). Similar to the rAAV2-retro-CAG-EGFP infected group, the expression of ferritin could be found in multiple brain regions other than the injection site CPu, such as the PFC (prefrontal cortex), HIP

(hippocampus), Ins (insular cortex), and BLA (basolateral amygdala). Most of those areas were directly connected to CPu (Tervo, et al., 2016). Secondly, the MRI signals were also compared in the whole brain of mice infected with these two virus vectors. In rAAV2-retro-CAG-Ferritin infected group, the hypointensity MRI signals (compared to the surrounding tissue) were observed at ferritin expressed regions (Fig. 1B, lower). Meanwhile, there was no discernible signal changes observed on the T2-weighted MRI image in the same regions with EGFP overexpression in the control group (Fig. 1B, upper).

In order to dissect the neural circuit, the resolution and SNR (signal to noise ratio) of the MRI image should be sufficient enough to distinguish the structural regions or even the sub-regions of the brain. The fluorescence and MRI images of similar brain structures were expanded to illustrate the details of ferritin-encoding AAV expression (Fig. 2). For fluorescence imaging, the red fluorescent was used to illustrate the expression of ferritin and the changes in MRI signal intensity were regard as the MRI contrast effect caused by ferritin expression and iron ions aggregation. Overlapping with a stereotaxic atlas of the mouse brain (Paxinos and Franklin), the location of ferritin could be plainly identified. Seven representative regions with obvious ferritin expression were collected for comparison, such as CPu, BLA, HIP, PFC, Tha (Thalamus), Ins and posterior HIP (Fig. 2B). Although the resolution and SNR of the MRI were much lower than fluorescence imaging, the same ferritin expressing regions could be observed by both MRI and fluorescence imaging. In addition, a more detailed brain partition map of the Ins region was used to explore whether subtle localized changes could be detected with *in vivo* MRI (Allen mouse brain atlas, http://atlas.brain-map.org/). Using this, the distribution of ferritin could be located in the fifth layer

of the insular cortex. Thus, the subtle localized changes could also be detected using the MRI method.

Long term observation of T2-weighted images with ferritin/EGFP expression

The longitudinal recording of rAAV2-retro-CAG-Ferritin and rAAV2-retro-CAG-EGFP infected groups were performed at different time points (0d, 10d, 30d, 60d) in the same animal using T2-weighted MRI (Supplementary material, Fig. S1). Based on the fluorescent imaging, the changes in MRI contrasts of the virus infected areas could be longitudinally monitored, such as PFC, BLA, Ins, and HIP. In the rAAV2-retro-CAG-Ferritin group, the changes in MRI contrast of those regions were directly observed after 30 days' infection and became stronger at 60 days. Meanwhile, there were no significant changes observed in the rAAV2-retro-CAG-EGFP group during the whole period of infection.

To quantitatively measure changes in MRI contrast, six regions (CPu, PFC, BLA, Ins, HIP and Tha) with ferritin/EGFP expression were selected as ROIs (regions of interest) based on an open source MRI template (TMBTA), and the other two regions CSF (cerebrospinal fluid) and SC (superior colliculus) were chosen for comparison due to limited ferritin/EGFP expression (Supplementary material, Fig. S2). Signal intensity normalization (with CSF) were performed before statistical comparison, and the one-way ANOVA (LSD post hoc test) method was used to evaluate changes in signal intensity in the T2-weighted images following the infection days (Supplementary material, Fig. S3). In the rAAV2-retro-CAG-Ferritin infected group (0d, N=14; 10d, N=11, 30d, N=8; 60d; N=5), statistical differences in MRI contrast were observed in the virus infected regions following virus infection, such as CPu, BLA, HIP, PFC, Ins and Tha ($p<0.05$), while no significant signal changes were detected in SC among different infection time points.

Moreover, there were no significant MRI signal changes in all these brain regions of the rAAV2-retro-CAG-EGFP group during the entire virus infected periods (0d, N=3; 10d, N=3, 30d, N=3, 60d; N=3).

Tracing CPu connected regions using changes in T2 relaxation time

To show the neural network *in vivo* with a whole-brain view, voxel-wise changes in T2 relaxation time were performed based on the normalized T2 relaxation time maps (Fig. 3A). The multi-echo T2-weighted images were converted to T2 relaxation time maps and then normalized to a standard space base on the transformation matrix of T2-weighted images. The differences between T2 relaxation time maps before injection (0d, Fig. 3B) and after injection (10d, 30d, 60d) were calculated voxel by voxel (Fig. 3B) and then filtered by a threshold of 4-15ms (Fig. 4).

The mapping of T2 relaxation time changes was compared with fluorescent images and T2-weighted images with similar brain structures. The comparisons among three different time points are illustrated (Fig. 4). Ten days after the injection, ferritin expression was observed in CPu and PFC with fluorescence imaging. The same regions were also detected in mapping of T2 relaxation time changes (10d), although the area was not as wide as the fluorescence imaging. Thirty days after infection, stronger signals were detected in PFC, CPu, Tha, BLA, HIP, and Ins from the fluorescence images, and wider signals were also found in PFC, CPu, BLA and Ins from the T2 relaxation time change mapping (30d). Sixty days after infection, the fluorescent signal spread across the whole-brain through the fluorescence imaging, particularly in the PFC, CPu, Tha, BLA, HIP and Ins. Consistently, the T2 relaxation time change mapping (60d) showed more similar labeled patterns compared to the fluorescence imaging. Overall, similar signal tendency was obtained from both the fluorescence imaging and the T2 relaxation time change mappings over the four infected periods.

189 Thus, the changes in T2 relaxation time can be used to identify *in vivo* the spreading area of the
190 virus in the brain over time.

191 Furthermore, the mapping of T2 relaxation time changes was utilized to investigate the CPu
192 connected regions with the *in vivo* MRI approach in the same animal at different time points after
193 the rAAV2-retro-CAG-Ferritin injection. The dynamic changes in MRI contrast were almost similar
194 in all individual subjects (Fig. 5). During the early stage of virus infection (10d), changes in T2
195 relaxation time was only observed at the injection site (CPu). Thirty days after the infection, more
196 regions showed distinct changes in all the subjects, such as PFC, HIP and BLA. After 60 days, most
197 of CPu single synapse connected regions were detected at the mapping of T2 relaxation time
198 changes, such as PFC, BLA, Ins and Tha. Although the signals were unstable during the early stage
199 of the infection, the changes in MRI contrast became stronger and more consistent after 60 days'
200 infection. Thus, this method could be a promising way for investigating the neural circuits in living
201 animals.

202 ***Quantification of T2 relaxation time changes in ferritin transduction regions***

203 The T2 relaxation time of ferritin overexpressed regions were further quantitatively measured
204 to assess the MRI signal change along with the infection periods. Six regions with ferritin
205 overexpression (CPu, BLA, HIP, Ins, PFC and Tha) and two regions without ferritin overexpression
206 (CSF and SC) were chosen for comparison (Supplementary material, Fig. S2). One-way ANOVA
207 (LSD post hoc test) was used to test for changes in T2 relaxation time among eight regions as well
208 as infection days (0d, N=14; 10d, N=11, 30d, N=8 60d; N=5). As shown in Fig. 6, there was no
209 significant change in the T2 relaxation time detected at CSF or SC where no ferritin was
210 overexpressed during the whole infection period ($p>0.05$, marked with same lowercase). Among

the other six regions, significant changes were only found at CPu after 10 days' infection ($p<0.05$, marked with different lowercase). When enough time was given for virus infection and ferritin expression, all six regions that ferritin overexpressed showed significant changes after 30 days' infection compared to 0 days' infection . More significant differences could be found in those regions after 60 days' infection time compared to 0 days' or 30 day'.

Discussion

Fluorescence imaging is one of the most commonly used method to visualize results of virus-based neural circuit tracing. However, the depth of fluorescence imaging is always limited by the light transmittance of animal tissues. Although much effort has been made to improve the detection depth of animal fluorescence imaging, it is difficult to show the labeled regions in living animals with a whole-brain view (Cook and Griffin, 2003; Frangioni, 2003; Helmchen and Denk, 2005; Zhu, et al., 2020). Herein, the retrograde virus vector AAV that express MRI contrast protein (ferritin) was used to illustrate the neural network in living animals. By injecting the virus into the CPu, we observed a network that directly connected to the CPu using *in vivo* MRI during three different periods after the virus injection. Moreover, the network was confirmed by the fluorescence imaging method. This method could be a powerful approach for exploring the neural circuits *in vivo*.

Resolution and SNR of MRI in dissecting neural circuits

Although MRI has the advantages of non-invasive and large imaging scale, the resolution and signal-to-noise ratio are much lower than fluorescence imaging. For fluorescence imaging, the resolution could reach 0.2 μm, and there is little interference in the background. Since the diameter of neurons is usually ~5μm, the fluorescence imaging can easily distinguish the neuron cells

(Cunnane, et al., 2019). In MRI it is hard to distinguish an individual neuron, due to limited resolution and SNR. However, the resolutions and SNR of MRI are sufficient to distinguish the brain regions for neuronal network detection. In the current study, the distributions of ferritin were always aggregated with significant boundaries, where the T2-weighted images could be utilized to distinguish the change in MRI contrast, such as BLA, Ins and HIP (Fig. 2). For the regions of PFC, CPu and Tha, it was not easy to distinguish the ferritin expressed regions from the T2-weighted images, due to the dispersive distribution of ferritin and the low signal intensity of the background. For these regions, the ROIs based statistics analysis of signal intensity and the changes in T2 relaxation time were capable of illustrating the MRI contrast effect caused by ferritin expression. There were also some regions with lower fluorescence where the ferritin expression was weak and sparse. For these regions, it was very difficult to distinguish the change in T2-weighted MRI signal intensity. This was probably caused by the low SNR and resolution of MRI, as the weak and sparse ferritin expressions were not sufficient enough to generate the detectable MRI contrast.

MRI signals at different infection times

In these experiments, ferritin was overexpressed in a CPu related network through a AAV2-retro virus vector, and three time points (10d, 30d, 60d) were selected to evaluate the ferritin expression and MRI signal. Different ferritin expressions and MRI signals were found at the three time points, and the characteristics of rAAV vector could be the main reason for the differences. The expression of AAV-carrying genes usually increases over time and reaches a plateau within 3-12 weeks (Tenenbaum, et al., 2004) and most of the AAV2-retro labeled results are obtained within 3-8 weeks as reported. Three weeks (Itoga, et al., 2019) and four weeks (Itoga, et al., 2019) are the

most commonly used waiting time for AAV2-retro expression and it is reported that more labeled information can be obtained using eight weeks waiting time rather than four weeks (Cunnane, et al., 2019).

Comparing ferritin expression and MRI signals, incomplete synchronization was detected between ferritin expression and MRI signal changes, especially in the early stage of the virus infection. The incomplete synchronization probably results from the characteristics of ferritin. Ferritin itself is not an MRI contrast agent and it influences the MRI signal by recruiting iron ions. There are several parameters influenced by the recruiting iron ions: the quantity of ferritin expressed in the infected region, the distribution of ferritin in cells, the iron enrichment in the intracellular environment, the supplement rate of iron ions and the degradation rate of ferritin. These differences could be the source of the incomplete synchronization between ferritin expression and MRI signals at 10 days and 30 days after the injection. Moreover, the difference between these two signals became smaller when the infection time reached 60 days, as enough time was given to enrich the iron ions. Further work is needed to shorten the time delay between ferritin expression and MRI contrast generation, such as supplementing iron ions.

Relationship between ferritin expression and MRI contrast

Although ferritin is reported to recruit iron ions and influence the MRI signals, there were many factors that influence the MRI signal changes. Ferritin is an autologous protein which is involved in inflammation reaction (Namaste, et al., 2017), the infection of AAV could hypothetically lead to regional inflammation which could also cause overexpression of ferritin (Vande Velde, et al., 2011). Therefore, the expression of ferritin in the infection of rAAV2-retro-CAG-EGFP was also investigated, and no obvious ferritin expression was found (Supplementary material, Fig. S4).

277 Furthermore, Prussian Blue staining was also utilized to verify the accumulation of Fe^{3+}. The blue
278 complexes were found at the regions where ferritin was overexpressed (Supplementary material,
279 Fig. S5). Thus, the change in MRI contrast mediated by infection of rAAV2-retro-CAG-Ferritin
280 might have been caused by ferritin overexpression and Fe^{3+} accumulation, rather than inflammation.
281 Degradation of ferritin could hypothetically cause ferroptosis (Xie, et al., 2016), leading to
282 cell death when a mass of unbound iron ions are released to the cell. However, overexpression of
283 ferritin can result in an iron-deficiency intracellular environment (Naumova and Vande Velde,
284 2018), which could lead to the suppression of ferroptosis (Hou, et al., 2016). Prior studies also
285 demonstrated that there is no obvious influence on cells when ferritin is overexpressed (Iordanova
286 and Ahrens, 2012; Iordanova, et al., 2013). Other than ferroptosis, changes in iron balance of the
287 surrounding tissue around the regions with ferritin overexpression are another potential concern.
288 While this may be a concern for short time periods, iron deficiency could be rebalanced by the
289 blood supply for long time tracing work (more than 10 days). Besides, the expression of apoptosis
290 and inflammation marker, caspase-3 and Iba1, were tested, and no noticeable abnormalities were
291 found in regions BLA, HIP and PFC, where ferritin was overexpressed (Fig. 7).
292 ***Perspective and limitations***
293 Using the ferritin encoding virus, we observed the structural neural network in living mice for
294 a long period after the virus injection. However, the entire neural network was presented in MRI
295 images 60 days after the virus injection. At earlier time points only part of the network was displayed.
296 Our future work could focus on shortening the latency time between the virus injection and MRI
297 detection. Although the ferritin used in our experiment is already an MRI contrast enhanced version
298 (Iordanova, et al., 2010), it is possible to make further improvements on the ferritin protein structure

for better MRI contrast effect. Besides, exogenous iron ions supplements could be a possible method for providing better MRI contrast effect in shorter latency time (Vande Velde, et al., 2011). In addition, the ferritin-encoding virus and *in vivo* MRI could be used to investigate the expression levels of optogenetic (e.g. ChR2) or chemogenetic (e.g. hM3Dq or hM4Di) proteins encoded by a virus. Through MRI imaging, it is easier to know the spread and expression levels of the virus that can guide further manipulation or operation. For non-human primates or other big laboratory animals which are expensive and used for many years, it is important to know the transfected functional protein expression level, especially after a long time. This new technique will make it much easier to operate and reduce the usage of non-human primates or big animals in brain research.

Materials and methods

Animal preparation

All animals involved in this study were treated in accordance with protocols approved by the Animal Ethics Committee at the Wuhan Institute of Physics and Mathematics, Chinese Academy of Sciences (approval number APM20016A). Male C57BL/6J mice (6–8 weeks old) were purchased from Hunan SJA Laboratory Animal Co., Ltd (Hunan, China) and allowed to acclimate three days before the experiment. All the animals were raised under 12h/12h light-dark cycle room with appropriate temperature, food and water were available *ad libitum*.

Construction of Virus

The rAAV2-retro-CAG-Ferritin and the rAAV2-retro-CAG-EGFP virus vector were packaged by a commercial company (BrainVTA, Wuhan, China). This virus will infect neurons in a network in a retrograde manner, moving from axon to soma. Briefly, the plasmids carrying exogenous genes

and AAV2-retro packaging components were cotransfected into 293T cells. After three days' cultivation, the AAV virions were collected and purified to a titer of 5-10*10^{12} vg/mL. In particular, the ferritin gene used in this experiment was a chimeric ferritin gene (L*H) which comes from the Mus musculus ferritin light chain and heavy chain (Gift from Prof. Xiaoming Li's lab in Zhejiang University).

Stereotaxic injection

Male C57BL/6J mice were anesthetized with 1.0% pentobarbital sodium (50 mg/kg) and fixed in a stereotaxic injection system (RWD, ShenZhen, China). The skull of each mouse was exposed after being locally anesthetized with lidocaine lincomycin gel (Xinya, Shanghai, China) and smeared with erythromycin eye ointment to prevent drying. A small hole about 1 mm in diameter was drilled in the skull to allow accessing the glass micropipette. The glass micropipette was stereotaxically injected into the CPu (Fig. 8, Caudate Putamen: 0.51 mm anterior to Bregma, 2 mm lateral from midline, 3.3 mm depth relative to Bregma) based on the stereotaxic coordinates of the mouse brain atlas (Paxinos and Franklin). Then, the virus suspension (2.8 μL) with titration of 5-10*10^{12} vg/mL was infused into the CPu at a rate of 0.14 μL/min. After the injection, the micropipette was kept at the injection site for 10 min to prevent reflux and then it was slowly withdrawn. The head skin was surgical sutured after treatment with lidocaine lincomycin gel. At the end of the experiment, the animal was recovered from anesthesia on the heating pad and returned to its home cage.

MRI scanning

The *in vivo* MRI experiment was performed using a 7.0 Tesla Biospec small animal magnetic resonance imaging system (Bruker, Ettlingen, Germany). The animals were initially anesthetized

with 4.0-5.0% isoflurane (RWD, Shenzhen, China) for induction and 1.0-1.5% for maintenance with a mixture of 30% O_2 and 70% N_2. The body temperature of animals was maintained with a thermostatic water cycle system under the animal bed. The breathing rate of the animal was monitored and maintained at 60±15 breaths/min to achieve the state of deep anesthesia. In addition, two ear bars and a tooth bar were used to minimize motion effect of MRI data acquisition. A 20cm birdcage coil was used for transmission, combined with a 20 mm surface coil for receiving (Bruker, Ettlingen, Germany). Multi-echo T2-weighted anatomical images were obtained using an MSME sequence (TR = 3000 ms; Effective TEs = 11, 22, 33, 44, 55, 66 ms; Number of Averages = 6; FOV = 17.5 *17.5 mm^2; Slice thickness = 0.5 mm; Spatial resolution, 0.137 mm *0.137 mm). The T2 relaxation time maps were obtained by processing the MSME image using Paravision 5.0 software (Bruker, Germany).

The expression of the rAAV2 in living animals can last for more than two months and its expression can reach a maximal level after 30 days (Cunnane, et al., 2019). Thus, MRI scans were performed at four time points to study the longitudinal effects of the virus infection. The time points were: the day before the virus injection (0d, n=14), 10 days (10d, n=11), 30 days (30d, n=8) and 60 days after injection (60d, n=5), respectively. The number of animals was reduced by 3 in each succeeding group as 3 animals were euthanized for the fluorescence study at each time point.

Fluorescence imaging and immunohistochemistry

Virus-infected mice were anesthetized with 1.0% pentobarbital sodium (50 mg/kg) and cardiac perfusion was applied with 0.9% saline followed by 4% paraformaldehyde solution. The brain was extracted and dehydrated with 30% (*w*/*v*) sucrose solution. After that, the dehydrated brain was

sectioned into 40 μm slices using freezing microtome (Leica, German) and one of twelve slices were selected for fluorescence imaging (approach to the MRI slice thickness 0.5 mm).

For the imaging of EGFP, the fluorescence imaging was directly performed. For the imaging of ferritin, caspase3, Iba1 or cell nucleus, immunohistochemistry staining was performed before the fluorescence imaging. For immunohistochemistry, the slices were first rinsed with PBS and then blocked with blocking solution buffer (PBS+0.3% TritonX-100+10% goat serum, 37℃, 1h). For ferritin staining, the rabbit anti-ferritin light chain antibody (Abcam, ab69090, UK) and Cy3-labeled goat anti-rabbit secondary antibody were utilized. For caspase3 staining, the rabbit anti anti-caspase3 antibody (Cell Signaling Technology, #9661) and 488-labeled goat anti-rabbit IgG were used. For Iba1 staining, the goat anti anti-Iba1 antibody (Abcam, ab5) and Cy3-labeled donkey anti goat antibody were utilized. For cell nucleus staining, the fluorescent dye DAPI was used.

For fluorescence imaging, the brain slices were transferred to microslide and scanned with an Olympus VS120 virtual microscopy slide scanning system (Olympus, Japan). For the filters, Leica U-MRFPHQ fluorescence mirror unit was used for the red fluorescence imaging, which included a 535-555nm excitation filter, a 575-625nm emission filter and a 565nm dichromatic mirror; Leica U-MWIBA3 fluorescence mirror unit was used for green fluorescence imaging, which included a 460-495nm excitation filter, a 510-550nm emission filter and a 505nm dichromatic mirror.

Furthermore, the Perls' Prussian Blue staining method was utilized to verify the accumulation of iron ions. It was performed following a previous study (Kim, et al., 2010) with a commercial staining kit (Solarbio, G1422, Beijing, China).

Data analysis

The T2-weighted images and T2 relaxation time maps were transformed to NIFTI format using Bru2anz (Bruker, Germany). MRI images were normalized to a homemade mouse MRI template using spm12 (www.fil.ion.ucl.ac.uk). The heavily T2-weighted images (TE/TR = 55ms/3000ms) were used to show the MRI signal change in ferritin expressed regions (Fig. 8).

For comparison between MRI images and fluorescence images, similar brain slices were chosen based on structural features and the slice position. The anterior commissure was used for feature recognition and the slice with the same axial distance from the anterior commissure was used for these comparisons. The MRI images and fluorescence images were matched with the mouse brain atlas.

For calculating the variation within T2 relaxation times, all the T2 relaxation time maps firstly used nonlinear transformation to determine a homemade mouse brain template with spm old-normalise. The T2 relaxation times mapping was obtained by comparing the T2 relaxation time map to the map from the same animal before injection (0d) (Fig. 3). The T2 relaxation time changes higher than 15ms or lower than 4ms were considered as outliers and deleted.

To quantitatively describe changes in T2 relaxation times, all the MRI images were normalized to a publicly available mouse brain template TMBTA (www.nitrc.org/projects/tmbta_2019) and smoothed. Six regions with ferritin expression (CPu, HIP, BLA, Ins, PFC, Tha) and two regions without ferritin expression (SC, CSF) were chosen as ROIs based on the TMBTA mouse brain atlas (Supplementary material, Fig. S2). The average T2 relaxation times for the eight ROIs were obtained and these average values were compared using one-way ANOVA. Least significant difference (LSD) was used for post hoc multiple comparisons, with statistical significance at $p<0.05$.

Data availability

All data and the implementation code in this article are available upon request from the corresponding author (jie.wang@wipm.ac.cn).

Conclusion

We developed a novel neural network tracing method using a combination of *in vivo* MRI and virus tracing techniques. It enabled the detection of the neural network in a living animal with a whole-brain view. Besides, we measured the virus infection progress over a period of time in the same animal. This technology provides a totally different perspective for our understanding of the neural network. It may lead to a different explanation of the brain network when combining the results of *in vivo* virus tracing and *in vivo* detection technologies. The technology may also benefit the neural circuit tracing in animals with larger-size brains, of which the whole-brain fluorescence imaging is tremendous amount of work, but easy when using MRI.

Author contributions: Fuqiang Xu, Jie Wang, Ning Zheng, Aoling Cai designed the research. Peng Su, Kunzhang Lin, Ling-Qiang Zhu gave advice on the research. Ning Zheng, Aoling Cai, Yang Wu performed experiments. Aoling Cai, Ning Zheng, Binbin Nie, Jinfeng Wu analyzed data. Aoling Cai, Jie Wang, Garth J. Thompson, Ning Zheng, Anne Manyande wrote the manuscript.

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Reference

Citraro, R., Russo, E., Ngomba, R.T., Nicoletti, F., Scicchitano, F., Whalley, B.J., Calignano, A., De Sarro, G. (2013) CB1 agonists, locally applied to the cortico-thalamic circuit of rats with genetic absence epilepsy, reduce epileptic manifestations. Epilepsy Res, 106:74-82.

Cook, S.H., Griffin, D.E. (2003) Luciferase imaging of a neurotropic viral infection in intact animals. J Virol, 77:5333-8.

Cunnane, K., Alvarado, A., Gwak, Y., Peters, C., Romero-Sandoval, E., Martin, T., Eisenach, J. (2019) Studying Pain Neural Circuits with Viral Vector rAAV2-Retro in the Brain. J Pain, 20:S36-S36.

Frangioni, J.V. (2003) In vivo near-infrared fluorescence imaging. Curr Opin Chem Biol, 7:626-34.

Grandjean, J., Canella, C., Anckaerts, C., Ayranci, G., Bougacha, S., Bienert, T., Buehlmann, D., Coletta, L., Gallino, D., Gass, N., Garin, C.M., Nadkarni, N.A., Hubner, N.S., Karatas, M., Komaki, Y., Kreitz, S., Mandino, F., Mechling, A.E., Sato, C., Sauer, K., Shah, D., Strobelt, S., Takata, N., Wank, I., Wu, T., Yahata, N., Yeow, L.Y., Yee, Y., Aoki, I., Chakravarty, M.M., Chang, W.T., Dhenain, M., von Elverfeldt, D., Harsan, L.A., Hess, A., Jiang, T., Keliris, G.A., Lerch, J.P., Meyer-Lindenberg, A., Okano, H., Rudin, M., Sartorius, A., Van der Linden, A., Verhoye, M., Weber-Fahr, W., Wenderoth, N., Zerbi, V., Gozzi, A. (2020) Common functional networks in the mouse brain revealed by multi-centre resting-state fMRI analysis. Neuroimage, 205:116278.

Helmchen, F., Denk, W. (2005) Deep tissue two-photon microscopy. Nat Methods, 2:932-40.

Hong, G., Diao, S., Chang, J., Antaris, A.L., Chen, C., Zhang, B., Zhao, S., Atochin, D.N., Huang, P.L., Andreasson, K.I., Kuo, C.J., Dai, H. (2014) Through-skull fluorescence imaging of the brain in a new near-infrared window. Nat Photonics, 8:723-730.

Hong, G.S., Antaris, A.L., Dai, H.J. (2017) Near-infrared fluorophores for biomedical imaging. Nat Biomed Eng, 1.

Hou, W., Xie, Y., Song, X., Sun, X., Lotze, M.T., Zeh, H.J., 3rd, Kang, R., Tang, D. (2016) Autophagy promotes ferroptosis by degradation of ferritin. Autophagy, 12:1425-8.

Iordanova, B., Ahrens, E.T. (2012) In vivo magnetic resonance imaging of ferritin-based reporter visualizes native neuroblast migration. Neuroimage, 59:1004-12.

Iordanova, B., Goins, W.F., Clawson, D.S., Hitchens, T.K., Ahrens, E.T. (2013) Quantification of HSV-1-mediated expression of the ferritin MRI reporter in the mouse brain. Gene Ther, 20:589-

96.
Iordanova, B., Robison, C.S., Ahrens, E.T. (2010) Design and characterization of a chimeric ferritin with enhanced iron loading and transverse NMR relaxation rate. J Biol Inorg Chem, 15:957-65.
Itoga, C.A., Chen, Y., Fateri, C., Echeverry, P.A., Lai, J.M., Delgado, J., Badhon, S., Short, A., Baram, T.Z., Xu, X. (2019) New viral-genetic mapping uncovers an enrichment of corticotropin-releasing hormone-expressing neuronal inputs to the nucleus accumbens from stress-related brain regions. J Comp Neurol, 527:2474-2487.
Kaplitt, M.G., Feigin, A., Tang, C., Fitzsimons, H.L., Mattis, P., Lawlor, P.A., Bland, R.J., Young, D., Strybing, K., Eidelberg, D., During, M.J. (2007) Safety and tolerability of gene therapy with an adeno-associated virus (AAV) borne GAD gene for Parkinson's disease: an open label, phase I trial. Lancet, 369:2097-105.
Kim, H.S., Cho, H.R., Choi, S.H., Woo, J.S., Moon, W.K. (2010) In vivo imaging of tumor transduced with bimodal lentiviral vector encoding human ferritin and green fluorescent protein on a 1.5T clinical magnetic resonance scanner. Cancer Res, 70:7315-24.
Li, X.F., Li, X.D., Deng, C.L., Dong, H.L., Zhang, Q.Y., Ye, Q., Ye, H.Q., Huang, X.Y., Deng, Y.Q., Zhang, B., Qin, C.F. (2017) Visualization of a neurotropic flavivirus infection in mouse reveals unique viscerotropism controlled by host type I interferon signaling. Theranostics, 7:912-925.
Namaste, S.M., Rohner, F., Huang, J., Bhushan, N.L., Flores-Ayala, R., Kupka, R., Mei, Z., Rawat, R., Williams, A.M., Raiten, D.J., Northrop-Clewes, C.A., Suchdev, P.S. (2017) Adjusting ferritin concentrations for inflammation: Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia (BRINDA) project. Am J Clin Nutr, 106:359S-371S.
Nassi, J.J., Cepko, C.L., Born, R.T., Beier, K.T. (2015) Neuroanatomy goes viral! Front Neuroanat, 9:80.
Naumova, A.V., Vande Velde, G. (2018) Genetically encoded iron-associated proteins as MRI reporters for molecular and cellular imaging. Wires Nanomed Nanobi, 10.
Pagani, M., Damiano, M., Galbusera, A., Tsaftaris, S.A., Gozzi, A. (2016) Semi-automated registration-based anatomical labelling, voxel based morphometry and cortical thickness mapping of the mouse brain. J Neurosci Meth, 267:62-73.
Rao, X., Wang, J. (2020) Neuronal Network Dissection with Neurotropic Virus Tracing. Neurosci Bull, 36:199-201.
Rozov, A., Jerecic, J., Sakmann, B., Burnashev, N. (2001) AMPA receptor channels with long-lasting desensitization in bipolar interneurons contribute to synaptic depression in a novel feedback circuit in layer 2/3 of rat neocortex. J Neurosci, 21:8062-71.
Tenenbaum, L., Chtarto, A., Lehtonen, E., Velu, T., Brotchi, J., Levivier, M. (2004) Recombinant AAV-mediated gene delivery to the central nervous system. J Gene Med, 6 Suppl 1:S212-22.
Tervo, D.G., Hwang, B.Y., Viswanathan, S., Gaj, T., Lavzin, M., Ritola, K.D., Lindo, S., Michael, S., Kuleshova, E., Ojala, D., Huang, C.C., Gerfen, C.R., Schiller, J., Dudman, J.T., Hantman, A.W., Looger, L.L., Schaffer, D.V., Karpova, A.Y. (2016) A Designer AAV Variant Permits Efficient Retrograde Access to Projection Neurons. Neuron, 92:372-382.
Ugolini, G. (2010) Advances in viral transneuronal tracing. J Neurosci Meth, 194:2-20.
Ullmann, J.F., Watson, C., Janke, A.L., Kurniawan, N.D., Reutens, D.C. (2013) A segmentation protocol and MRI atlas of the C57BL/6J mouse neocortex. Neuroimage, 78:196-203.

Van Leemput, K., Bakkour, A., Benner, T., Wiggins, G., Wald, L.L., Augustinack, J., Dickerson, B.C., Golland, P., Fischl, B. (2009) Automated segmentation of hippocampal subfields from ultra-high resolution in vivo MRI. Hippocampus, 19:549-57.

Vande Velde, G., Rangarajan, J.R., Toelen, J., Dresselaers, T., Ibrahimi, A., Krylychkina, O., Vreys, R., Van der Linden, A., Maes, F., Debyser, Z., Himmelreich, U., Baekelandt, V. (2011) Evaluation of the specificity and sensitivity of ferritin as an MRI reporter gene in the mouse brain using lentiviral and adeno-associated viral vectors. Gene Ther, 18:594-605.

Wei, P., Liu, N., Zhang, Z., Liu, X., Tang, Y., He, X., Wu, B., Zhou, Z., Liu, Y., Li, J., Zhang, Y., Zhou, X., Xu, L., Chen, L., Bi, G., Hu, X., Xu, F., Wang, L. (2015) Processing of visually evoked innate fear by a non-canonical thalamic pathway. Nat Commun, 6:6756.

Wei, Y.C., Wang, S.R., Jiao, Z.L., Zhang, W., Lin, J.K., Li, X.Y., Li, S.S., Zhang, X., Xu, X.H. (2018) Medial preoptic area in mice is capable of mediating sexually dimorphic behaviors regardless of gender. Nat Commun, 9:279.

Wu, E.X., Wong, K.K., Andrassy, M., Tang, H. (2003) High-resolution in vivo CBV mapping with MRI in wild-type mice. Magn Reson Med, 49:765-70.

Wu, Q., Ono, K., Suzuki, H., Eguchi, M., Yamaguchi, S., Sawada, M. (2018) Visualization of Arc promoter-driven neuronal activity by magnetic resonance imaging. Neurosci Lett, 666:92-97.

Xie, Y., Hou, W., Song, X., Yu, Y., Huang, J., Sun, X., Kang, R., Tang, D. (2016) Ferroptosis: process and function. Cell Death Differ, 23:369-79.

Zhang, Z., Liu, Q., Wen, P., Zhang, J., Rao, X., Zhou, Z., Zhang, H., He, X., Li, J., Zhou, Z., Xu, X., Zhang, X., Luo, R., Lv, G., Li, H., Cao, P., Wang, L., Xu, F. (2017) Activation of the dopaminergic pathway from VTA to the medial olfactory tubercle generates odor-preference and reward. Elife, 6.

Zheng, N., Su, P., Liu, Y., Wang, H., Nie, B., Fang, X., Xu, Y., Lin, K., Lv, P., He, X., Guo, Y., Shan, B., Manyande, A., Wang, J., Xu, F. (2019) Detection of neural connections with ex vivo MRI using a ferritin-encoding trans-synaptic virus. Neuroimage, 197:133-142.

Zheng, N., Wang, Z.Z., Wang, S.W., Yang, F.J., Zhu, X.T., Lu, C., Manyande, A., Rao, X.P., Xu, F.Q. (2020) Co-localization of two-color rAAV2-retro confirms the dispersion characteristics of efferent projections of mitral cells in mouse accessory olfactory bulb. Zool Res, 41:148-156.

Zhu, J., Yu, T., Li, Y., Xu, J., Qi, Y., Yao, Y., Ma, Y., Wan, P., Chen, Z., Li, X., Gong, H., Luo, Q., Zhu, D. (2020) MACS: Rapid Aqueous Clearing System for 3D Mapping of Intact Organs. Adv Sci (Weinh), 7:1903185.

Figure Legend

Fig. 1. Detection of EGFP/Ferritin expression with fluorescence imaging and T2-weighted MRI (TE=55ms), 60 days after the virus injection. A: Virus genomes of rAAV2-retro-CAG-EGFP and rAAV2-retro-CAG-Ferritin, Ftl1: Mus musculus ferritin light chain, Fth1: Mus musculus ferritin heavy chain. B: Upper, fluorescence images (green, EGFP) and corresponding MRI images (grey) of one representative mouse brain infected with rAAV2-retro-CAG-EGFP; Lower, fluorescence images (red, Ferritin) and corresponding MRI images (grey) of one representative mouse brain infected with rAAV2-retro-CAG-Ferritin.

Fig. 2. Signal comparison of the fluorescence imaging and MRI with brain region segmentation. A: the sketch map of virus infected regions after the rAAV2-retro injection (shown in fluorescence image). B: The distribution of Ferritin expression (red) and MRI signal changes (dark) compared at seven regions, CPu, BLA, HIP, PFC, Tha, Ins and post HIP. The mouse brain stereotaxic atlas is overlapped (white) to distinguish the brain structure.

Fig. 3. The schematic diagram of the processing of multi-Echo T2-weighted images, and the calculation of T2 relaxation time change mapping. A: The multi-Echo T2-weighted images were firstly transformed to the T2 relaxation time map and then normalized to a template. The normalized T2 relaxation time maps (right) were subtracted with the map of 0d (left) and then screened with a threshold to form a T2 relaxation time change mapping. B: The voxel-by-voxel T2 relaxation time change mappings in different infection periods.

Fig. 4. The comparison of fluorescence image, T2 relaxation change mapping and T2-weighted image on a different infection day. Three time points after injection are shown (10d, 30d, 60d). Fluorescence images (top) are displayed to show the location and expression quantity of Ferritin at different time points. Corresponding images of the T2 relaxation time change mapping (middle) and the T2-weighted images (bottom) are displayed for comparison. The voxels in T2 relaxation time change mappings are shown with pseudo color (red-yellow) when the value is between 4ms and 15ms. Data was obtained from three different representative mice, as the fluorescence imaging was obtained from brain slices.

Fig. 5. The longitudinal study of the rAAV2-retro-CAG-Ferritin infection at three different time points (10d, 30d, 60d) using in vivo MRI. The change in T2 relaxation times before versus after virus injection are used to represent the infected regions of the virus at three time points. The voxels in T2 relaxation time change mappings are shown with pseudo color (red-yellow) when the value is between 4ms and 12ms.

Fig. 6. Statistics analysis of T2 relaxation times at different time points after rAAV2-retro-CAG-Ferritin injection (0d, 10d, 30d, 60d). The T2 relaxation times of six ferritin expressed regions (CPu_R, BLA_R, HIP_R, Ins_R, PFC_R and Tha_R) and two negative control regions without ferritin expressed (CSF and SC) were extracted for comparison. Note: Significant changes among the four time points were calculated using one-way ANOVA with LSD post hoc test, and significant differences between each time point are illustrated with lowercase letters a, b, c, d (a different letter represented $p<0.05$ and the same letter represented $p>0.05$).

589

590 **Fig. 7.** The expression of caspase-3 and Iba1 was investigated at the regions that ferritin was
591 overexpressed (red, left). For caspase-3 staining (green, middle), obvious caspase-3 expression can only
592 be observed at the injection site (CPu), and no signal was found at the other three regions (BLA, HIP,
593 and PFC). For Iba1 staining (red, right), no abnormality was observed in the morphology and distribution
594 of microglia.

595

596 **Fig. 8**. Illustration of the schedule of the experiment.