

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

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35

36 **Abstract**

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

55

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

57

58 **Introduction**

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

67 Currently, neurotropic viral vectors have been widely used to investigate the neural networks.
68 As a series of artificially modified neurotropic viruses, neurotropic viral vectors can transport
69 exogenous genes along the synapses-connected neural networks. Numerous virus vectors were
70 constructed to dissect the structure of neural networks after genetic modifications, such as herpes
71 simplex virus (HSV), pseudorabies virus (PRV), rabies virus (RV), *etc.* (Nassi, et al., 2015; Rao and
72 Wang, 2020; Ugolini, 2010). However, most of them can only be used for *ex vivo* imaging due to
73 their virulence. Recombinant adeno-associated virus (rAAV) vectors are effective tools for
74 exogenous gene delivery for living animal studies due to their advantages of high-level transgene
75 expression and low cell toxicity (Kaplitt, et al., 2007). Recently an artificially-evolved AAV series
76 vector (rAAV2-retro) was introduced to mediate retrograde access to neurons (Tervo, et al., 2016).
77 It is a powerful tool to image neural circuits when combined with the fluorescent protein gene
78 (Zheng, et al., 2020). However, due to limitation of the fluorescent imaging depth, it is hard to
79 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 ($1/T_2$) 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

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

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

112

113 **Results**

114 ***MRI signal changes of the regions with ferritin transduction***

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

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

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

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

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

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

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

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

170 ***Tracing CPu connected regions using changes in T2 relaxation time***

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

177 The mapping of T2 relaxation time changes was compared with fluorescent images and T2-
178 weighted images with similar brain structures. The comparisons among three different time points
179 are illustrated (Fig. 4). Ten days after the injection, ferritin expression was observed in CPu and
180 PFC with fluorescence imaging. The same regions were also detected in mapping of T2 relaxation
181 time changes (10d), although the area was not as wide as the fluorescence imaging. Thirty days after
182 infection, stronger signals were detected in PFC, CPu, Tha, BLA, HIP, and Ins from the fluorescence
183 images, and wider signals were also found in PFC, CPu, BLA and Ins from the T2 relaxation time
184 change mapping (30d). Sixty days after infection, the fluorescent signal spread across the whole-
185 brain through the fluorescence imaging, particularly in the PFC, CPu, Tha, BLA, HIP and Ins.
186 Consistently, the T2 relaxation time change mapping (60d) showed more similar labeled patterns
187 compared to the fluorescence imaging. Overall, similar signal tendency was obtained from both the
188 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

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

216

217 **Discussion**

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

228 ***Resolution and SNR of MRI in dissecting neural circuits***

229 Although MRI has the advantages of non-invasive and large imaging scale, the resolution and
230 signal-to-noise ratio are much lower than fluorescence imaging. For fluorescence imaging, the
231 resolution could reach $0.2 \mu\text{m}$, and there is little interference in the background. Since the diameter
232 of neurons is usually $\sim 5 \mu\text{m}$, the fluorescence imaging can easily distinguish the neuron cells

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

246

247 ***MRI signals at different infection times***

248 In these experiments, ferritin was overexpressed in a CPu related network through a AAV2-
249 retro virus vector, and three time points (10d, 30d, 60d) were selected to evaluate the ferritin
250 expression and MRI signal. Different ferritin expressions and MRI signals were found at the three
251 time points, and the characteristics of rAAV vector could be the main reason for the differences. The
252 expression of AAV-carrying genes usually increases over time and reaches a plateau within 3-12
253 weeks (Tenenbaum, et al., 2004) and most of the AAV2-retro labeled results are obtained within 3-
254 8 weeks as reported. Three weeks (Itoga, et al., 2019) and four weeks (Itoga, et al., 2019) are the

255 most commonly used waiting time for AAV2-retro expression and it is reported that more labeled
256 information can be obtained using eight weeks waiting time rather than four weeks (Cunnane, et al.,
257 2019).

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

270 ***Relationship between ferritin expression and MRI contrast***

271 Although ferritin is reported to recruit iron ions and influence the MRI signals, there were
272 many factors that influence the MRI signal changes. Ferritin is an autologous protein which is
273 involved in inflammation reaction (Namaste, et al., 2017), the infection of AAV could hypothetically
274 lead to regional inflammation which could also cause overexpression of ferritin (Vande Velde, et al.,
275 2011). Therefore, the expression of ferritin in the infection of rAAV2-retro-CAG-EGFP was also
276 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 Fe³⁺. 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 Fe³⁺ 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

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

308

309 **Materials and methods**

310 *Animal preparation*

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

317 *Construction of Virus*

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

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

326 ***Stereotaxic injection***

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

340 ***MRI scanning***

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

343 with 4.0-5.0% isoflurane (RWD, Shenzhen, China) for induction and 1.0-1.5% for maintenance with
344 a mixture of 30% O₂ and 70% N₂. The body temperature of animals was maintained with a
345 thermostatic water cycle system under the animal bed. The breathing rate of the animal was
346 monitored and maintained at 60±15 breaths/min to achieve the state of deep anesthesia. In addition,
347 two ear bars and a tooth bar were used to minimize motion effect of MRI data acquisition. A 20cm
348 birdcage coil was used for transmission, combined with a 20 mm surface coil for receiving (Bruker,
349 Ettlingen, Germany). Multi-echo T2-weighted anatomical images were obtained using an MSME
350 sequence (TR = 3000 ms; Effective TEs = 11, 22, 33, 44, 55, 66 ms; Number of Averages = 6; FOV
351 = 17.5 *17.5 mm²; Slice thickness = 0.5 mm; Spatial resolution, 0.137 mm *0.137 mm). The T2
352 relaxation time maps were obtained by processing the MSME image using Paravision 5.0 software
353 (Bruker, Germany).

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

360 ***Fluorescence imaging and immunohistochemistry***

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

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

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

375 For fluorescence imaging, the brain slices were transferred to microslide and scanned with an
376 Olympus VS120 virtual microscopy slide scanning system (Olympus, Japan). For the filters, Leica
377 U-MRFPHQ fluorescence mirror unit was used for the red fluorescence imaging, which included a
378 535-555nm excitation filter, a 575-625nm emission filter and a 565nm dichromatic mirror; Leica
379 U-MWIBA3 fluorescence mirror unit was used for green fluorescence imaging, which included a
380 460-495nm excitation filter, a 510-550nm emission filter and a 505nm dichromatic mirror.

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

384 ***Data analysis***

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

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

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

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

406 ***Data availability***

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

409

410 **Conclusion**

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

419

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421 Su, Kunzhang Lin, Ling-Qiang Zhu gave advice on the research. Ning Zheng, Aoling Cai, Yang Wu
422 performed experiments. Aoling Cai, Ning Zheng, Binbin Nie, Jinfeng Wu analyzed data. Aoling Cai,
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434

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544

545 **Figure Legend**

546

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

554

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

560

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

567

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

575

576 **Fig. 5.** The longitudinal study of the rAAV2-retro-CAG-Ferritin infection at three different time points
577 (10d, 30d, 60d) using in vivo MRI. The change in T2 relaxation times before versus after virus injection
578 are used to represent the infected regions of the virus at three time points. The voxels in T2 relaxation
579 time change mappings are shown with pseudo color (red-yellow) when the value is between 4ms and
580 12ms.

581

582 **Fig. 6.** Statistics analysis of T2 relaxation times at different time points after rAAV2-retro-CAG-Ferritin
583 injection (0d, 10d, 30d, 60d). The T2 relaxation times of six ferritin expressed regions (CPu_R, BLA_R,
584 HIP_R, Ins_R, PFC_R and Tha_R) and two negative control regions without ferritin expressed (CSF and
585 SC) were extracted for comparison. Note: Significant changes among the four time points were calculated
586 using one-way ANOVA with LSD post hoc test, and significant differences between each time point are
587 illustrated with lowercase letters a, b, c, d (a different letter represented $p < 0.05$ and the same letter
588 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.