1 **FRMPD2: a novel GluN2A-interacting scaffold protein in synaptic** 2 **excitatory transmission**

3 Xi Lu¹, Haiqing Zhang¹, Xin Tian¹, Xuefeng Wang^{1, 2*}.

4 **Affiliations:**

5 1: Department of Neurology, Chongqing Key Laboratory of Neurology, The First
6 Affiliated Hospital of Chongqing Medical University, 1 Youyi Road, Chongqing
7 400016, China

8 2: Center of Epilepsy, Beijing Institute for Brain Disorders, 10 Xitoutiao, Youanmen,
9 Fengtai District, Beijing 100069, China.

10 *: The corresponding author: Xuefeng Wang, Email: wxfcqmu@163.com

11 Address correspondence to Xuefeng Wang, Department of Neurology, The First
12 Affiliated Hospital, Chongqing Medical University, 1 Youyi Road, Chongqing
13 400016, China.

14

15

16

17 **Abstract**

18 The scaffold proteins FRMPD2 is localized at the basolateral membranes of polarized
 19 epithelial cells and associated with tight junction formation. But the expression and
 20 function of FRMPD2 in vivo remain unknown. Here, we found that postsynaptic
 21 FRMPD2 effectively alters the neuronal excitability by regulating
 22 NMDARs-component excitatory synaptic transmission in adult hippocampus.
 23 FRMPD2 anchors the NMDAR subunit GluN2A at the synapses without changing
 24 the total NMDAR expression. By using of Co-IP, Pull Down and SPR, the second
 25 PDZ (PDZ2) domain of FRMPD2 direct and binds to the C-terminus of GluN2A. Our
 26 study discover that a novel scaffold protein FRMPD2 regulates synaptic excitatory in
 27 a PDZ2 domain-dependent manner in adult hippocampus.

28

29 **Keywords:** excitatory synapses, NMDAR, PDZ domain, scaffold protein

30 **Introduction**

31 Scaffold proteins contain several modular protein interaction domains within
 32 their structure, and they bind to various interacting proteins, such as ion channels,
 33 neurotransmitter receptors, adhesion molecule, microtubule, filamentous actin
 34 (F-actin)(Anzai et al., 2002; Durand et al., 2012; Elias and Nicoll, 2007;
 35 Hruska-Hageman et al., 2002; Kim and Sheng, 2004; Sala et al., 2001; Schultz et al.,
 36 1998). They strongly contribute to the organization of synaptic signalling complexes,
 37 and the clustering of surface receptors and ion channels, and they participate in the
 38 dynamic trafficking of vesicle transport and coordinate cytoskeletal dynamics(Feng
 39 and Zhang, 2009). FRMPD2 (also named Gm626 in mice) is a novel scaffold protein
 40 that consists of an N-terminal KIND domain, a Ferm (band 4.1, ezrin, radixin, and
 41 moesin) domain and three C-terminal PDZ (PSD-95/Discs-large/ZO1)
 42 domains(Lipinski et al., 2012; Stenzel et al., 2009). FRMPD2 is reported to localize
 43 in the basolateral membranes of polarized epithelial cells and is associated with tight
 44 junction formation and immune response(Lipinski et al., 2012; Stenzel et al., 2009).
 45 However, there are few studies investigating FRMPD2 in vivo and the expression and
 46 function of FRMPD2 in nervous system remain unknown.

47 Inspired by multiple domains in its structure, FRMPD2 is predicted to play
 48 a critical role in neuronal excitation. PDZ domain-containing proteins are involved in
 49 neuronal excitatory by trafficking neurotransmitter receptors to plasma
 50 membrane(Kim and Sheng, 2004). Thus, FRMPD2 may be implicated in the
 51 regulation of neuronal excitation in a PDZ- dependent manner.

52 In this paper, we first reported that postsynaptic scaffold protein FRMPD2
53 activated neuronal excitability in adult hippocampus by mediating the
54 N-methyl-d-aspartic acid (NMDA) receptors-component excitatory synaptic
55 transmission. FRMPD2 anchored the GluN2A subunit of NMDA receptors
56 (NMDARs) to the synapses by directly binding the second PDZ (PDZ2) domain to
57 the C-terminus of GluN2A. Our study shed light on a novel scaffold protein
58 FRMPD2 mediate synaptic excitatory via directly anchoring synapses GluN2A
59 subunits.

60 **Results**

61 **1. Expression patterns of the FRMPD2 protein.**

62 The FRMPD2 protein was expressed in a restricted manner in some organs
63 including the brain, eyes, testes and lungs (Fig 1A), and was enriched in a more
64 widespread manner in various brain regions, including the cortex, hippocampus and
65 cerebellum, in addition to the ependymal cells lining the ventricles (Fig 1B, C).

66 When immunofluorescence staining was performed in mouse cortex and the
67 hippocampus Cornu Ammonis 1 (CA1) region, FRMPD2 was localized in
68 microtubule-associated protein 2 (MAP2)-positive neuronal dendrites and another
69 major scaffold protein, PSD95 (postsynaptic marker) (Fig 1D, E). Synaptosomes in
70 the hippocampus were isolated by ultracentrifugation, and the FRMPD2 protein was
71 detected in the postsynaptic density (PSD) fraction, but not in the presynaptic fraction
72 or non-synaptic synaptosomal fraction (Fig 1F).

73 **2. Effects of FRMPD2 on NMDA receptor-mediated excitatory synaptic** 74 **transmission**

75 PDZ domain-containing proteins are reported to be involved in neuronal
76 excitatory (Kim and Sheng, 2004). We tested that whether postsynaptic FRMPD2
77 containing three PDZ domains affects neuronal excitatory. Whole cell patch-clamp
78 electrophysiology was performed in CA1 pyramidal neurons in mouse hippocampal
79 slices at 14 days after lentivirus vector (LV) injection of mice that was used to
80 knockdown or overexpress FRMPD2. LV-encoding green fluorescent protein (GFP)
81 was detected in the mouse hippocampus (Fig 2A). At 2 and 6 weeks post-LV injection,

82 the FRMPD2 protein levels of the hippocampus in FRMPD2 knockdown mice
83 (shFRMPD2) was almost 40 percent less than in scramble controls (shFRMPD2-Scr).
84 FRMPD2 protein levels were significantly higher in shFRMPD2-Res mice that were
85 injected with shRNA plus overexpression LV in the hippocampus than in FRMPD2
86 knockdown mice. The FRMPD2 protein in FRMPD2 overexpression mice
87 (FRMPD2-OE) was 1.9-2.1 times higher than in negative controls (FRMPD2-OE NC)
88 (Fig 2B). These findings suggest that FRMPD2 protein levels in the hippocampus
89 were effectively modulated by LV injection.

90 We firstly analyzed the effect of FRMPD2 on spontaneous action potential (AP)
91 in CA1 pyramidal neurons. The frequency of spontaneous AP in FRMPD2
92 knockdown mice reduced by 71 percent compared with the scramble control. AP
93 frequency of FRMPD2 overexpression mice was 2.28 times faster than in the
94 negative control (Fig 3A). To further explore the contribution of glutamate excitatory
95 receptors and/or inhibitory synaptic inputs contribute to the increase in neuronal
96 hyperactivity induced by FRMPD2, α -amino-3-hydroxy-5-methyl-4-isoxazole
97 propionic acid (AMPA) receptor (AMPA) component or NMDAR component
98 mEPSCs, and miniature inhibitory postsynaptic currents (mIPSCs) of pyramidal
99 neurons in the CA1 region of the hippocampus were recorded. The two types of
100 ionotropic glutamate receptors, AMPARs and NMDARs, present at postsynaptic
101 membrane of excitatory synapses mediate almost all synaptic transmission in the
102 brain (Bredt and Nicoll, 2003; Valtschanoff and Weinberg, 2001). There was no
103 significant difference in AMPAR mEPSCs (Fig 3B) or mIPSCs (Fig 3C) after

FRMPD2 knockdown or overexpression. However, the mEPSC amplitude, but not the frequency, of NMDAR mEPSCs was decreased in FRMPD2 knockdown mice compared with scramble controls, and increased in FRMPD2 overexpression mice compared with negative controls (Fig 4A).

To verify the changes of mEPSCs, NMDAR-mediated evoked EPSCs (eEPSCs) were measured. Firstly, the input-output curve of NMDAR eEPSCs (Fig 4B) verified that FRMPD2 enhanced the postsynaptic efficacy of NMDAR responses (Shen et al., 2010). As shown in Fig 4C, the amplitudes of NMDAR currents were significantly lower in the FRMPD2 knockdown than in the scramble controls. There was no significant difference in evoked AMPAR currents between the FRMPD2 knockdown and overexpression groups, in line with data of AMPAR component mEPSCs. In particular, to determine whether GluN2A and/or GluN2B component currents contributed to the observed changes in NMDAR eEPSCs, selective antagonist for GluN2A (NVP-AAM077) and GluN2B (Ro25-6981) were used (Fig 4D). The amplitude of GluN2A-sensitive currents of the FRMPD2 knockdowns was decreased by 65 percent compared with the scramble groups, and amplitude of GluN2A currents of overexpressing FRMPD2 mice was 2.38 times higher than of the negative controls. Interesting, the GluN2B component-mediated currents were not changed after FRMPD2 knockdown or overexpression. These results suggested that FRMPD2 strengthens GluN2A-mediated NMDARs synaptic transmission.

Because presynaptic release at postsynaptic glutamate receptors also contribute to synaptic excitatory transmission, we analyzed the paired-pulse ratio (PPR), a

reliable index of the contribution of presynaptic vesicles release to excitatory postsynaptic currents(Zucker and Regehr, 2002). There was no significant difference in PPR values between the FRMPD2 knockdown and overexpression results and those in the corresponding control groups (Fig 4E), indicating that presynaptic vesicles release is not responsible for the FRMPD2-mediated changes in mEPSCs

3. Effects of FRMPD2 anchoring GluN2A at synapses.

To correlate electrophysiological changes with biochemical alterations in synaptic NMDAR, we performed immunoblotting using hippocampal synaptosomal and total lysates. As shown in Fig 5A, the synaptic GluN2A and GluN2B were significantly fewer in the FRMPD2 knockdown mice than in the scramble mice, and higher in the FRMPD2 overexpression mice than in the negative controls. The total protein levels of GluN2A, GluN2B and GluN1 were not affected by FRMPD2. Immunofluorescence staining was also used to test for co-localization between FRMPD2 and GluN2A at postsynaptic membranes, which were marked by PSD-95 in hippocampal CA1 areas (Fig 5B).

The extreme C-terminus of the NMDAR subunits provides a mechanism for trafficking NMDAR subunits in postsynaptic membrane by interacting with PDZ proteins (Bard et al., 2010; Kim and Sheng, 2004). We performed co-immunoprecipitation (Co-IP) and GST pull-down assays to detect whether the three PDZ domains of FRMPD2 interact with NMDAR subunits. In vivo, co-immunoprecipitation of FRMPD2 with GluN1, GluN2A and GluN2B were detected in the lysates of mice hippocampus (Fig 5C). A GST fusion protein bearing

148 the PDZ2 domain but not the first PDZ or third PDZ domain pulled down
149 endogenous GluN1, GluN2A and GluN2B from mice hippocampus in vivo. The
150 full-length GluN1, GluN2A and GluN2B proteins tagging with GFP were generated
151 in translation experiments in vitro, GST-PDZ2 pulled down full-length GluN2A and
152 GluN2B, but not GluN1 (Fig 5D). However, the GST-PDZ2 fusion protein did not
153 pull down GluN2A proteins from the cells transfected with a form of GFP-GluN2A
154 that lacked a set of ten C-terminal amino acids (Fig 5D).

155 The binding abilities of FRMPD2-isolated PDZ2 domain to the GluN2A and
156 GluN2B C-terminus was measured using surface plasmon resonance (SPR) analyses.
157 The binding capacity of the FRMPD2 PDZ2 domain to bind to the wild type GluN2A
158 C-terminus (KKMPSIESDV) and wild type GluN2B C-terminus (EKLSSIESDV)
159 yielded a K_D (dissociation constant) value of 0.31 μ M and 0.25mM, respectively (Fig
160 5E,F), whereas no binding was observed between the mutant GluN2A C terminus
161 (KKMPSIEADK) (Fig 5G) and the FRMPD2 PDZ2. These results supported the
162 direct interaction of C terminus of GluN2A to PDZ2 of FRMPD2.

163 To further explore potential sites for binding between GluN2A and FRMPD2,
164 we first obtained the crystal X-ray structure of the PDZ2 domain(Fig 6A) of the
165 mouse homologue of FRMPD2 (PDB ID: 5ZDS) (Table 1). The crystal structure was
166 solved by PHENIX with the template PDZ2 domain structure of tyrosine phosphatase
167 PTP-BL (PDB ID: 1VJ6) (Fig 6B) with 63% identity(Adams et al., 2010; Gianni et
168 al., 2006). In the template structure (Fig 6C), there was a peptide that is a C-terminal
169 (HSGSYLVTSV) of tumor suppressor protein APC, binding to β 2 strand and α 2 helix.

170 The residues V(0) and T(-2) of the peptide formed critical hydrophobic interactions
171 with residues L25, I27, L85, V29, and V65 of the template PDZ2 structure(Gianni et
172 al., 2006). These residues were highly conserved in the FRMPD2 PDZ2 crystal
173 structure, as shown by the alignment (Fig 6D).

174 The results shown so far reveal that FRMPD2 affects NMDAR-mediated
175 excitatory postsynaptic currents by anchoring GluN2A subunits at postsynaptic
176 membranes. This activity is achieved by an interaction of the FRMPD2 PDZ2 domain
177 to the GluN2A C-terminus.

178

179 **Discussion**

180 In our report, we identified a novel scaffold protein FRMPD2, and explored its
181 physiological contribution to the modulation of excitatory synaptic
182 electrophysiological function.

183 FRMPD2 was essential for NMDA-component, but not AMPA-component
184 excitatory transmission in adult hippocampus. It is seems that the binding of
185 glutamate receptors by FRMPD2 appears to change with glutamate receptor-specific
186 manner(Beique et al., 2006; Elias et al., 2008). Our results showed that synaptic
187 FRMPD2 mediated neurotransmission by trafficking GluN2A subunits on synapses in
188 adult hippocampus. NMDA receptors are composed of obligatory GluN1 subunit
189 co-assembled with GluN2A or GluN2B subunit (GluN1/GluN2A or GluN1/GluN2B).
190 The identity of the GluN2A and/or GluN2B subunits determines the biophysical and
191 pharmacological properties of NMDARs (Bard et al., 2010; Sultan et al., 2015). Our

192 results of Co-IP, GST-pull-down and SPR assays identified the second PDZ domain
193 of FRMPD2 as a novel binding partner of GluN2A and GluN2B *in vivo* and *in vitro*.
194 Although the FRMPD2 PDZ2 did not directly bind to GluN1, the obligatory GluN1
195 subunit of GluN1/2A complex was, expectedly, detectable by Co-IP of FRMPD2.
196 Meanwhile, the GluN1 protein of synaptosome was changed after FRMPD2
197 knockdown or overexpression, which was accompanied by the alteration of GluN2A
198 subunits. In addition, the binding capacity of PDZ2 to GluN2A subunit was 1000
199 times higher than that to GluN2B subunit. It is probably that the binding of PDZ2 to
200 GluN2B is flexible and instant. In addition, the GluN2B-containing NMDARs are
201 located at extrasynaptic sites and involved in neurotoxicity in adult hippocampal
202 neurons, GluN2A-containing NMDARs mainly resided at synaptic sites and involved
203 in neurotransmission (Parsons and Raymond, 2014).

204 In summary, the results presented here suggest a critical role of novel scaffold
205 protein FRMPD2 in regulating NMDA receptors anchoring, which is critical for
206 regulation of glutamatergic synapses transmission. The dysregulation of this scaffold
207 protein contributed to the impaired excitatory conductance.

208 **Methods and materials**

209 **DNA constructs**

210 To construct the following GST fusion proteins, the PDZ domain of mouse
211 FRMPD2 (Uniprot: A0A140LI67) was subcloned into pGEX4T-1 with a GST tag:
212 GST-PDZ1 (amino acids 786-872) and GST-PDZ2 (amino acids 934-1027),
213 GST-PDZ3 (amino acids 1071-1159).

214 The lentivirus vector (LV) constructs were manufactured by Genechem Co.
215 Biotechnology (Shanghai City, China). The mouse FRMPD2 (NCBI Gene: 268729)
216 short hairpin RNA (shRNA) knockdown construct targeted 5'-3'
217 CTGAATCTTTCTCTTGCAT and was subcloned it into the LV GV248 (GeneChem,
218 China), which contained green fluorescent protein (GFP). A scramble shRNA (5'-3'
219 CTTTCTAACGTGTCGACT) was used as a negative control for the FRMPD2
220 shRNA. The complete mouse cDNA sequence for FRMPD2 (XM_017316244)
221 (FRMPD2 overexpression, FRMPD2 OE) and the GFP sequence were inserted into
222 the LV GV358 (GeneChem, China).

223

224 **Antibodies**

225 The following antibodies were used: FRMPD2 (rabbit, Sigma;rabbit, Origene),
226 Flag(mouse, Sigma), GST(rabbit, Invitrogen), GFP (rabbit, Invitrogen),
227 GluN2A(rabbit, Proteintech), GluN2B(mouse, Proteintech; rabbit, Abcam),
228 GluN1(rabbit, Abcam), MAP2(guinea pig, SYSY), GFAP(mouse, Sigma),
229 PSD-95(guinea pig, SYSY; mouse, Millipore), VGLUT1(guinea pig, SYSY),

230 synaptophysin(rabbit, Abcam), syntaxin 1a(rabbit, Abcam).

231

232 **Cell line cultures and transfection**

233 HEK293T and SH-SY5Y cells were maintained in DMEM (Gibco)
 234 supplemented with 10% fetal bovine serum (Gibco), 100 U/mL penicillin and 100
 235 µg/mL streptomycin (Gibco) at 37°C in 5% CO₂. The cells were transfected using
 236 Lipofectamine 2000 (Invitrogen)(Klein et al., 2017). At 4 h after transfection, the
 237 cells were rinsed with warm PBS and incubated in culture medium containing 10%
 238 fetal bovine serum.

239

240 **Intrahippocampal injection of LV**

241 Adult C57/BL6 male mice were obtained from the Experimental Animal
 242 Center of Chongqing Medical University. Intrahippocampal injection of LV was
 243 performed using a stereotaxic apparatus (Stoelting Co. Ltd Wood Dale, IL, USA).
 244 Briefly, the mice were anesthetized with pentobarbital (80mg/kg, intraperitoneal) and
 245 placed on stereotaxic apparatus. The reference points were bregma for the
 246 anterior-posterior axis, the midline for the medial-lateral axis and the dura mater for
 247 the dorsal-ventral axis with a tooth bar. LV particles were injected bilaterally into the
 248 dorsal hippocampus through a glass pipette (0.2 µl/min) attached to a glass
 249 microsyringe(Shangguan et al., 2017). The mice were allowed to recover for 2 weeks
 250 after the vector injections.

251

252 **Patch clamp recordings**

253 Brain slices were prepared as previously reported(Chen et al., 2006; Kim et al.,
254 2001). Briefly, C57/BL6 mice were anesthetized with pentobarbital. Brain slices (300
255 μm) were prepared with a Leica (Germany) VP1200S Vibratome and incubated in
256 artificial cerebral spinal fluid (ACSF) (119 mM NaCl, 26 mM NaHCO_3 , 2.5 mM KCl,
257 1 mM MgCl_2 , 1.25 mM NaH_2PO_4 , 2 mM CaCl_2 and 25 mM glucose, pH 7.4, 310
258 mOsm) bubbled with 5% CO_2 and 95% O_2 for at least 1 h at room temperature before
259 recording. Whole-cell recording was performed as described previously(Liu et al.,
260 2005). Glass microelectrodes (Sutter, USA) were shaped by a pipette puller (P-97,
261 Sutter, USA) to a final resistance of 3–5 M Ω when the pipette was filled with internal
262 solution. A multi-clamp 700B amplifier (Axon, USA) was used for the recordings.
263 Signals were sampled at 10 kHz and filtered at 2 kHz. A stable baseline was obtained
264 for at least 5 min prior to recording and data were discarded when the access
265 resistance (15–20 M Ω) was changed by 20% at the end of recording. Mini Analysis
266 6.0.1 (Synaptosoft) and Clamp Fit 10.3 software (Axon) were used to analyze the
267 recorded data.

268 For action potential (AP) recording, glass pipettes were filled with the following
269 internal solution: 17.5 mM KCl, 0.5 mM EGTA, 122.5 Mm K-gluconate, 10 mM
270 HEPES, and 4 mM ATP, pH adjusted to 7.2 with KOH [20]. The internal solution
271 used to record excitatory post-synaptic currents (EPSCs) contained: 17.5 mM CsCl,
272 10 mM HEPES, 4 mM ATP, 0.5 mM EGTA, 132.5 mM Cs-gluconate, and 5 mM
273 QX-314. The internal solution used to record inhibitory post-synaptic currents (IPSCs)

274 contained: 100 mM CsCl, 1 mM MgCl₂, 1 mM EGTA, 30 mM
275 N-methyl-D-glucamine, 10 mM HEPES, 5 mM MgATP 0.5 mM Na₂GTP and 12 mM
276 phosphocreatine.

277 Spontaneous activity was assessed by observing the neuron under current clamp
278 for 1 min. Tetrodotoxin (TTX, 1 μM), bicuculline (10 μM),
279 6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX, 20 μM) and D-serine (10 μM)
280 were added to Mg-free-ACSF to record NMDAR mEPSCs that held at -60 mV. TTX
281 (1 μM), bicuculline (10 μM) and amino-5-phosphonopentanoate (AP-V, 40 μM) were
282 added to ACSF to record AMPA mEPSCs. TTX(1 μM), DNQX(20 μM) and APV(40
283 μM) were added to ACSF to record mIPSCs that held at -70 mV (Shangguan et al.,
284 2017).

285 Evoked EPSCs (eEPSCs) currents were generated with a 40μs pulse (0.1 Hz)
286 from a stimulated isolation unit controlled by an AMPI generator (Master-8, USA). A
287 bipolar stimulation electrode (FHC) was located ~100 μm rostral to the recording
288 electrode in the same layer(Chen et al., 2008; Tang et al., 2015). The eEPSCs at -70
289 mV holding potential in the present of PTX (100 μM) was considered as the
290 AMPAR-mediated current. Neurons were then held at +40 mV and the amplitude of
291 the eEPSCs at 50 ms post-stimulus was interpreted as the NMDAR-mediated
292 current(Etherton et al., 2009; Etherton et al., 2011). Input-output curve of NMDAR
293 eEPSCs were recorded in response to a series of stimulation intensities (pA).
294 Paired-pulse ratio (PPR) recordings were voltage-clamped at +40 mV and obtained
295 using a paired-pulse protocol of two stimuli at an inter-pulse interval of 50 ms. PPR

values were defined as the ratio of the second peak amplitude to the first peak amplitude. The highly selective GluN2B subunit antagonist Ro25-6981 (Ro, 0.5 μ M) was applied to assess GluN2A-sensitive eEPSCs, and the selective GluN2A antagonist NVP-AAM077(NVP, 0.4 μ M) was added to record GluN2B component eEPSCs(Bartlett et al., 2007).

301

302 **Western blotting**

Samples boiled with 4X sample buffer at 95°C for 5 min, and then separated on 8-10% SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes (Millipore). The target proteins were immunoblotted with primary antibodies overnight at 4°C and then incubated with HRP-conjugated secondary antibodies. The blots were imaged and quantified using a Fusion Imaging System. The quantitative densitometric values of the proteins were normalized to that of GAPDH or beta-actin.

309

310 **Immunofluorescence**

For animal tissues, brain were post-fixed at 4°C for 24 h. Floating slices (50 μ m in thickness) were permeabilized with 0.5% Triton X-100 and blocked in goat serum. The slices were then incubated with primary antibodies overnight at 4°C followed by incubation with secondary fluorescent conjugated antibodies at room temperature for 2 h. Images were captured using laser-scanning confocal microscopy (Nikon A1+R Microsystems) on an Olympus IX 70 inverted microscope (Olympus) equipped with a Fluoview FVX confocal scanning head. Areas of overlap and fluorescence intensity

318 were analyzed using Image Pro Plus 6.0.

319

320 **Sub-synaptic fractionation**

321 Sub-synaptic fractionation was performed as previously described(Phillips et al.,
322 2001). To obtain synaptosomes, adult mouse hippocampus were homogenized in
323 solution buffer (0.32 M sucrose, 0.1 mM CaCl_2 , 1 mM MgCl_2 , 0.1 mM PMSF) and
324 brought to a final sucrose concentration of 1.25 M. Protease inhibitors were used in
325 all purification steps. The homogenate was overlaid with 1.0 M sucrose and
326 centrifuged (100,000 g, 3 h, 4°C). Synaptosomes were collected at the 1 M/1.25 M
327 interface.

328 For sub-synaptic fractionation, synaptosomes were sequentially extracted with
329 1% Triton X-100 first at pH 6.0 and then at pH 8.0. Synaptosomes (described above)
330 were diluted 1:10 with 0.1 mM CaCl_2 to a final concentration of 20 mM Tris, pH 6.0
331 and 1% Triton X-100. After the solution was incubated for 30 min and then
332 centrifuged 40,000 g for 30 min at 4°C. The supernatant (presynaptic fraction) were
333 precipitated with 10 volumes of acetone at -20°C and recovered by centrifugation
334 (20,000 g; 30 min, 4°C). The pellet containing insoluble postsynaptic density (PSD)
335 was resuspended in 10 volumes solution buffer (1% Triton X-100, 20 μM Tris, pH
336 8.0, 4°C), incubated for 30 min, and centrifuged (40,000 g, 30 min, 4°C).

337

338 **GST-pull-down and co-immunoprecipitation assays.**

339 The methods used to produce the GST fusion proteins in bacteria have been

340 previously reported (Anzai et al., 2002; Anzai et al., 2004). GST-pull-down assays
341 were carried out using a GST Protein Interaction Pull-Down Kit (Pierce) according to
342 the manufacturer's instructions. Briefly, the bacterial lysate containing the fusion
343 proteins was sonicated and centrifuged, and the supernatant was used for protein
344 binding assays. In vitro translation was facilitated using a plasmid carrying
345 full-length GluN2A, GluN2B or GluN1 (Addgene) that was transfected into
346 HEK293T cells. The translated total proteins obtained from cells and the GST fusion
347 proteins were incubated together with GST glutathione agarose resin. The protein
348 complexes were then eluted by glutathione and analyzed by Western blotting using
349 GST, GFP, GluN2A, GluN2B or GluN1 antibodies.

350 For co-immunoprecipitation assays, the extracts contained in the crude
351 synaptosomal fraction obtained from adult mouse hippocampal tissues were prepared
352 with anti-GluN2A, anti-GluN2B, anti-GluN1 or anti-FRMPD2 antibodies or IgG
353 (rabbit, Abcam) (Control) at 4°C for 12 h before they were incubated with protein
354 A/G agarose beads (Beyotime). The immunoprecipitated mixture was immunoblotted
355 with GluN2A, GluN2B, GluN1 or FRMPD2 antibodies.

356

357 **Surface Plasmon Resonance (SPR) assays.**

358 SPR biosensor analysis was used to verify binding between peptides and the
359 PDZ2 domain of FRMPD2 protein using a Biacore 8K instrument (GE Healthcare),
360 as previously described (Zhang et al., 2014). Proteins were immobilized on S CM5
361 chips using amine-coupling chemistry. Binding experiments were performed in

362 running buffer (10 mM PBS, pH 7, 0.05% P20) at 25°C with a flow rate of 30 µL/min.
363 Gradient concentrations of PDZ2 proteins were diluted to 50 ug/ml in sodium
364 acetate(10 mM, pH 5.5) and injected into the channel for 120 s, followed by
365 disassociation for 120 s. RU values were collected at 10 Hz and all the experimental
366 data were globally analyzed in a steady-state model within Biacore 8K Evaluation
367 software.

368

369 **Study approval**

370 All protocols of animal studies were approved by the Commission of Chongqing
371 Medical University for the ethics of experiments on animals and conducted in
372 accordance with international standards.

373

374 **Crystallization**

375 GST-PDZ2 (amino acids 934-1027) was expressed in Escherichia coli,
376 BL21(DE3) cells. Cells pellet was sonicated and centrifuged at 5000 rpm. The
377 supernatant was subjected to GST column purification (10 mM Tris, 10 mM GSH,
378 pH 7.4). Pooled fractions were dialyzed with thrombin and passed through a Q-HP
379 column (20 mM Tris, 0-1 M NaCl, 5% glycerol, pH 8.0). Finally, protein fractions
380 were further purified by Superdex 75 columns (20 mM Tris, 5% glycerol, 150 mM
381 NaCl, 2 mM DTT pH 7.4) for crystallization.

382 Screen kits, including the JCSG-CORE-I-IV (Qiagen) and HR2-144 Index
383 (Hampton), were used to perform crystallization screen with the hanging drop

method at 18 °C. The protein (15 mg/ml) was mixed with an equivalent volume (1 µl) of screen buffer. The crystal was grown in 20% PEG3350 and 0.2 M sodium thiocyanate. A single crystal was transferred to the crystallization buffer containing an additional 20% glycerol and flash frozen with liquid nitrogen. The crystal diffracted well at BL19U (Shanghai Synchrotron Radiation Facility, SSRF, China). In all, images for 180° were collected. The data were processed by HKL3000 (Table 1). The crystal structure was refined by PHENIX and Coot. The structure was refined to 1.80 Å with R factor 19.76% and R free 24.76%.

Statistical analysis

All averaged data are presented as the means ± SD, and all graphs were prepared using GraphPad Prism 4 software (La Jolla, CA). For independent-samples, Student's t-test was used. Repeated measures ANOVA followed by post hoc t-tests was used to measure differences between two groups at multiple time points. All tests were two-sided. n indicates the number of cells/slices or independent experiments and was used to calculate the degree of freedom. All samples included in each experiment were analyzed in triplicate.

401 **Acknowledgements**

402 This work was supported by grants from the National Natural Science
403 Foundation of China (No. 81271445, 81471319, 81301109, and 81301110) and the
404 National Clinical Key Specialty Construction Foundation of China.

405 **Author contributions**

406 X. L. and XF. W. conceived and designed the experiments. HQ. Z. and X.
 407 L. performed the experiments. X. L. collected and analyzed the data. X. L. and X.T.
 408 wrote the manuscript. All authors contributed to preparation of the paper and
 409 approved the final contributions.

410 The authors have declared that no conflict of interest exists.

References

- Adams, P.D., Afonine, P.V., Bunkoczi, G., Chen, V.B., Davis, I.W., Echols, N., Headd, J.J., Hung, L.W., Kapral, G.J., Grosse-Kunstleve, R.W., *et al.* (2010). PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta crystallographica Section D, Biological crystallography* *66*, 213-221.
- Anzai, N., Deval, E., Schaefer, L., Friend, V., Lazdunski, M., and Lingueglia, E. (2002). The multivalent PDZ domain-containing protein CIPP is a partner of acid-sensing ion channel 3 in sensory neurons. *The Journal of biological chemistry* *277*, 16655-16661.
- Anzai, N., Miyazaki, H., Noshiro, R., Khamdang, S., Chairoungdua, A., Shin, H.J., Enomoto, A., Sakamoto, S., Hirata, T., Tomita, K., *et al.* (2004). The multivalent PDZ domain-containing protein PDZK1 regulates transport activity of renal urate-anion exchanger URAT1 via its C terminus. *The Journal of biological chemistry* *279*, 45942-45950.
- Bard, L., Sainlos, M., Bouchet, D., Cousins, S., Mikasova, L., Breillat, C., Stephenson, F.A., Imperiali, B., Choquet, D., and Groc, L. (2010). Dynamic and specific interaction between synaptic NR2-NMDA receptor and PDZ proteins. *Proceedings of the National Academy of Sciences of the United States of America* *107*, 19561-19566.
- Bartlett, T.E., Bannister, N.J., Collett, V.J., Dargan, S.L., Massey, P.V., Bortolotto, Z.A., Fitzjohn, S.M., Bashir, Z.I., Collingridge, G.L., and Lodge, D. (2007). Differential roles of NR2A and NR2B-containing NMDA receptors in LTP and LTD in the CA1 region of two-week old rat hippocampus. *Neuropharmacology* *52*, 60-70.
- Beique, J.C., Lin, D.T., Kang, M.G., Aizawa, H., Takamiya, K., and Huganir, R.L. (2006). Synapse-specific regulation of AMPA receptor function by PSD-95. *Proceedings of the National Academy of Sciences of the United States of America* *103*, 19535-19540.
- Bredt, D.S., and Nicoll, R.A. (2003). AMPA receptor trafficking at excitatory synapses. *Neuron* *40*, 361-379.
- Chen, G., Chen, P., Tan, H., Ma, D., Dou, F., Feng, J., and Yan, Z. (2008). Regulation of the NMDA receptor-mediated synaptic response by acetylcholinesterase inhibitors and its impairment in an animal model of Alzheimer's disease. *Neurobiology of aging* *29*, 1795-1804.
- Chen, G., Kittler, J.T., Moss, S.J., and Yan, Z. (2006). Dopamine D3 receptors regulate GABAA receptor function through a phospho-dependent endocytosis mechanism in nucleus accumbens. *The Journal of neuroscience : the official journal of the Society for Neuroscience* *26*, 2513-2521.
- Durand, C.M., Perroy, J., Loll, F., Perrais, D., Fagni, L., Bourgeron, T., Montcouquiol, M., and Sans, N. (2012). SHANK3 mutations identified in autism lead to modification of dendritic spine morphology via an actin-dependent mechanism. *Molecular psychiatry* *17*, 71-84.
- Elias, G.M., Elias, L.A., Apostolides, P.F., Kriegstein, A.R., and Nicoll, R.A. (2008). Differential trafficking of AMPA and NMDA receptors by SAP102 and PSD-95 underlies synapse development. *Proceedings of the National Academy of Sciences of the United States of America* *105*, 20953-20958.
- Elias, G.M., and Nicoll, R.A. (2007). Synaptic trafficking of glutamate receptors by MAGUK scaffolding proteins. *Trends in cell biology* *17*, 343-352.
- Etherton, M.R., Blaiss, C.A., Powell, C.M., and Sudhof, T.C. (2009). Mouse neurexin-1alpha deletion causes correlated electrophysiological and behavioral changes consistent with cognitive impairments. *Proceedings of the National Academy of Sciences of the United States of America* *106*, 17998-18003.
- Etherton, M.R., Tabuchi, K., Sharma, M., Ko, J., and Sudhof, T.C. (2011). An autism-associated point

454 mutation in the neuroligin cytoplasmic tail selectively impairs AMPA receptor-mediated synaptic
455 transmission in hippocampus. *The EMBO journal* 30, 2908-2919.

456 Feng, W., and Zhang, M. (2009). Organization and dynamics of PDZ-domain-related supramodules in
457 the postsynaptic density. *Nature reviews Neuroscience* 10, 87-99.

458 Gianni, S., Walma, T., Arcovito, A., Calosci, N., Bellelli, A., Engstrom, A., Travaglini-Allocatelli, C.,
459 Brunori, M., Jemth, P., and Vuister, G.W. (2006). Demonstration of long-range interactions in a PDZ
460 domain by NMR, kinetics, and protein engineering. *Structure (London, England : 1993)* 14, 1801-1809.

461 Hruska-Hageman, A.M., Wemmie, J.A., Price, M.P., and Welsh, M.J. (2002). Interaction of the synaptic
462 protein PICK1 (protein interacting with C kinase 1) with the non-voltage gated sodium channels BNC1
463 (brain Na⁺ channel 1) and ASIC (acid-sensing ion channel). *The Biochemical journal* 361, 443-450.

464 Kim, C.H., Chung, H.J., Lee, H.K., and Huganir, R.L. (2001). Interaction of the AMPA receptor subunit
465 GluR2/3 with PDZ domains regulates hippocampal long-term depression. *Proceedings of the National*
466 *Academy of Sciences of the United States of America* 98, 11725-11730.

467 Kim, E., and Sheng, M. (2004). PDZ domain proteins of synapses. *Nature reviews Neuroscience* 5,
468 771-781.

469 Klein, Z.A., Takahashi, H., Ma, M., Stagi, M., Zhou, M., Lam, T.T., and Strittmatter, S.M. (2017). Loss of
470 TMEM106B Ameliorates Lysosomal and Frontotemporal Dementia-Related Phenotypes in
471 Progranulin-Deficient Mice. *Neuron* 95, 281-296.e286.

472 Lipinski, S., Grabe, N., Jacobs, G., Billmann-Born, S., Till, A., Hasler, R., Aden, K., Paulsen, M., Arlt, A.,
473 Kraemer, L., *et al.* (2012). RNAi screening identifies mediators of NOD2 signaling: implications for
474 spatial specificity of MDP recognition. *Proceedings of the National Academy of Sciences of the United*
475 *States of America* 109, 21426-21431.

476 Liu, Q.S., Pu, L., and Poo, M.M. (2005). Repeated cocaine exposure in vivo facilitates LTP induction in
477 midbrain dopamine neurons. *Nature* 437, 1027-1031.

478 Parsons, M.P., and Raymond, L.A. (2014). Extrasynaptic NMDA receptor involvement in central
479 nervous system disorders. *Neuron* 82, 279-293.

480 Phillips, G.R., Huang, J.K., Wang, Y., Tanaka, H., Shapiro, L., Zhang, W., Shan, W.S., Arndt, K., Frank, M.,
481 Gordon, R.E., *et al.* (2001). The presynaptic particle web: ultrastructure, composition, dissolution, and
482 reconstitution. *Neuron* 32, 63-77.

483 Sala, C., Piech, V., Wilson, N.R., Passafaro, M., Liu, G., and Sheng, M. (2001). Regulation of dendritic
484 spine morphology and synaptic function by Shank and Homer. *Neuron* 31, 115-130.

485 Schultz, J., Hoffmuller, U., Krause, G., Ashurst, J., Macias, M.J., Schmieder, P., Schneider-Mergener, J.,
486 and Oshkinat, H. (1998). Specific interactions between the syntrophin PDZ domain and voltage-gated
487 sodium channels. *Nature structural biology* 5, 19-24.

488 Shangguan, Y., Xu, X., Ganbat, B., Li, Y., Wang, W., Yang, Y., Lu, X., Du, C., Tian, X., and Wang, X. (2017).
489 CNTNAP4 Impacts Epilepsy Through GABAA Receptors Regulation: Evidence From Temporal Lobe
490 Epilepsy Patients and Mouse Models. *Cerebral cortex (New York, NY : 1991)*, 1-14.

491 Shen, G., Van Sickle, B.J., and Tietz, E.I. (2010). Calcium/calmodulin-dependent protein kinase II
492 mediates hippocampal glutamatergic plasticity during benzodiazepine withdrawal.

493 *Neuropsychopharmacology : official publication of the American College of*
494 *Neuropsychopharmacology* 35, 1897-1909.

495 Stenzel, N., Fetzer, C.P., Heumann, R., and Erdmann, K.S. (2009). PDZ-domain-directed basolateral
496 targeting of the peripheral membrane protein FRMPD2 in epithelial cells. *Journal of cell science* 122,
497 3374-3384.

498 Sultan, S., Li, L., Moss, J., Petrelli, F., Casse, F., Gebara, E., Lopatar, J., Pfrieger, F.W., Bezzi, P.,
499 Bischofberger, J., *et al.* (2015). Synaptic Integration of Adult-Born Hippocampal Neurons Is Locally
500 Controlled by Astrocytes. *Neuron* 88, 957-972.
501 Tang, B., Luo, D., Yang, J., Xu, X.Y., Zhu, B.L., Wang, X.F., Yan, Z., and Chen, G.J. (2015). Modulation of
502 AMPA receptor mediated current by nicotinic acetylcholine receptor in layer I neurons of rat
503 prefrontal cortex. *Scientific reports* 5, 14099.
504 Valtschanoff, J.G., and Weinberg, R.J. (2001). Laminar organization of the NMDA receptor complex
505 within the postsynaptic density. *The Journal of neuroscience : the official journal of the Society for*
506 *Neuroscience* 21, 1211-1217.
507 Zhang, C., Wan, X., Zheng, X., Shao, X., Liu, Q., Zhang, Q., and Qian, Y. (2014). Dual-functional
508 nanoparticles targeting amyloid plaques in the brains of Alzheimer's disease mice. *Biomaterials* 35,
509 456-465.
510 Zucker, R.S., and Regehr, W.G. (2002). Short-term synaptic plasticity. *Annual review of physiology* 64,
511 355-405.

512

513 Figures

514

515 Fig 1. Expression patterns of the FRMPD2 protein.

516 A, FRMPD2 protein is located in the mouse brain, eyes, testes and lungs. B, The
517 brain-specific expression of the FRMPD2 protein was revealed in adult mouse tissue
518 homogenates. C, The FRMPD2 protein has a widespread distribution pattern in adult
519 mouse brain slices. Bar, 500 μ m. D, FRMPD2 was present in MAP2-positive
520 neuronal dendrites but not in GFAP-positive glial cells as determined by

immunofluorescence staining. Bar, 40 μ m. E, FRMPD2 co-localized with the postsynaptic marker PSD95 but not with the presynaptic marker VGLUT1 in the mouse hippocampus and cortex. Bar, 40 μ m. F, Immunoblot of the synapse fraction (Syn) showing that FRMPD2 was preferentially distributed in the postsynaptic fraction (Post) rather than in the presynaptic (Pre) or non-synaptic synaptosomal proteins (NSSP) fraction. Anti-synaphophysin, anti-syntaxin 1a and anti-PSD95 were used as markers for ultrasynaptic fraction to verify that the purity of the preparation was higher than 90%. The blots were representative of 4-6 analyses.

529

530

Fig 2. Alteration of FRMPD2 protein levels in the hippocampus by LV injection.

A, Lentiviral vector (LV)-encoding green fluorescent protein (GFP) was detected in the mouse hippocampus following the injection of LV. Bar, 250 μ m. B, Hippocampal FRMPD2 protein expression levels were detected in FRMPD2 knockdown or overexpression mice on the 2 and 6 weeks after LV injection. Data were shown as mean $\pm$ SD and analyzed by Student's t test, n=5 mice, *p< 0.05.

537

538

539 **Fig 3. Effects of FRMPD2 on electrophysiology of CA1 neurons in mouse**
540 **hippocampal brain slices.**

541 A, The frequency of spontaneous AP was compared after FRMPD2 knockdown or
542 overexpression. Bar, 1 s, 25 mV. B, C, FRMPD2 had no significant effect on the
543 amplitude or frequency of AMPAR mEPSCs (B) and mIPSCs (C). Bar, 1 s, 20 pA.
544 Data were shown as mean±SD and analyzed by Student's *t* test, n=6-7 neurons of 3
545 mice, ***p*< 0.01.

546

547

Fig 4. Effects of FRMPD2 on NMDA receptor-mediated excitatory synaptic transmission.

A, Quantitative analysis of amplitude and frequency of NMDAR mEPSCs in CA1 pyramidal cells of hippocampus after FRMPD2 knockdown or overexpression. Bar, 1 s, 20 pA. B, The input-output curves of NMDAR eEPSCs were recorded with gradual increasing intensities. Bar, 25 ms, 50 pA. C, Amplitude of NMDAR, AMPAR component eEPSCs and AMPA/NMDA ratio were compared. Bar, 25 ms, 25 pA. D, Quantitative analysis of the amplitude of GluN2A- and GluN2B-sensitive eEPSCs. Bar, 25 ms, 25 pA. E, Knocking down or overexpressing FRMPD2 did not alter the paired-pulse ratio (PPR) values. Bar, 25 ms, 50 pA. Data of were presented as the mean $\pm$ SD or median with interquartile range. Data of A, C, D, E, were analyzed by Student's *t* test, data of B was analyzed by repeated measures ANOVA, n=5-7 neurons from 3 mice. **p<0.01. *p<0.05.

562

563 **Fig 5. Effects of FRMPD2 anchoring GluN2A at synapses.**

564 A, Quantitative analysis of GluN2A, GluN2B and GluN1 expressions of total

565 hippocampus tissue and synaptic homogenates after FRMPD2 knockdown or

566 overexpression. The data were shown as the mean $\pm$ SD and analyzed by Student's *t*

567 test, **p* < 0.05. B, Immunofluorescence staining in the mouse hippocampus CA1 area

568 showed that FRMPD2 co-localizes with GluN2A at the postsynaptic density, which

569 was marked by PSD95. Bar, 40 μ m. C, FRMPD2 and GluN2A, GluN2B and GluN1

570 were co-immunoprecipitated from mouse hippocampal tissues. D, GST pull-down
571 assay showing that the GST-PDZ2 fusion protein but not the PDZ1 or PDZ3 fusion
572 proteins pulled down endogenous GluN2A from mouse hippocampal tissues, and
573 exogenous full-length GFP-GluN2A from HEK293 cells but did not pull down the
574 deletion variant GFP-GluN2A from HEK293 cells transfected with GFP-GluN2A that
575 lacked ten amino acids at the C-terminus. E-G, The ability of the isolated FRMPD2
576 PDZ2 domain to bind to the GluN2A and GluN2B C-terminus was measured using
577 SPR. Peptides sequences of C-termini of wild type GluN2A (E), wild type GluN2B
578 (F) and mutant GluN2A (G) were used in this study. Sensorgrams were shown to
579 illustrate the binding of increasing concentrations of the PDZ2 domain to peptides
580 and steady-state equilibrium responses from the sensorgrams were shown plotted
581 against the injected PDZ2 concentrations. K_D : dissociation constant.

582

583

584

585 **Fig 6. Crystal structure of FRMPD2 PDZ2 domain.**

586 A, The crystal structure of a compact globular fold in the ligand-free FRMPD2 PDZ2
 587 domain (PDB ID: 5ZDS) was shown. B, The crystal structure of the FRMPD2 PDZ2
 588 domain (green) was shown aligned to that of the PDZ2-peptide template structure
 589 (cyan and magenta) (PDB ID: 1VJ6). C, A template of PDZ2-peptide (cyan and
 590 magenta) interaction (PDB ID: 1VJ6). D, The binding pocket alignment between
 591 FRMPD2 PDZ2 crystal structure (green) and PDZ2 template (cyan).
 592
 593

594 **Table 1. Data collection and refinement statistics of PDZ2 of FRMPD2.**

	PDZ2
Data collection	
Wavelength(Å)	0.9786
Resolution range(Å)	50-1.58 (1.64-1.58) ^a
Space group	P 2 ₁ 2 ₁ 2
Unit cell dimensions	
a, b, c(Å)	32.200, 48.179, 56.845
α, β, γ(°)	90, 90, 90
No. of reflections	80345
No. unique reflections	12699 (1248)
Multiplicity	6.3 (6.4)
Completeness (%)	99.7 (100)
Mean I/σ (I)	22.52(7.70)
R-meas	0.065(0.268)
Refinement	
Resolution range(Å)	50-1.8 (1.86-1.8)
No. of reflections	8610 (849)
No. of reflections used for R-free	860 (85)
R-work	0.1980 (0.2202)
R-free	0.2347 (0.2630)
No. of non-hydrogen atoms	810

Macromolecules	658
Protein residues	88
R.m.s.d.	
Bond lengths(Å)	0.005
Bond angles(°)	0.83
Ramachandranplot(%)	
Allowed regions	1.1
Outliers	0
Average B-factor	13.71
Macromolecules	11.25
Solvent	24.37

595 ^aStatistics for the highest-resolution shell are shown in parentheses. No., number,
596 r.m.s.d., root mean squared deviation.
597