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1 Astroglial-Kir4.1 in Lateral Habenula Drives Neuronal Bursts to

2 Mediate Depression

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29 **Keywords:** depression, lateral habenula, bursting, Kir4.1, inward-rectifying potassium
30 channel, astrocyte, neuron-glia interaction, potassium buffering, firing mode

32

Summary

33

34 **Enhanced bursting activity of the lateral habenula (LHb) neurons is essential in driving**
35 **depressive-like behaviors, but the cause of this increased bursts remained unknown.**
36 **Here using high-throughput quantitative proteomic screen, we identified an astroglial**
37 **potassium channel, Kir4.1, to be upregulated in the LHb of animal models of depression.**
38 **Kir4.1 in the LHb shows a distinct expression pattern on the astrocytic membrane**
39 **processes tightly wrapping around the neuronal soma. Electrophysiology and modeling**
40 **data demonstrate that the level of Kir4.1 on astrocytes tightly regulates the degree of**
41 **membrane hyperpolarization and the amount of burst activity of LHb neurons.**
42 **Astrocyte-specific gain and loss of Kir4.1 functions in the LHb bidirectionally regulate**
43 **neuronal bursting and depressive-like symptoms. Together, these results reveal a new**
44 **form of glial-neural interaction in setting neuronal firing mode in a devastating**
45 **psychiatric disease, and discover the therapeutic potential of targeting LHb Kir4.1 for**
46 **treating major depression.**

47

48 A major breakthrough in neuroscience has been the discovery that astrocytes intimately interact
49 with neurons to support and regulate a series of essential functions to foster brain information
50 processing¹⁻⁷. A wealth of investigations has focused on the astroglial-neural interactions at the
51 tripartite synapses, where astrocyte processes tightly wrap around pre- and post-synaptic sites⁸.
52 In contrast, not as much attention has been placed on astroglial-neural interaction in proximity to
53 neuronal soma^{9,10}. Particularly, how astrocytes regulate intrinsic firing patterns of neurons, and
54 what structural basis may underlie this regulation, are much less explored.

55

56 Despite the surging interest of lateral habenula (LHb) in negative emotion¹¹⁻²⁰, only limited
57 attention has been given to astrocytes and their potential roles in LHb hyperfunction in
58 depression²¹. In the accompanying paper, we demonstrate that bursting activity of LHb
59 neurons are greatly enhanced in animal models of depression. LHb burst drives depressive-
60 like behaviors and is a prominent target of the rapid antidepressant ketamine. However, the
61 cause of this enhanced burst of LHb neurons remains unsolved.

62

63 **Results**

64 **Kir4.1 is upregulated in LHb of animal models of depression**

65 In an unbiased, high-throughput, quantitative proteomic screening to compare habenular
66 protein expression of congenitally learned helpless (cLH) rats²² and wild-type SD rats, we
67 identified Kir4.1 to be highly upregulated in the LHb of cLH rats¹¹ (1.69-fold of wild-type
68 control, p = 0.02, Student's *t*-test, Fig. 1a). Western blot analysis confirmed that Kir4.1 had a
69 significant increase (1.44-fold, p = 0.009, Paired *t*-test) in the membrane protein extraction of
70 cLH habenulae (Fig. 1b). In contrast, another astrocyte-specific protein, glial fibrillary acidic
71 protein (GFAP), did not show any change in expression (Extended Data Fig. 1a), indicating
72 there was no astrogliosis.

73

74 To test whether Kir4.1 upregulation is universal in depression, we examined an additional rat
75 model of depression, the LPS (lipopolysaccharide)-induced depression²³. One week of LPS
76 injection (0.5mg/kg, i.p.) in 3-month-old Wistar rats was sufficient to cause strong
77 depressive-like phenotype in the forced swim test (FST, Extended Data Fig. 2e)²³ and sucrose
78 preference test (SPT)²³. The Kir4.1 level was also significantly increased in the LPS-induced
79 rats (1.87-fold, p < 0.0001, Paired *t*-test, Fig. 1c). Quantitative real-time PCR revealed an
80 increase (1.2-fold, p = 0.015, Paired *t*-test) of Kir4.1 mRNA level in cLH habenulae (Fig. 1d),
81 suggesting that at least part of the protein level change is due to transcriptional upregulation.

82

83 **Age-dependent increase of LHb astrocytic Kir4.1 current and depression onset in cLH
84 rats**

85 Kir4.1 is a principal component of the glial Kir channel and is largely responsible for
86 mediating the K⁺ conductance and setting the resting membrane potential (RMP) of
87 astrocytes²⁴. To confirm that Kir4.1 function is indeed upregulated, we performed whole-cell
88 patch clamp onto the astrocytes in brain slices made from the LHb of cLH or SD rats.
89 Astrocytes were distinguished from neurons by their small (5–10 µm) oval shaped somata
90 and electrophysiological features including a relatively hyperpolarized RMP (-74 ± 1 mV), a
91 low input resistance R_{in} (47 ± 6 MΩ), a linear I–V relationship and an absence of action
92 potentials in response to depolarizing current injections (Extended Data Fig. 2a-d)²⁵⁻²⁷.
93 Biocytin filling and absence of NeuN co-staining confirmed that cells fitting the above
94 criteria were indeed astrocytes (Extended Data Fig.3). We then bath applied Ba²⁺ (BaCl₂, 100
95 µM), which selectively blocks Kir channels at sub-mM concentrations, to isolate Kir4.1
96 current²⁵ (Extended Data Fig. 2a, c). The Ba²⁺-sensitive current displayed a reversal potential
97 close to E_k (-90mV)^{25,28} (Fig. 1e, f), indicating that it represents the K⁺ conductance. We

98 found that Ba²⁺-sensitive currents in LHb astrocytes were almost doubled in cLH rats (Fig.
99 1e), as well as in LPS-treated rats (Extended Data Fig. 2f), at the age of P60-90.

100
101 Interestingly, the increase of Kir4.1 current and protein level was not obvious at P30 (Fig. 1f,
102 Extended data Fig. 1b). At this age, cLH rats did not yet show depressive-like phenotypes in
103 both the FST (Fig. 1g) and learned helpless test (LHT, Fig. 1h), suggesting that the
104 upregulation of Kir4.1 level is concomitant with the developmental onset of the depressive-
105 like symptoms.

106
107 **Kir4.1 are expressed on astrocytic processes tightly wrapping around neuronal soma**
108 As an inwardly rectifying K⁺ channel, Kir4.1 has been strongly implicated in buffering
109 excess extracellular K⁺ in tripartite synapses^{24,29-31}. Conventional model of K⁺ buffering
110 suggests Kir4.1 to be highly expressed in astrocytic endfeet surrounding synapses³²⁻³⁴.
111 Surprisingly, with immunohistochemistry co-labeling, Kir4.1 staining in LHb appeared to
112 overlap with the neuronal marker NeuN at low magnification (20X, Extended Data Fig. 4a),
113 although in the same brain slice Kir4.1 staining patterns in hippocampus were typical
114 astrocytic-looking (Extended Data Fig. 4b). However, higher magnification imaging with
115 single layer scanning (0.76μm per layer) revealed that Kir4.1 staining enveloped NeuN
116 signals (Fig. 2a). To confirm that Kir4.1 indeed locates within astrocytes but not neurons in
117 LHb, we separately knocked it out in either neurons or astrocytes by injecting AAV virus
118 expressing the cre recombinase under either the neuronal promoter CaMKII or glial promoter
119 GFAP (gfaABC1D)^{26,35} into Kir4.1^{f/f} floxed mice²⁵. The staining of Kir4.1 remained intact
120 with neuronal knock-out, but was completely eliminated with astrocytic knock-out (Fig. 2b).
121 Electron microscopy imaging revealed that Kir4.1-positive gold particles were distributed
122 encircling the membrane of neuronal cell bodies (Fig. 2c, Extended Data Fig. 5a, b), as well

123 as in the synapses (Extended Data Fig. 5d). Consistently, whole-cell-patch recordings showed
124 that Ba²⁺-sensitive currents were absent in neurons but abundant in astrocytes in the LHb (Fig.
125 2d, Extended Data Fig. 6). Together these results suggest that Kir4.1 is mainly expressed in
126 astrocytic processes tightly wrapping around neuronal soma and synapses in the LHb.

127

128 **Overexpression of kir4.1 in LHb astrocytes increases neuronal bursts and causes strong
129 depressive-like behaviors**

130 To test the consequence of Kir4.1 upregulation in LHb, we used adeno-associated viruses of
131 the 2/5 serotype (AAV2/5) that preferentially target astrocytes²⁶ together with the human
132 GFAP (gfaABC1D) promoter^{26,35} to deliver GFP-tagged Kir4.1 channels (AAV-
133 GFAP::Kir4.1) or AAV-GFAP::GFP as a control (Fig. 3a). 14 days after bilateral injection in
134 the LHb at P50, AAV2/5-mediated viral transfection led to Kir4.1 and GFP expression in
135 astrocytes throughout the LHb (Fig. 3b). The specificity of the viral infection in astrocytes
136 was verified by co-immunostaining of NeuN and GFP: only 0.3% NeuN+ cells (n = 2668)
137 were infected by this virus (Extended Data Fig.7). We made whole-cell recordings from
138 either astrocytes or neurons surrounding the viral-transfected astrocytes in coronal LHb slices
139 (Fig. 3c-e). The RMPs of both astrocytes and neurons were more hyperpolarized (Fig. 3d, e)
140 and the percentage of bursting neurons were significantly higher (Fig.3f) in mice infected
141 with AAV-GFAP::Kir4.1 than with AAV-GFAP::GFP.

142

143 We then assayed depressive-like phenotypes and found that mice with AAV-GFAP::Kir4.1
144 infection in the LHb displayed severe depressive-like behaviors, including increased
145 immobile duration ($p = 0.0002$, Unpaired *t*-test) and decreased latency to immobility ($p =$
146 0.001 , Unpaired *t*-test) in FST (Fig. 3g), and decreased sucrose preference in the SPT ($p <$

147 0.0001, Unpaired *t*-test, Fig. 3h), while the general locomotion was unchanged (Extended
148 Data Fig. 8a-b).

149

150 **Kir4.1-mediated K buffering regulates neuronal RMP and bursting activity**

151 How does an astrocytic potassium channel regulate RMP and burst firing of the LHb neuron?
152 We hypothesize that within the highly confined extracellular space between neuronal soma
153 and Kir4.1-positive astrocytic processes (Fig. 2), the constantly-released K⁺ from
154 intrinsically active LHb neurons is quickly cleared by astrocytes through a Kir4.1-dependent
155 mechanism. Accordingly, we predict that blockade of Kir4.1 should compromise K⁺ spatial
156 buffering, resulting in increased extracellular K⁺ (Kout), and according to Nernst Equation,
157 depolarized neuronal RMPs (modeled in Extended Data Fig. 9). Consistent with this
158 prediction, blocking Kir4.1 with BaCl₂ depolarized the RMPs of LHb neurons, after about 10
159 min bath perfusion of BaCl₂ (Fig. 4a). The amount of changes in RMP positively correlated
160 with the original firing rates of neurons (Fig. 4b), indicating that the more active the neuron is,
161 the larger contribution the K⁺ buffering to its RMP. Similar amount of RMP change was
162 induced when BaCl₂ was applied in presence of synaptic transmission blockers (picrotoxin,
163 NBQX and AP5, Extended Data Fig. 10), suggesting that Kir4.1-dependent regulation of
164 LHb neuronal RMP occurs mostly at neuronal cell bodies instead of at synaptic sites.
165 Consequent to the RMP change, perfusion of BaCl₂ caused a dramatic increase of firing
166 frequency until the neuron reached a sustained plateau of a tetanus response and stopped
167 firing (Fig. 4c).

168

169 Vice versa, upregulation of Kir4.1 or stop neuronal firing should decrease extracellular K⁺
170 (Kout) and hyperpolarize neuronal RMPs. Indeed, overexpression of Kir4.1 in astrocytes (Fig.
171 3e) or blocking neuronal APs by TTX (Fig. 4d, e) both caused hyperpolarization of LHb

172 neurons. Overexpression of Kir4.1 in astrocytes also increased neuronal bursts (Fig. 3g).
173 Finally, to assess a causal relationship between Kout and firing mode, we made current-
174 clamp recordings of LHb neurons while lowering Kout from 2.75mM to 1.4mM (Fig. 4f).
175 This led to lowered neuronal RMP by 13.7 +/- 0.5mV and a direct shift of originally tonic-
176 firing neurons (8 out of 15) into bursting mode (Fig. 4g). Consequently, percentage of
177 bursting neurons was increased from 8% to 23% ($p < 0.0001$, Chi-square test, Fig. 4h). In
178 summary, by increasing astrocytic Kir4.1 expression or decreasing the extracellular K+
179 concentration, we were able to phenocopy in WT animals several key neuronal properties
180 observed in the LHb of animal models of depression, namely hyperpolarized RMPs and
181 enhanced bursts. These results indicate that enhanced capacity of extracellular K clearance
182 due to Kir4.1 overexpression may underlie the neuronal hyperpolarization required for burst
183 initiation.

184

185 **Loss-of-function of Kir4.1 in LHb decreases neuronal bursting and rescues depressive-
186 like phenotypes**

187 Next, to determine whether loss-of-function of Kir4.1 in the LHb may reduce neuronal bursts
188 and reverse depressive phenotypes, we devised two strategies, by using AAV2/5 viral vectors
189 to express either a short hairpin RNA (shRNA) to knock down the level of Kir4.1, or a
190 dominant negative construct to block its function in the LHb of cLH rats (Fig. 5a). We tested
191 six shRNAs specifically targeting the Kir4.1 transcript in cell culture and chose the one with
192 most efficient knock-down efficiency (Fig. 5b and Extended Data Fig. 11a) for viral package.
193 To avoid an off-target effect of shRNA, we also used a dominant-negative form of Kir4.1,
194 dnKir4.1, containing a GYG to AAA point mutation at the channel pore, which blocks K+
195 channels³⁶ (Extended Data Fig. 11b, 12, Fig. 5c). We first examined the effect of Kir4.1-
196 shRNA on glial and neural electrophysiological properties. In astrocytes infected with the

197 AAV-H1::Kir4.1-shRNA, we observed a dramatic change of I-V relation (Fig. 5d), a 32 mV
198 depolarization compared with non-infected astrocytes ($p < 0.0001$, One-way ANOVA) and
199 41mV depolarization compared with ctrl-shRNA-infected astrocytes ($p < 0.0001$, One-way
200 ANOVA, Fig. 5e), consistent with the literature that Kir4.1 is majorly responsible for setting
201 the astrocytic RMPs²⁵. In neurons infected with the AAV-H1::Kir4.1-shRNA, the RMPs did
202 not differ from neighboring non-infected neurons (because neurons do not express Kir4.1
203 endogenously, Fig. 5f). However, RMPs of neighboring LHb neurons from AAV-H1::Kir4.1-
204 shRNA-infected brain slices were overall more depolarized than RMPs of those from AAV-
205 H1::Ctrl-shRNA-infected rats (-43 ± 2mV vs. -53 ± 2.7mV, $p = 0.006$, One-way ANOVA,
206 Fig. 5f), suggesting that knock-down of Kir4.1 in astrocytes had a global impact on RMPs of
207 neighboring neurons. Similar effects were observed in AAV-GFAP::dnKir4.1 infected LHb
208 slices (Extended Data Fig. 11c-f). Most importantly, bursting activity in the LHb of cLH rats
209 were significantly eliminated by AAV-H1::Kir4.1-shRNA (Fig. 5g) and AAV-
210 GFAP::dnKir4.1 (Extended Data Fig. 11f) infection.

211
212 Behaviorally, infection of AAV-H1::Kir4.1-shRNA or AAV-GFAP::dnKir4.1 had a
213 pronounced effect on rescuing the depressive-like phenotypes of cLH rats in three depression
214 paradigms: reducing the immobility time ($p = 0.01$, Unpaired *t*-test, Fig. 5h) and increasing
215 latency to immobility ($p = 0.0018$, Unpaired *t*-test, Fig. 5h) in FST, increasing bar pressing
216 number in the LHT ($p = 0.0004$, Unpaired *t*-test, Fig. 5i, j), and increasing the sucrose
217 preference score in SPT ($p = 0.04$, Unpaired *t*-test, Fig. 5k). The behavior scores in LHT
218 clearly correlated with those in FST (Fig. 5l).

219
220 Here we discover an important function of Kir4.1 at the highly specialized neuron-glia
221 interface in the LHb in regulating neuronal RMP and firing pattern. During depression, the

222 upregulation of Kir4.1 may cause enhanced capacity of extracellular K clearance, leading to a
223 decrease of K_{out} and neuronal hyperpolarization (Fig. 5m). As demonstrated in our
224 accompanying manuscript, neuronal hyperpolarization may de-inactivate T-type voltage-
225 sensitive calcium channels (T-VSCCs) which in turn initiate NMDAR-dependent bursts to
226 cause a stronger suppression of the downstream monoaminergic centers (Fig. 5m). These
227 results may inspire the development of novel treatments of major depression targeting on
228 maladaptive neuron-glia interactions in the LHb.

229

230

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232

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243

244 **Author contributions**

245 H.H. and Y.C. designed the study. Y.C. performed the *in vitro* patch recordings; Y.Y.
246 performed the biochemistry and immunohistochemistry experiments; Y.C., Y.Y., Y.D. and
247 K.S. performed viral injections and behavioral experiments; Z.N., A.F. and H.B. established

248 the biophysical model; S.M. assisted cell culture experiments; G.C. and S.W. conducted the
249 EM experiments; Y.S. contributed Kir4.1^{f/f} floxed mice; S.T. and Y.L. constructed plasmids.
250 H.H. wrote the manuscript with the assistance of Y.C., Y.Y. and Z.N..
251

252 **Methods**

253

254 **Animals.** Male cLH rats (3-4 weeks or 8-12 weeks of age) and age-matched male Sprague
255 Dawley rats (SLAC Laboratory Animal Co., Shanghai) were used. The cLH rats were
256 screened by learned helpless test^{1,2} for breeding as previously described¹. Male Wistar rats
257 (SLAC Laboratory Animal Co., Shanghai, 12 weeks) were used for establishing the LPS-
258 induced depressive-like rat model. Male adult (7-8 weeks of age) C57BL/6 mice (SLAC) or
259 Kir4.1^{f/f} floxed mice (originally obtained from Dr. Ken McCarthy at University of North
260 Carolina) were used for virus injection the immunohistochemistry experiments. Animals
261 were group-housed two/cage for rats and four/cage for mice under a 12-h light-dark cycle
262 (light on from 7 a.m. to 7 p.m.) with free access to food and water *ad libitum*. All animal
263 studies and experimental procedures were approved by the Animal Care and Use Committee
264 of the animal facility at Zhejiang University.

265

266 **Western Blot.** The habenular membrane fraction and whole protein was extracted as
267 previously described¹¹. Animals were anesthetized using 10% chloral hydrate, and habenular
268 tissue was quickly dissected from the brain and homogenized in lysis buffer (320 mM
269 sucrose, 4 mM HEPES pH7.4, 1 mM MgCl₂ and 0.5 mM CaCl₂, 5 mM NaF, 1 mM Na₃VO₄,
270 EDTA-free, Protease Inhibitor cocktail tablets (Roche) on ice. The lysis buffer used for
271 extracting the total protein of HEK293TN cell contained 50mM Tris (pH 7.4), 150mM NaCl,
272 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and Protease Inhibitor cocktail tablets
273 (Roche). After protein concentration measurement by BCA assay, 10~20μg proteins for each
274 lane was separated on a 10% SDS-PAGE gel and transferred for western blot analysis. Anti-
275 Kir4.1 (1:1000, Alomone labs), anti-GFAP (1:1000, Sigma) and anti-tubulin (1:5000, Bio-
276 Rad) antibodies, and high sensitive ECL reagent (GE Healthcare) were used. All the bands
277 were analyzed with Quantity one or Image J.

278 **Immunohistochemistry.** Animals were anesthetized using 10% chloral hydrate, and then
279 perfused transcardially with ice-cold PBS (pH 7.4) followed by 4% paraformaldehyde. After
280 overnight post fix in 4% paraformaldehyde solution, brains were cryoprotected in 30%
281 sucrose for 1 day (for mice) or 3 days (for rats). Coronal sections (40 μ m) were cut on a
282 microtome (Leica) and collected in PBS and stored at 4°C for further using. The antibodies
283 used were rabbit anti-Kir4.1 extracellular peptide (1:200, Alomone labs), mouse anti-GFAP
284 (1:500, Sigma), mouse anti-NeuN (1:500, Millipore), Rabbit anti NeuN (1:500, Millipore),
285 mouse anti-S100b (1:500, Sigma), chicken anti-GFP (1:1000, Abcam), Mouse anti-Flag
286 (1:1000, Beyotime), Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 488 goat anti-chicken
287 IgG, Alexa Fluor 546 goat anti-mouse IgG (all 1:1000, Invitrogen). Specifically, for Kir4.1
288 staining, the rabbit anti-Kir4.1 extracellular peptide antibody was incubated for 48-72 h and
289 the other primary antibodies were incubated for 36-48 h. For the antibody absorption
290 experiments, the rabbit anti-Kir4.1 extracellular peptide antibody was pre-adsorbed with the
291 Kir4.1 antigen by mixing at the weight ratio of 1:2 for 24 h. Slices were counterstained with
292 Hoechst in the final incubation step to check the injection site. Fluorescent image acquisition
293 was performed with an Olympus Fluoview FV1000 confocal microscope and a Nikon A1
294 confocal microscope.

295

296 **Cell transfection and cell culture.** HEK293 cells (gift from Jianhong Luo) were used for the
297 electrophysiology recording and HEK293TN cells (Taitool Bioscience, China) were used for
298 western blot analysis. Cells used in this study were authenticated and checked for
299 mycoplasma contamination. The plasmids used were pAAV-Ubi-Kir4.1-2A-eGFP, pAAV-
300 Ubi-dnKir4.1 (GYG to AAA)-2A-eGFP, pAAV-CAG-eGFP, pAAV-H1-Kir4.1-shRNA-
301 CAG-eGFP and pAAV-H1-Luciferase-shRNA-CAG-eGFP. HEK293 cells were trypsinized
302 for 5 min using 0.25% trypsin (wt/vol), and replated onto glass coverslips in fresh

303 DMEM:F12 medium (Life Technologies) containing 1 mM nonessential amino acids (Gibco),
304 1 mM sodium pyruvate (Gibco), and 10% FBS (Hyclone). Cells were transfected using
305 Lipofectamine 2000 (Invitrogen) according to standard protocols, then cultured for 5-6 h in
306 well plates at 37 °C in a humid atmosphere of 5% CO₂ and 95% air. Cells were cultured for
307 another 48 h before western blot or electrophysiology experiments.

308

309 **Plasmid constructs.** The pAAV-Ubi-Kir4.1-2A-eGFP plasmid was assembled by
310 homologous recombination of AAV backbone linearized from the AAV-Ubi-CaMKII-2A-
311 eGFP plasmid¹¹ by PCR and Kir4.1 amplified from pZac2.1- gfaABC1D-EGFP-Kir4.1
312 plasmid (AddGene). The pAAV-Ubi-dnKir4.1 (GYG to AAA)-2A-eGFP plasmid was made
313 by PCR-based mutagenesis using pAAV-Ubi-Kir4.1-2A-eGFP as a backbone (Fw: 5'-
314 AAAGATGGCCGGAGCAACGTGAGAATGGAGCATATTGCT-3'; Rev: 5'-
315 GCTGATGTAGCGGAAGGCAGGCGGCAATGGTGGTCTGGGATTCGAGGGA-3'). The
316 pAAV-gfaABC1D- dnKir4.1 (GYG to AAA)-2A-EGFP was assembled by homologous
317 recombination of pZac2.1 gfaABC1D backbone linearized from the pZac2.1- gfaABC1D-
318 EGFP-Kir4.1 plasmid (AddGene) by PCR and Kir4.1dn-2A-EGFP sequence amplified from
319 pAAV-Ubi-dnKir4.1 (GYG to AAA)-2A-eGFP plasmid. The pAAV-H1-Kir4.1-shRNA-
320 CAG-eGFP was constructed using a vector (provide by Taitool Bioscience, China), which
321 contains a CAG promoter driving eGFP and a H1 promoter driving shRNA expression. We
322 designed 6 shRNA sequences by RNAi designer online software (Invitrogen) as indicated
323 below:

- 324 1) 5'-GGACGACCTTCATTGACAT-3';
325 2) 5'-GCTACAAGCTTCTGCTCTTCT-3';
326 3) 5'-GCTCTTCTGCCAACCTTAC-3';
327 4) 5'-CCGGAACCTTCCTTGCAAA-3';

328 5) 5'-GCGTAAGAGTCTCCTCATTGG-3'

329 6) 5'-GCCCTTAGTGTGCGCATTA-3'.

330 We then tested the knock-down efficiency by Western blot of Kir4.1 from HEK293TN cells
331 which were co-transfected with Flag-tagged-Kir4.1 plasmid (pAAV-CMV-betaGlobin-
332 Kir4.1-eGFP-3Flag) and each of the six shRNA plasmids. Based on our western blot result
333 (Extended Figure 11a), we chose the fifth sequence, 5'-GCGTAAGAGTCTCCTCATTGG-
334 3', for Kir4.1-shRNA virus package.

335

336 **Electron microscopic immunohistochemistry.** Four mice were deeply anesthetized with
337 1% sodium pentobarbital intraperitoneally (50 mg/kg body weight) and perfused
338 transcardially with 20ml saline, followed by 40ml ice-cold mixture of 4% paraformaldehyde
339 and 0.05% glutaraldehyde in 0.1M PB for 1 h. Brainstems were removed and postfixed by
340 immersion in the same fixative for 4 h at 4°C. Serial coronal sections of 50 µm thickness
341 were prepared with a vibratome (VT 1000S, Leica), and approximately 18-20 sections,
342 including the LHb region, were collected from each brain.

343

344 Kir4.1 was detected by the immunogold-silver staining. Briefly, sections were blocked with
345 blocking buffer (5% BSA, 5% NGS and 0.05% Triton X-100 in PBS), and then incubated
346 overnight in the primary antibodies of rabbit anti-Kir4.1 (1:200) diluted with solution
347 containing 1% BSA, 1% NGS and 0.05% Triton X-100. The secondary antibody was anti-
348 rabbit IgG conjugated to 1.4 nm gold particles (1:100, Nanoprobes, Stony Brook, NY) for 4 h.
349 After rinsing, sections were post fixed in 2% glutaraldehyde in PBS for 45 min. Silver
350 enhancement was performed in the dark with HQ Silver Kit (Nanoprobes) for visualization of
351 Kir4.1 immunoreactivity. Before and after the silver enhancement step, sections were rinsed
352 several times with de-ionized water.

353

354 Immuno-labeled sections were fixed with 0.5% osmium tetroxide in 0.1 M PB for 1 h,
355 dehydrated in graded ethanol series, then in propylene oxide, and finally flat-embedded in
356 Epon 812 between sheets of plastic. After polymerization, acrylic sheets were then peeled
357 from the polymerized resin, and flat-embedded sections were examined under the light
358 microscope. Three to four sections containing Kir4.1 immunoreactivity in the LHb were
359 selected from each section, trimmed under a stereomicroscope, and glued onto blank resin
360 stubs. Serial ultrathin sections were cut with an Ultramicrotome (Leica EM UC6, Germany)
361 using a diamond knife (Diatome, PA) and mounted on formvar-coated mesh grids (6-8
362 sections/grid). They were then counterstained with uranyl acetate and lead citrate, and
363 observed under a JEM-1230 electron microscope (JEOL LTD, Japan) equipped with CCD
364 camera and its application software (832 SC1000, Gatan, PA).

365

366 **Stereotaxic surgery and virus injection.** cLH rats (postnatal 50-60 days) were deeply
367 anesthetized by using 4% pentobarbital. Mice (postnatal 50-60 days) were deeply
368 anesthetized by ketamine (100 mg/kg of body weight) and xylazine (8 mg/kg). Animals were
369 placed on a stereotactic frame (RWD Instruments, China). A small volume of virus was
370 injected into bilateral LHb (for rats LHb: AP, -3.7 mm from bregma; ML, ±0.7 mm; DV, -
371 4.55 mm from the brain surface; for mice: AP, -1.72 mm from bregma; ML, ±0.46 mm; DV,
372 -2.62 mm from the brain surface) using a pulled glass capillary with a pressure microinjector
373 (Picospritzer III, Parker, USA) at a slow rate of 0.1 µl/min. After the injection was completed,
374 the capillary was left for an additional 10 minutes before it was then slowly withdrawn
375 completely. After surgery, animals were recovered from anesthesia under a heat pad.

376

377 AAV-CaMKII:EGFP-Cre (AAV2/1-CamKII-HI-EGFP-Cre, 0.2 μ l, bilateral into LHb,
378 University of Pennsylvania vector core, Pennsylvania, USA), AAV2/5-GFAP::EGFP-Cre
379 (AAV2/5-gfaABC1D-EGFP-Cre, titer: 4.74×10^{12} v.g./ml, dilution: 1:2, 0.2 μ l, bilateral into
380 LHb, Taitool Bioscience, China), AAV-GFAP::Kir4.1 (AAV2/5-gfaABC1D-EGFP-Kir4.1,
381 titer: 9.19×10^{12} v.g./ml, dilution: 1:5, 0.2 μ l, bilateral into LHb, Taitool Bioscience, China),
382 AAV-GFAP::GFP (AAV2/5-gfaABC1D-EGFP, titer: 1.61×10^{13} v.g./ml, dilution: 1:5, 0.2 μ l,
383 bilateral into LHb, University of Massachusetts, Guangping Gao Lab, USA), AAV-
384 H1::Kir4.1-shRNA (AAV2/5-H1-Kir4.1-shRNA-CAG-EGFP, titer: 3.04×10^{13} v.g./ml,
385 dilution: 1:10, 0.2 μ l, bilateral into LHb, Taitool Bioscience, China), AAV-H1::Ctrl-shRNA
386 (AAV2/5-H1-Luciferase-shRNA-CAG-EGFP, titer: 1.46×10^{13} v.g./ml, dilution: 1:5, 0.2 μ l,
387 bilateral into LHb, Taitool Bioscience, China), AAV2/5-gfaABC1D-dnKir4.1-2A-EGFP
388 (GYG to AAA) (titer: 4.15×10^{13} v.g./ml, 0.2 μ l, bilateral into LHb, Taitool Bioscience,
389 China). All viral vectors were aliquot and stored at -80 °C until use.
390

391 Depression model and Behavior assay

392 **LPS-induced depression.** The LPS-induced depression model was conducted as previously
393 described²². Wistar male rats (3 months) were used for the experiments. LPS (Sigma, L-
394 2880) dissolved in sterile 0.9% saline was intraperitoneally injected into Wistar rats, at a
395 dosage of 0.5 mg/kg. This dosage was used to stimulate a subclinical infection without
396 inducing obvious inflammation and other apparent impairments in animals. Saline or LPS
397 was injected between 09:30 and 10:30 a.m. daily for 7 days. The forced swim test was
398 performed 24 hours after the last injection. The habenular tissue was dissected 24 hours after
399 the behavioral test and eletrophysiology experiments.
400

401 **Learned helpless test (LHT).** Male juvenile (P30) or adult (P90) cLH rats were tested in a
402 lever-pressing task to evaluate the learned helpless (LH) phenotype¹¹. A cue-light-illuminated
403 lever in the shock chamber was presented, which can terminate the shock when rats pressed
404 the lever. 15 escapable shocks (0.8 mA) were delivered with a 24 s inter-shock interval. Each
405 shock lasted up to 60 s unless the rat pressed the lever to terminate the shock. Out of the 15
406 trials, rats which failed to press the lever for more than 10 trials were defined as “learned
407 helplessness” (LH), and rats with less than 5 failures were defined as “non-learned
408 helplessness” (NLH).

409

410 **Forced swim test (FST).** Animals were individually placed in a cylinder (12 cm diameter, 25
411 cm height for mice; 20 cm diameter, 50 cm height for rats) of water (23-25 °C) and swam for
412 6 min under normal light³. Water depth was set to prevent animals from touching the bottom
413 by tails and hind limbs. Animal behaviors were videotaped from the side. The immobility
414 time during the last 4 min test was counted offline by an observer blind of the animal
415 treatments. Immobility was defined as time when animals remained floating or motionless
416 with only movements necessary for keeping balance in the water. For rats, an additional pre-
417 test was conducted 24 h before the test, during which rats were individually placed in a
418 cylinder of water with conditions described above for 15 min.

419

420 **Sucrose preference test (SPT).** Animals were single housed and habituated with two bottles
421 of water for 2 days, followed by two bottles of 2% sucrose for 2 days³. Animals were then
422 water deprived for 24 h and then exposed to one bottle of 2% sucrose and one bottle of water
423 for 2 h in the dark phase. Bottle positions were switched after 1 h. Total consumption of each
424 fluid was measured and sucrose preference was defined as the ratio of sucrose consumption
425 divided by total consumptions of water and sucrose.

426 **Open field test (OFT).** Animals were placed in the center of an arena (40 cm x 40 cm x 40.5
427 cm for mice; and 100 cm x 100 cm x 40 cm for rats) in a room with dim light for 10 min. A
428 video camera positioned directly above the arena was used to track the movement of each
429 animal (Any-maze, Stoelting, US).

430

431 **LHb brain slice preparation.** Rats (P25-30 or P60-90) and mice (P90) were anesthetized
432 with isoflurane and 10% chloral hydrate, and then perfused with 20 ml ice-cold ACSF
433 (oxygenated with 95 % O₂ + 5% CO₂) containing (mM): 125 NaCl, 2.5 KCl, 25 NaHCO₃,
434 1.25 NaH₂PO₄, 1 MgCl₂ and 25 Glucose, with 1 mM pyruvate added. The brain was removed
435 as quickly as possible after decapitation and put into chilled and oxygenated ACSF. Coronal
436 slices containing habenular (350μm- and 300μm- thickness for rats and mice, respectively)
437 were sectioned in cold ACSF by a Leica 2000 vibratome and then transferred to ACSF at
438 32°C for incubation and recovery for 1h and then transferred to room temperature. ACSF was
439 continuously gassed with 95% O₂ and 5% CO₂. Slices were allowed to recover for at least 1
440 hour before recording.

441

442 **In vitro electrophysiological recording.** For LHb neuron recordings, currents were
443 measured under whole-cell patch clamp using pipettes with a typical resistance of 5–6 MΩ
444 filled with internal solution containing (mM) 105 K-Gluconate, 30 KCl, 4 Mg-ATP, 0.3 Na-
445 GTP, 0.3 EGTA, 10 HEPES and 10 Na-phosphocreatine, with pH set to 7.35. For the
446 biocytin filling, 5mg/ml biocytin was added in the internal solution. The external ACSF
447 solution contained (in mM) 125 NaCl, 2.5 KCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 1 MgCl₂ and 25
448 Glucose. Cells were visualized with infrared optics on an upright microscope (BX51WI,
449 Olympus). A MultiClamp 700B amplifier and pCLAMP10 software were used for electro-
450 physiology (Axon Instruments). The series resistance and capacitance was compensated

451 automatically after stable Giga seal were formed. The spontaneous neuronal activity was
452 recorded under current-clamp ($I = 0$ pA) for consecutive 60 s. RMP was determined during
453 the silent period of the neuronal spontaneous activity.

454

455 To test TTX (1 μ M, Sigma) and BaCl₂ (100 μ M, Sigma) effect onto neuronal RMP and
456 spontaneous activity, baselines were recorded for at least for 3 min. Drug were then perfused,
457 the arrival of the drug was precisely indicated with a bubble that was pre-added before the
458 transition from normal ACSF to drug added ACSF. TTX effect on LHb neuronal RMP and
459 spontaneous activity was stabilized after several minutes while BaCl₂ effect on neuronal RMP
460 and spontaneous activity took more than 10 min to stabilize. We thus analyzed the drug effect
461 of TTX and BaCl₂ at the time point of 5min and 15 min respectively.

462

463 **Astrocytic whole-cell patch clamp and Kir4.1 current isolation.** Astrocytes were
464 distinguished from neurons by their small (5-10 μ m) oval-shaped somata and by distinct
465 electrophysiological features: a hyperpolarized RMP and a low input resistance, a linear I-V
466 relationship and an absence of action potentials in response to increased injection currents.
467 BaCl₂ (100 μ M, Sigma) were applied to isolate Kir4.1 current which is subtracted from the IV
468 curve recorded from -120mV to 0mV. Biocytin (Sigma) 5mg/ml was dissolved into the
469 patch-clamp pipette solution. After electrophysiological characterization, cells are held for at
470 least 30 minutes in voltage clamp and constantly injected with a hyperpolarization current
471 (500 ms, 50 pA, 0.5 Hz, 30 min) to allow biocytin filling (performed at 34 °C). Subsequently,
472 slices were fixed overnight in 4% paraformaldehyde at 4 °C. The antibodies used were rabbit
473 anti-NeuN (1:500, Millipore), Alexa Fluor 546 donkey anti-rabbit IgG (1:1000, Invitrogen),
474 Cy2-conjugated Streptavidin (1:1000, Jackson ImmunoResearch). Fluorescent image
475 acquisition was performed with a Nikon A1 confocal microscope.

476 **Tri-compartment model.**

477 Tri-compartment model was constructed including the neuron, the astrocyte and the
478 extracellular space. The model is based on ionic fluxes between three compartments. Na^+ and
479 K^+ voltage-gated channel, and leak channel were recruited into the neuron as:

480 $dV_N/dt = (I_{app} - I_K - I_{Na} - I_{Leak,N})/c_N$

481 where V_N is the neuronal membrane potential, c_N is the neuronal capacitance. I_{app} is the
482 external current applied to the neuron. I_{Na} and I_K are the fast Na^+ and K^+ currents responsible
483 for the generation of action potentials; $I_{Leak,N}$ are responsible for the neuronal resting
484 membrane potential. Kir4.1 channel on the extracellular and vessel side and leak channel
485 were recruited into the astrocyte as:

486 $dV_A/dt = (-I_{Kir} - I_{Kir,vess} - I_{Leak,A})/c_A$

487 where V_A is the astrocytic membrane potential, c_A is the astrocytic capacitance. I_{Kir} are
488 outward during the resting state, while become inward when K^+ equilibrium potential
489 exceeds the astrocytic membrane potential. $I_{Kir,vess}$ accounts for the K^+ outflow from the
490 astrocyte from the vessel side. $I_{Leak,A}$ are responsible for the astrocytic resting membrane
491 potential.

492 The equations of the K^+ in the three compartments are described by:

493 $d[K^+]_o/dt = (I_{kir} + I_k)/(F \cdot vol_0) - 2 \cdot (i_{pump,N} + i_{pump,A}) - d_{K_o} \cdot ([K^+]_o - K_{O,o})$

494 $d[K^+]_N/dt = -I_k/(F \cdot vol_N) + 2 \cdot i_{pump,N} \cdot vol_0/vol_N - d_{K_N} \cdot ([K^+]_N - K_{N,o})$

495 $d[K^+]_A/dt = (-I_{kir} - I_{kir,vess})/(F \cdot vol_A) + 2 \cdot i_{pump,A} \cdot vol_0/vol_A - d_{K_A} \cdot ([K^+]_A - K_{A,o})$

496 The extracellular K^+ is mainly contributed by I_K . Released extracellular K^+ from the neuron
497 is taken up by through Kir4.1 channels and Na^+/K^+ pump.

498 The equations of the Na^+ in the three compartments are described by:

499 $d[Na^+]_o/dt = I_{Na}/(F \cdot vol_0) + 3 \cdot (i_{pump,N} + i_{pump,A}) - d_{Na_o} \cdot ([Na^+]_o - Na_{O,o})$

500 $d[Na^+]_N/dt = -I_{Na}/(F \cdot vol_N) - 3 \cdot i_{pump,N} \cdot vol_0/vol_N - d_{Na_N} \cdot ([Na^+]_N - Na_{N,o})$

501 $d[Na^+]_A/dt = -3 \cdot i_{pump,A} \cdot vol_0/vol_A - d_{Na_A} \cdot ([Na^+]_A - Na_{A,o})$

502 See supplementary information for detailed formulas.

503

504 **Statistical analyses.** Required sample sizes were estimated based on our past experience
505 performing similar experiments. Animals were randomly assigned to treatment groups.
506 Analyses were performed in a manner blinded to treatment assignments in all behavioral
507 experiments. Statistical analyses were performed using GraphPad Prism software v6. By pre-
508 established criteria, values were excluded from the analyses if the viral injection or drug
509 delivering sites were out of LHb. All statistical tests were two-tailed, and significance was
510 assigned at $P < 0.05$. Normality and equal variances between group samples were assessed
511 using the D'Agostino & Pearson omnibus normality test and Brown–Forsythe tests
512 respectively. When normality and equal variance between sample groups was achieved, one-
513 way ANOVAs (followed by Bonferroni's multiple comparisons test), or t test were used.
514 Where normality or equal variance of samples failed, Mann-Whitney U test were performed.
515 Linear regression test, Chi-square test was used inappropriate situations. The sample sizes,
516 specific statistical tests used, and the main effects of our statistical analyses for each
517 experiment are reported in Supplementary Table 1.

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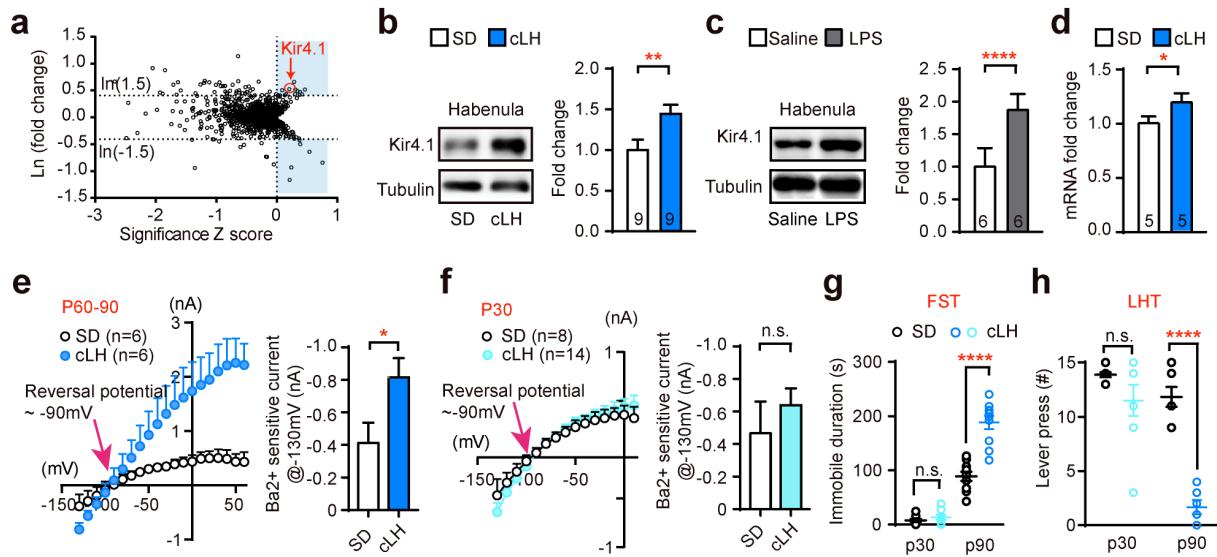


Figure 1 | Kir4.1 is upregulated in LHB of animal models of depression.

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a, Volcano plot of high-throughput proteomic screen identifies differentially expressed proteins in habenula of cLH rats. Ln (fold change) was Ln transformed value of the normalized protein ratio of cLH and control. Significance Z score was calculated as the average normalized ratio minus two folds of standard deviation. Proteins in the shaded areas have more than 50% significant change¹¹. Kir4.1 is one of the eight upregulated proteins identified. Dash lines indicate fold change of 50%.

b, c, Western blot analysis showing upregulation of Kir4.1 protein in membrane fraction of habenula of cLH (**b**) and LPS-induced depression rat models (**c**). Tubulin is used as loading control. Numbers in the bars indicate the number of animals used.

d, QPCR analysis of Kir4.1 mRNA in habenula.

e, f, I-V plot and bar graph showing Ba²⁺-sensitive current in cLH rats and their wild type controls at the age of P60-90 (**e**) and P30 (**f**).

g, h, Age-dependent forced swim (FST, **g**) and learned helpless (LHT, **h**) phenotypes of cLH rats. Low number of lever press and high immobility time indicate depressive-like phenotype in P90 cLH rats.

Data are means \pm SEM. *P < 0.05, **P < 0.01, ****P < 0.0001; n.s., not significant (See Supplementary Table 1 for statistical analysis and n numbers).

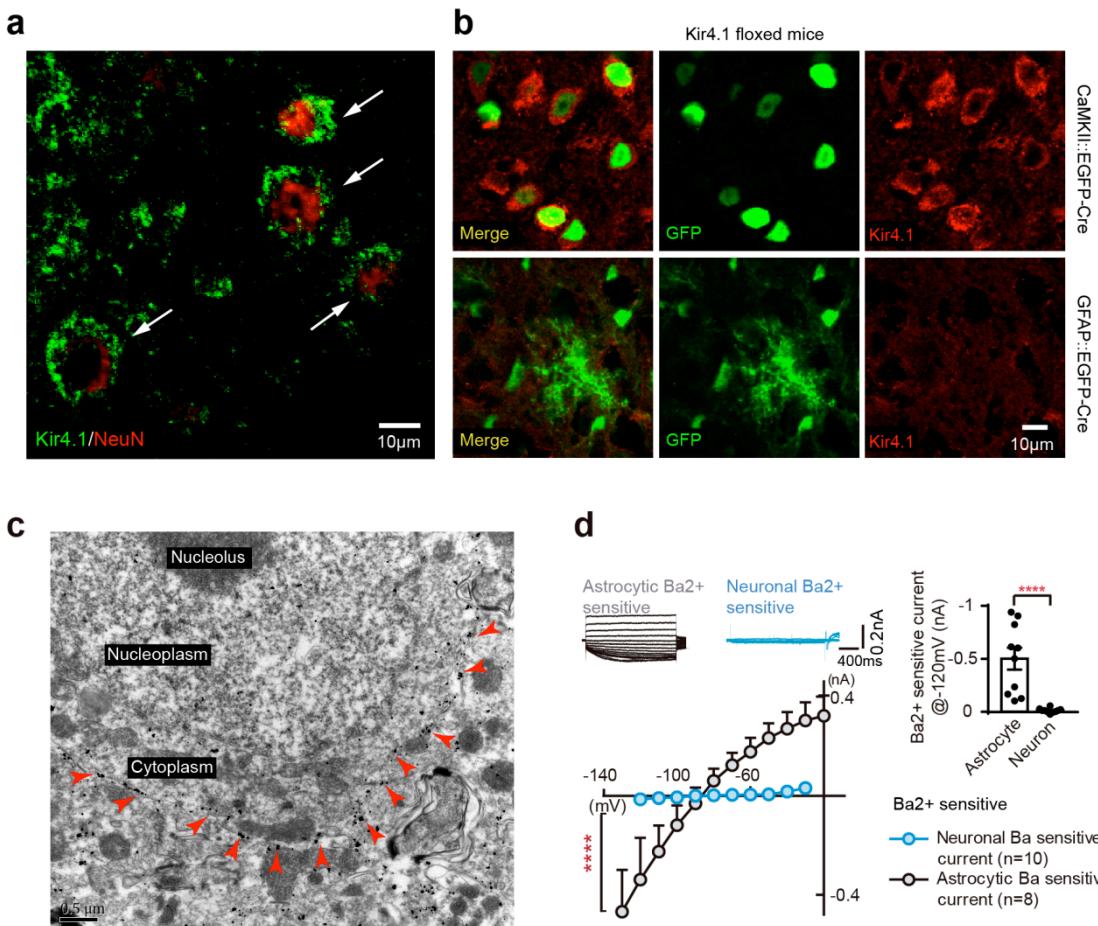


Figure 2 | Kir4.1 is expressed on astrocytic processes tightly wrapping around neuronal soma in Lhb.

a, Immunohistochemistry signals of Kir4.1 envelope neuronal soma as indicated by white arrows.
 b, The pan-soma Kir4.1 signals remain intact in Lhb of Kir4.1^{f/f} floxed mice injected with AAV2/1-CaMKII::EGFP-Cre, but are eliminated when injected with AAV2/5-GFAP::EGFP-Cre (GFAP: human astrocyte-specific GFAP promoter, gfaABC1D).
 c, Immunogold electron microscopy of Kir4.1. Red arrows indicate gold signals surrounding a neuronal soma.
 d, I-V plots of the Ba²⁺ sensitive Kir4.1 current recorded in Lhb astrocytes and neurons, with representative traces shown in up-left and statistic bar graph of current recorded when cells are held at -120mV shown in up-right.

Data are means ± SEM. ****P < 0.0001 (See Supplementary Table 1 for statistical analysis and n numbers).

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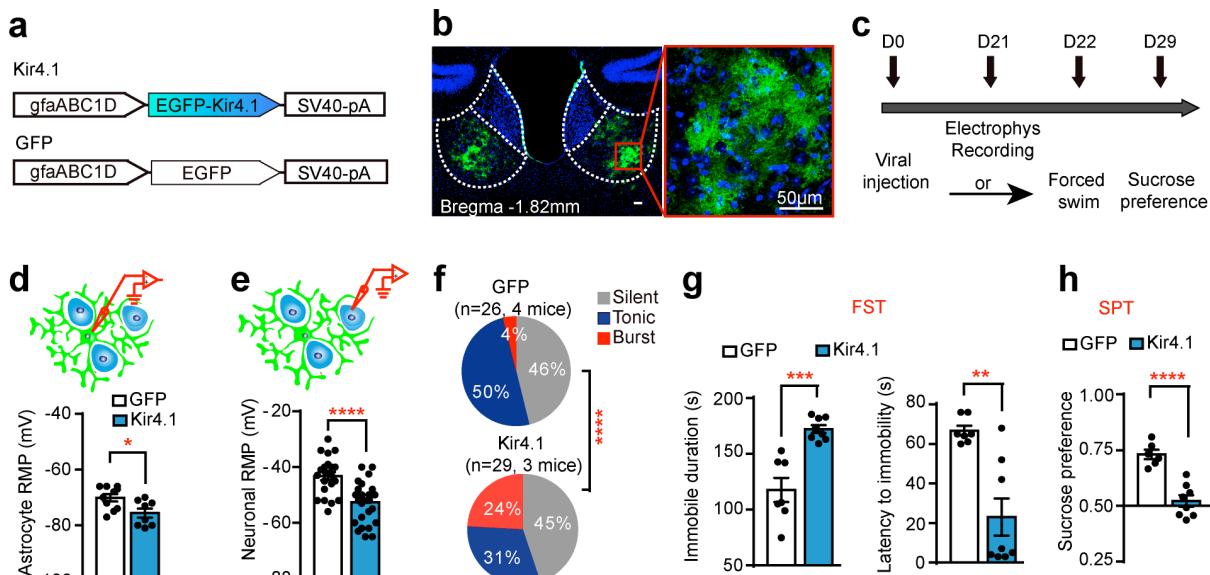


Figure 3 | Astrocytic kir4.1 overexpression increases neuronal bursts in LHB and causes depressive-like phenotypes.

666 a, Schematics of AAV vectors engineered to overexpress Kir4.1 or a GFP control and under a GFAP
667 promoter.

668 b, Illustration of bilateral viral injection of AAV-GFAP::Kir4.1 in mouse LHB (stained with antibody
669 against GFP and Hoechst).

670 c, Experimental paradigm for electrophysiology and behavioral testing.

671 d-f, Astrocytic overexpression of Kir4.1 decreases RMPs of both astrocytes (d) and neurons (e) and
672 increases the bursting population in neurons (f).

673 g, h, Behavioral effects of expressing various viral constructs in LHB in forced swim test (FST) (h) and
674 sucrose preference test (SPT) (i).

675 Data are means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (See Supplementary Table
676 1 for statistical analysis and n numbers).

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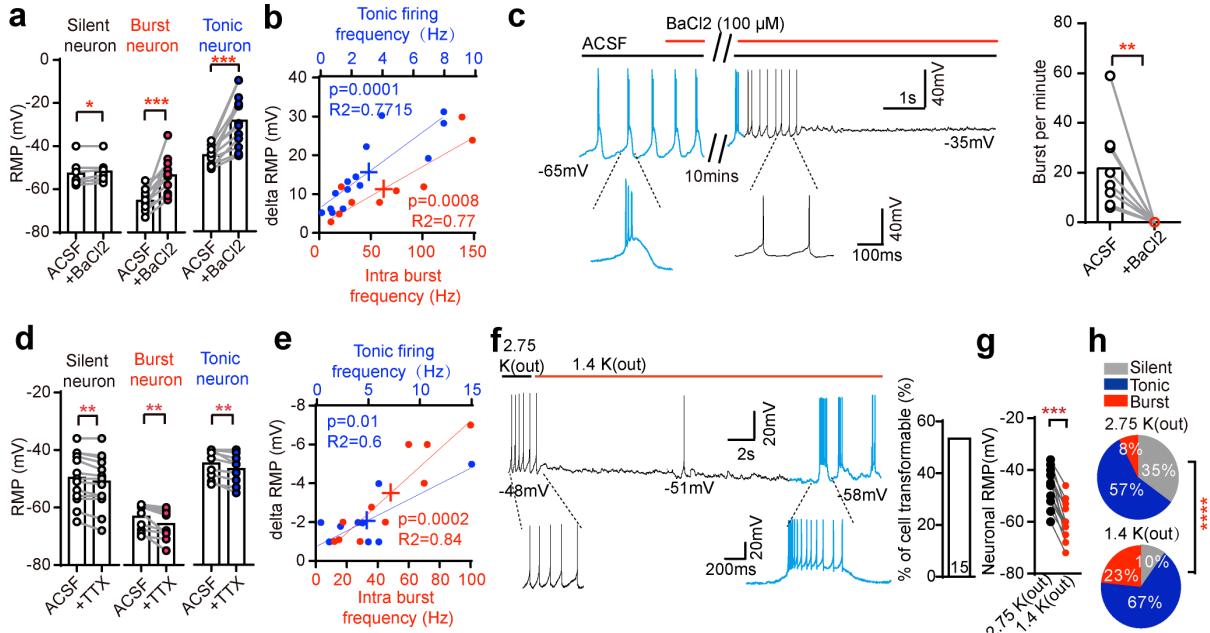


Figure 4 | Kir4.1-dependent potassium buffering regulates neuronal RMP and bursting in LHB.

a, d, Changes of neuronal RMPs caused by BaCl_2 (100 μM , a) or TTX (1 μM , d) in different neuronal types. RMPs are measured at 15min or 5min after perfusion of BaCl_2 or TTX, respectively, when the RMPs stabilize.

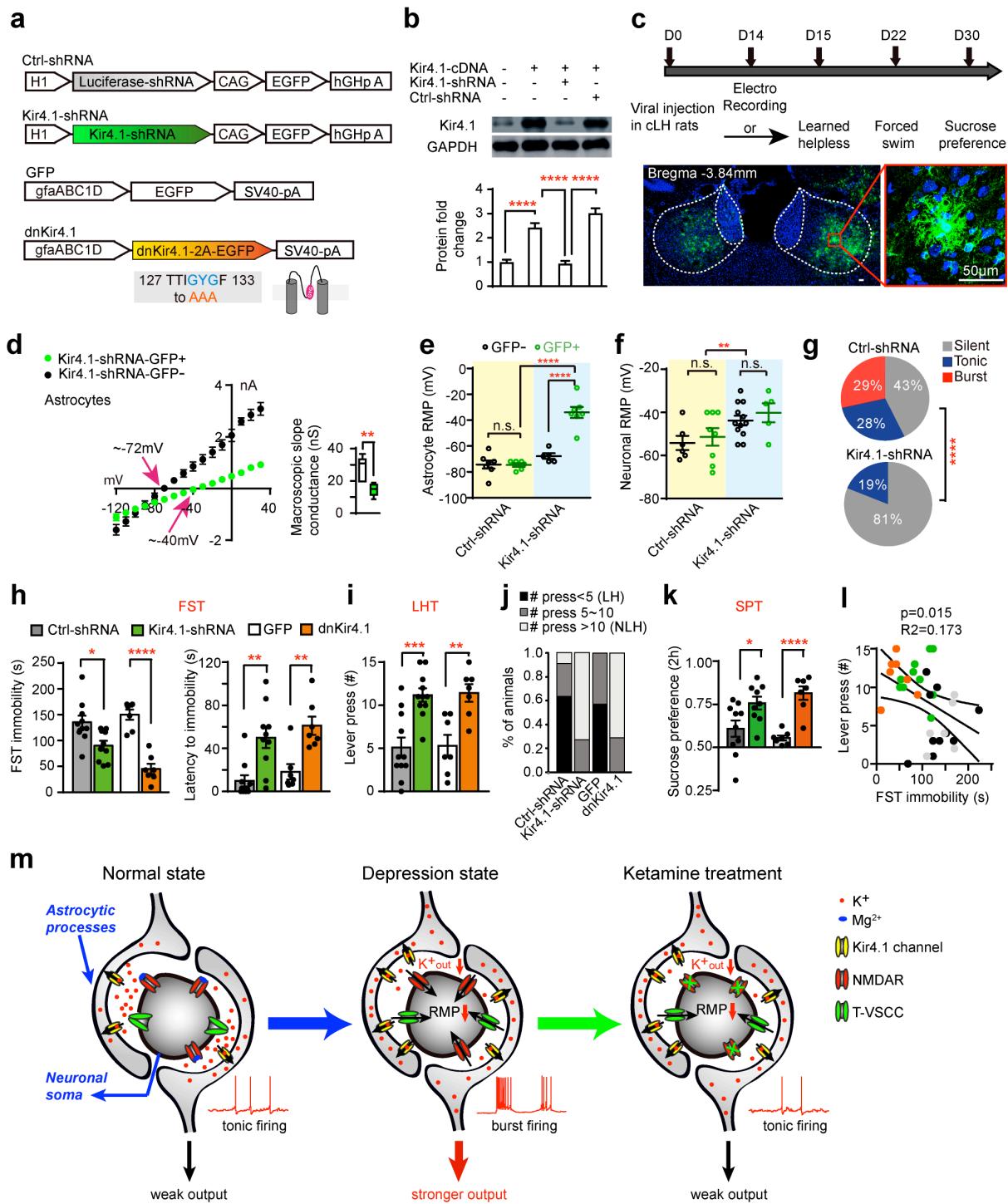
b, e, Changes of neuronal RMPs after BaCl_2 (b) or TTX (e) treatments correlate with original firing rates of tonic-firing neurons (blue) or intra-burst frequencies of bursting neurons (red).

c, Representative trace (left) and bar graph (right) showing BaCl_2 effect (sampled at 15mins after drug perfusion) onto bursting neurons. Spikes in bursting mode are marked in blue. Spikes in tonic-firing mode are marked in black.

f, Example (left) and percentage of LHB neurons transforming from tonic- to burst- firing mode after $\text{K}^{(\text{out})}$ is switched from normal (2.75 mM) to half (1.4 mM) (sampled at 1min after drug perfusion).

g, h, Lowering $\text{K}^{(\text{out})}$ to half decreases neuronal RMPs (g) and increases bursting neural population (h).

Data are means \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, n.s., not significant (See Supplementary Table 1 for statistical analysis and n numbers).



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Figure 5 | Loss of function of Kir4.1 in Lhb decreases neuronal bursting and rescues depressive-like phenotypes of cLH rats.

710 a, Schematics of the AAV vector engineered to overexpress shRNA or dominant negative form of
711 Kir4.1. H1: human H1 promoter. CAG: The CMV early enhancer/chicken beta actin promoter. Three
712 point mutations in the dnKir4.1 and their locations are indicated.
713 b, Western blot and quantification showing efficient knock-down of Kir4.1 by shRNA in HEK293T cells.
714 c, Experimental paradigm for behavioral testing of cLH rats infected by virus (top) and illustration of
715 bilateral expression of AAV-GFAP::dnKir4.1 in Lhb (stained with antibody against GFP and Hoechst).
716 d-g, AAV-Kir4.1-shRNA caused a shift of reverse potential from -72mV to -40mV in astrocytes (d),
717 depolarization of astrocytes (e) and neurons in viral infected region (f), and abolished neuronal
718 bursting (g). Floating bars for membrane slope conductance in d is calculated from the I/V plots
719 (between -120 and +40 mV).
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722 **h-k**, Behavioral effects of expressing various viral constructs in the LHB of cLH rats in FST (**h**), LHT (**i**,
723 **j**) and SPT (**k**). **j**: Percentage of rats in each category. LH: learned helpless rats with <5 lever presses;
724 NLH, non-learned helpless rats with >10 lever presses.

725 **I**, FST immobility is highly correlated with learned helpless phenotype.

726 **m**, A model for mechanisms of depression and ketamine treatment at LHB. Upregulation of Kir4.1 on
727 astrocytic processes surrounding neuronal soma leads to enhanced K⁺ buffering at the tight neuron-
728 glia junction, decreased K_{out} and hyperpolarized neuronal RMP. Consequently, de-inactivation of T-
729 VSCCs initiates NMDAR-dependent bursts, causing a stronger output of LHB to trigger depression.
730 Ketamine blockade of NMDARs stops bursts and relieves depression.

731 Data are means ± SEM.*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, n.s., not significant (See
732 Supplementary Table 1 for statistical analysis and n numbers).

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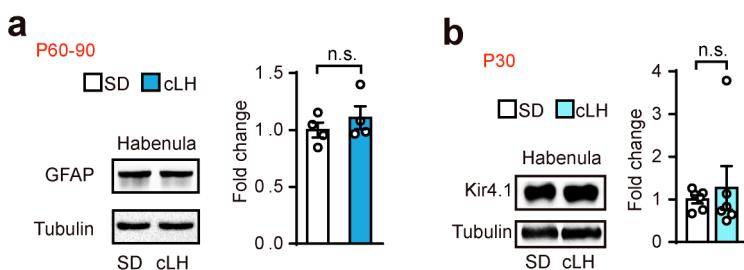
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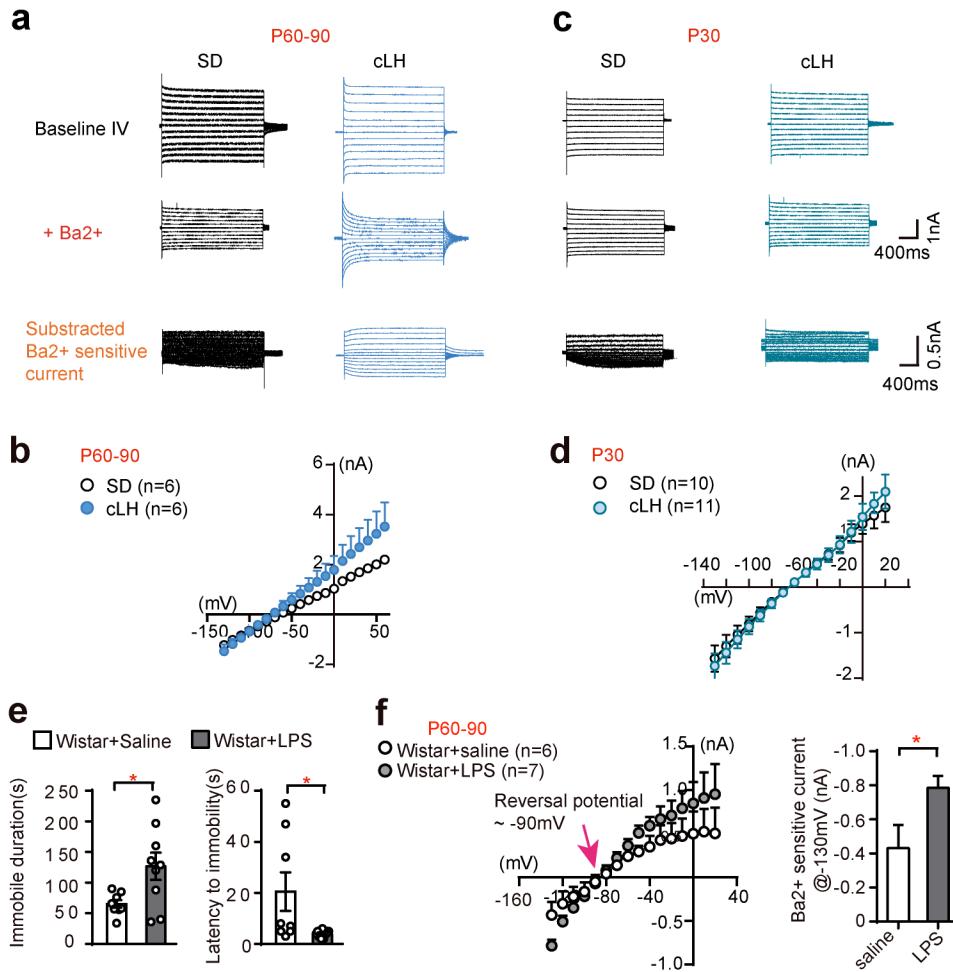
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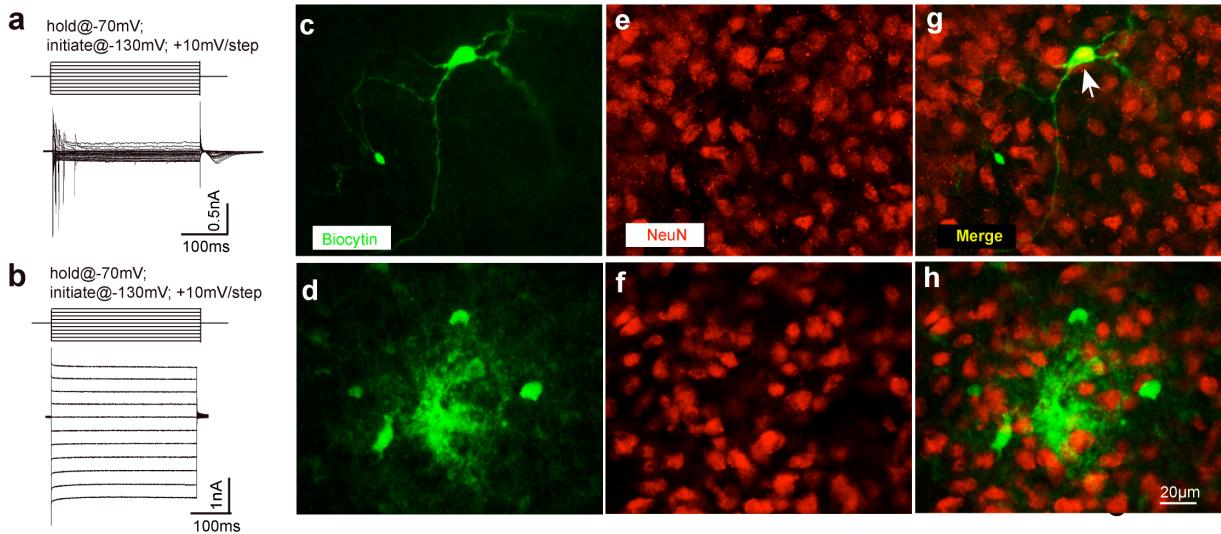
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771 **Extended Data Figure 1 | Kir4.1 is unchanged in p30 cLH rats.**772 a, Western blot analysis showing no change of GFAP protein in habenula of cLH rats at the age of
773 P60-90.774 b, Western blot analysis showing no change of Kir4.1 protein in membrane fraction of habenula in
775 cLH rats at the age of P30.776 Data are means \pm SEM. n.s., not significant (See Supplementary Table 1 for statistical analysis and n
777 numbers).



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Extended Data Figure 3 | Biocytin intercellular filling and double staining with NeuN confirm the identity of electrophysiologically identified neurons and astrocytes.

a, b, A neuron (a) and an astrocyte (b) in LHB slices are first identified based on their specific morphology (astrocytes: 5–10 μm diameter; neuron: ~15 μm diameter) and physiological properties. The neuron fires at a depolarizing voltage step (a), whereas the astrocyte shows a steady-state I–V relationship and a lack of spiking activity (b). After electrophysiological characterization, cells are held for at least 30 minutes in voltage clamp and constantly injected with a hyperpolarization current (500 ms, 50 pA, 0.5 Hz, 30 min) to allow biocytin filling.

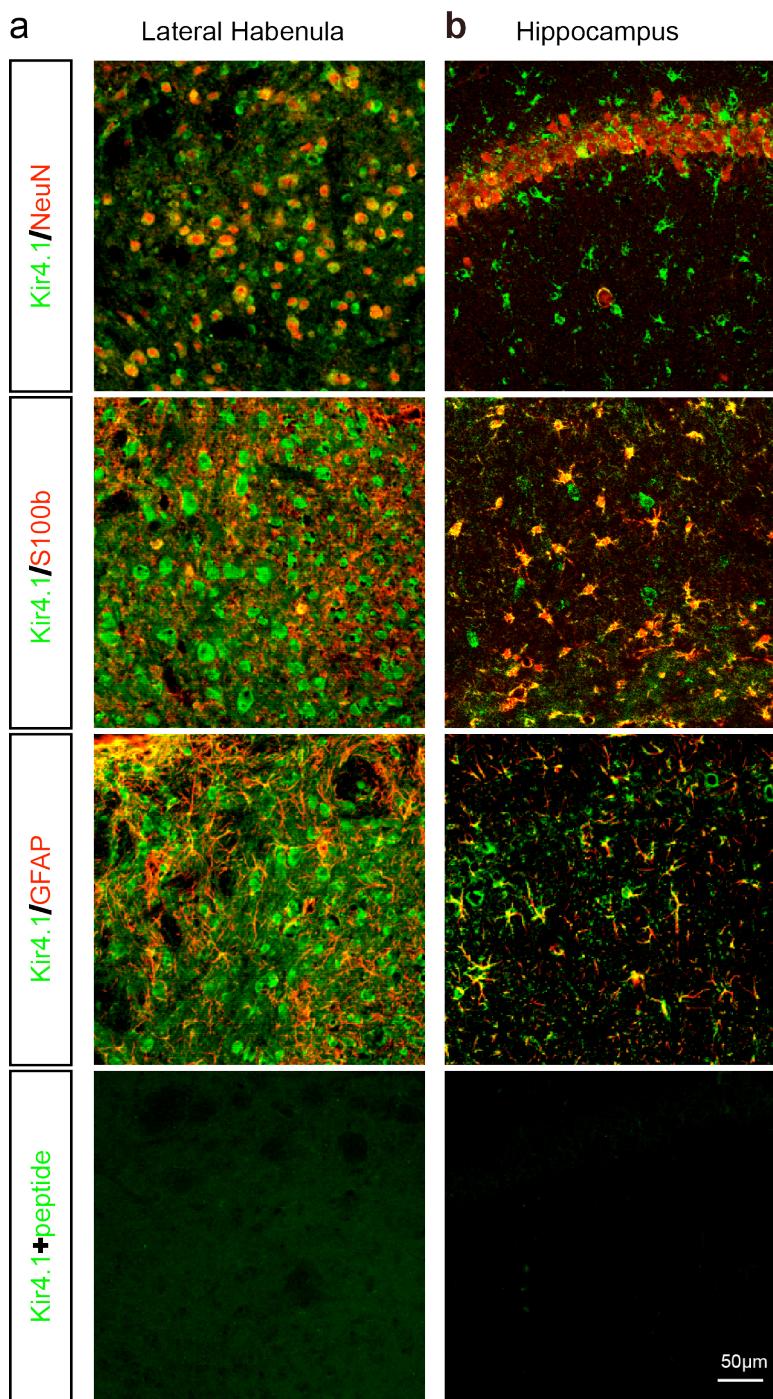
c–h, Biocytin labeled neurons and astrocytes subsequently confirmed by co-labeling with NeuN.

c, d, Biocytin signals in a single neuron (c) or a group of astrocytes due to diffusion through gap junctions (d).

e, f, NeuN signals.

g, h, Colabeling of NeuN with the neuron (indicated by white arrow, g) but not astrocytes (h). Note that all biocytin filled neurons ($n = 18$) show colabel with NeuN and all biocytin filled astrocytes ($n = 11$) do not colabel with NeuN.

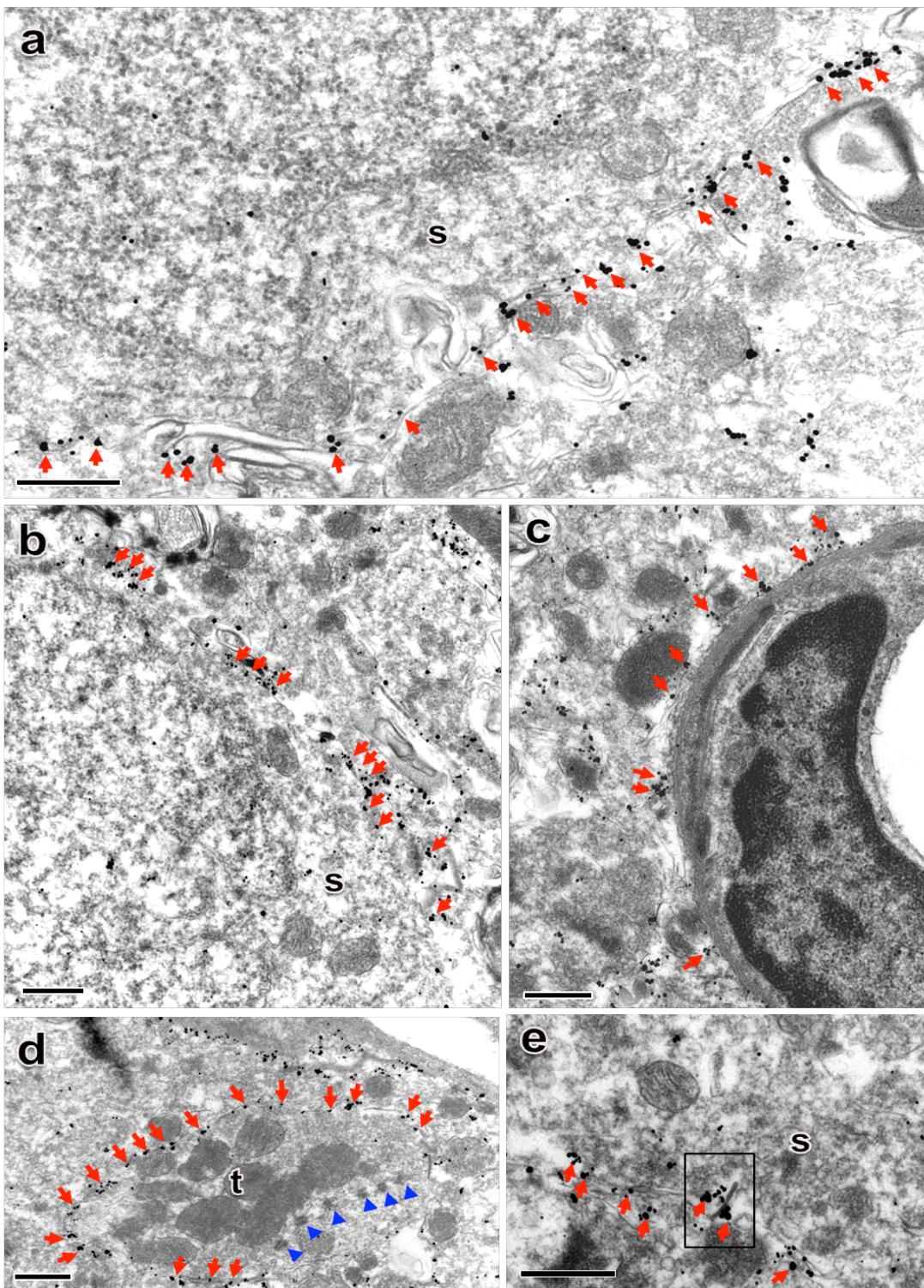
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Extended Data Figure 4 | Expression pattern of Kir4.1 in Lhb and hippocampus.

a, b, Kir4.1 co-immunostaining with neuronal marker (NeuN) or astrocytic marker (S100b and GFAP) in Lhb (**a**) or hippocampus (**b**). Bottom two panels are staining with the same kir4.1 antibody pre-incubated with the antigen peptide, demonstrating the specificity of Kir4.1 antibody.



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Extended Data Figure 5 | Electron microscopy immunohistochemistry of Kir4.1 staining.

849 a-c, Many Kir4.1 immunograins (arrows) surround the neuronal soma (a, b) and the

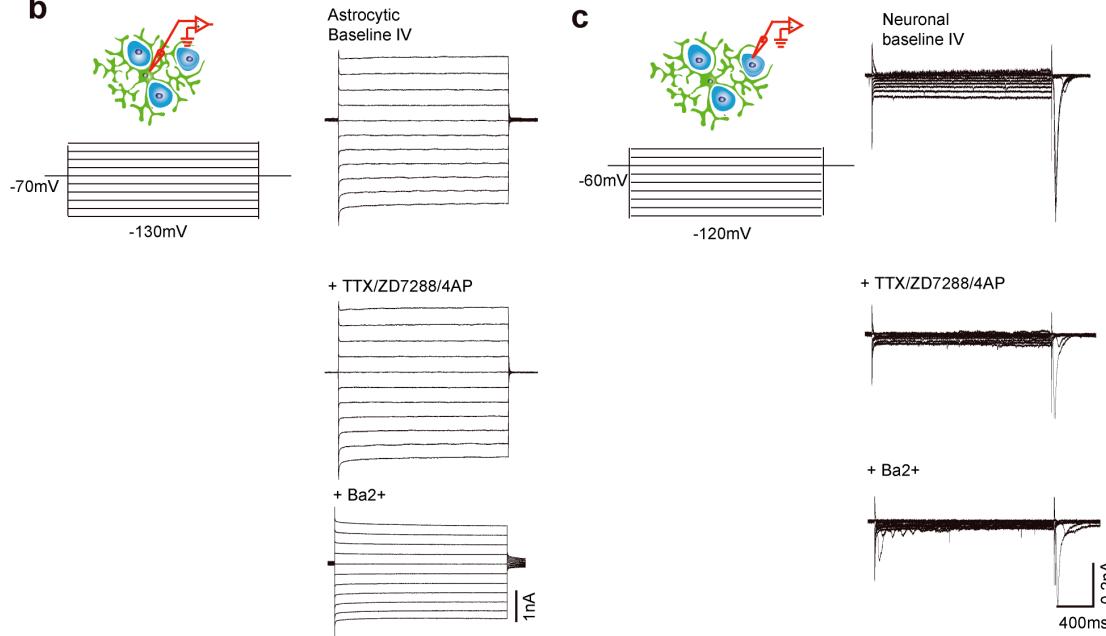
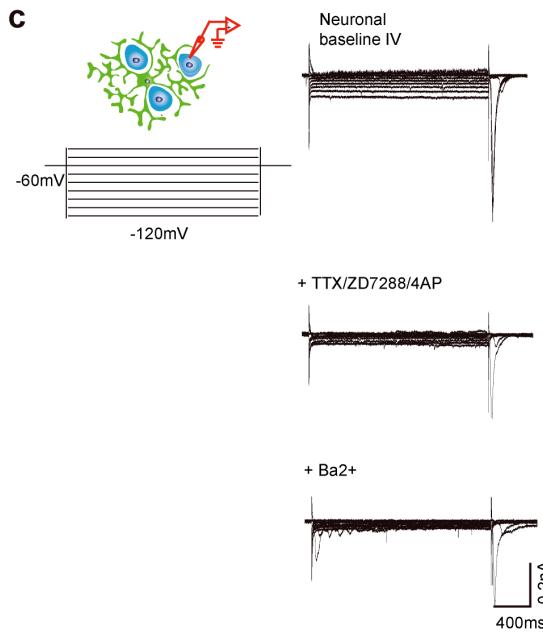
850 vascular endothelial cell (c).

851 852 d, The Kir4.1 grains (arrows) also surround the axon-dendritic synapses, but are rare near the

853 synaptic zones as indicated by the postsynaptic densities (arrowheads).

854 e, Insert shows Kir4.1 immunograins near a gap junction.

855 s: neuronal soma; t: axon terminal. Scale bars= 0.5 um.

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a**b****c**

Extended Data Figure 6 | Kir4.1 is expressed in astrocytes but not neurons in LHB.

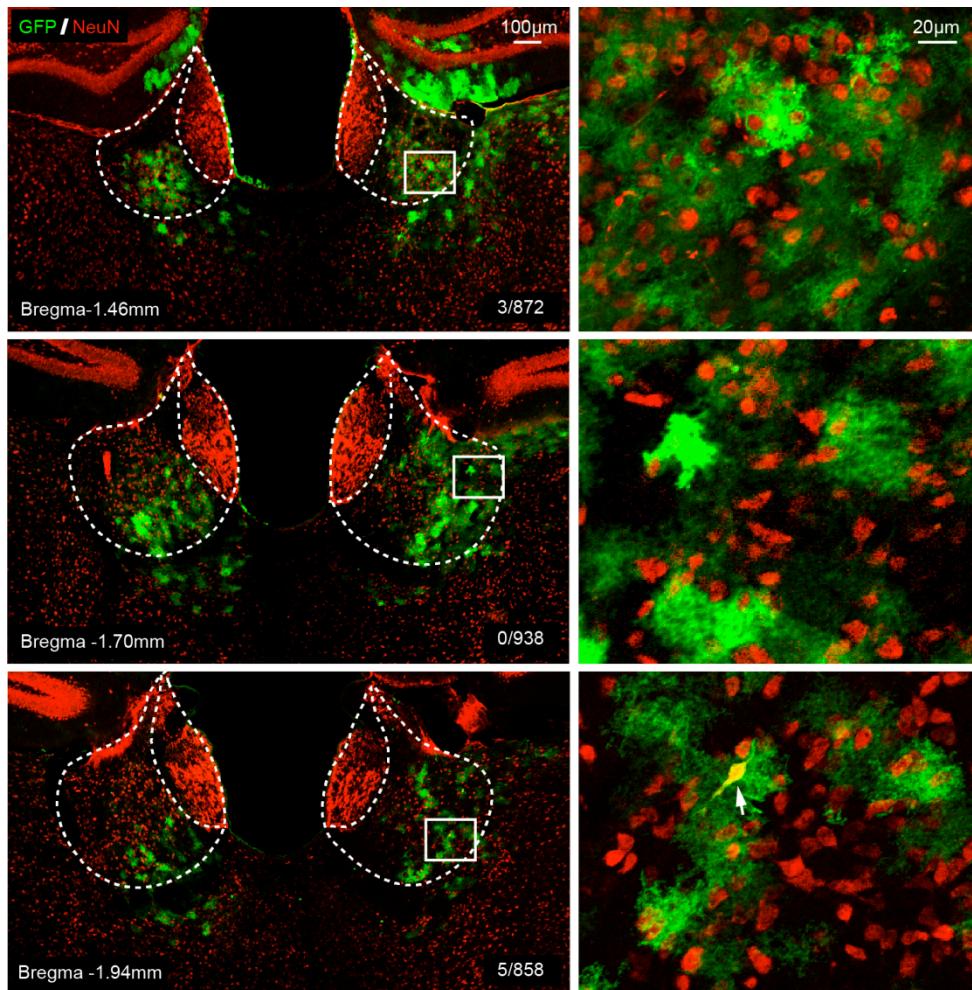
a, Schematics showing sequence of drug application and recording after a neuron or astrocyte is patched.

b, Representative traces showing a linear I-V curve in a typical astrocyte under voltage steps (-130mV to -30 mV, step by 10mV, 2s duration, holding at -70mV, protocol demonstrated on left) (upper panel). I-V curves of the same cell after addition of TTX (1uM), ZD7288 (50uM) and 4AP (1mM, middle), and further addition of Ba²⁺ (100uM, bottom) are shown below.

c, Representative traces showing a non-linear I-V curve in a typical neuron under voltage steps (-130mV to -30 mV, step by 10mV, 2s duration, holding at -70mV, protocol demonstrated on left) (upper panel). I-V curves of the same cell after addition of TTX, ZD7288 and 4AP (middle), and further addition of Ba²⁺ (bottom) are shown below.

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Extended Data Figure 7 | Characterization of cell-type specificity of GFAP promoter driven expression of Kir4.1 in mouse LHB

Double immunofluorescence for mouse NeuN (red) and GFP (green) in the coronal section of LHB brain slices infected with AAV-GFAP::Kir4.1 (AAV2/5-gfaABC1D-EGFP-Kir4.1) virus. Left are examples of anterior, middle and posterior coronal section of LHB. Numbers in the bottom right corner are the number of merged cells/ number of NeuN+ cells in the viral-infected area. Right are zoom-in images of the white square area in left. Note that there is only one infected neuron, as indicated by the white arrow, in all three fields of view.

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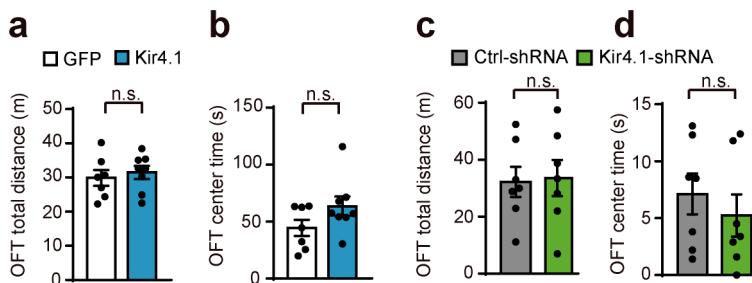
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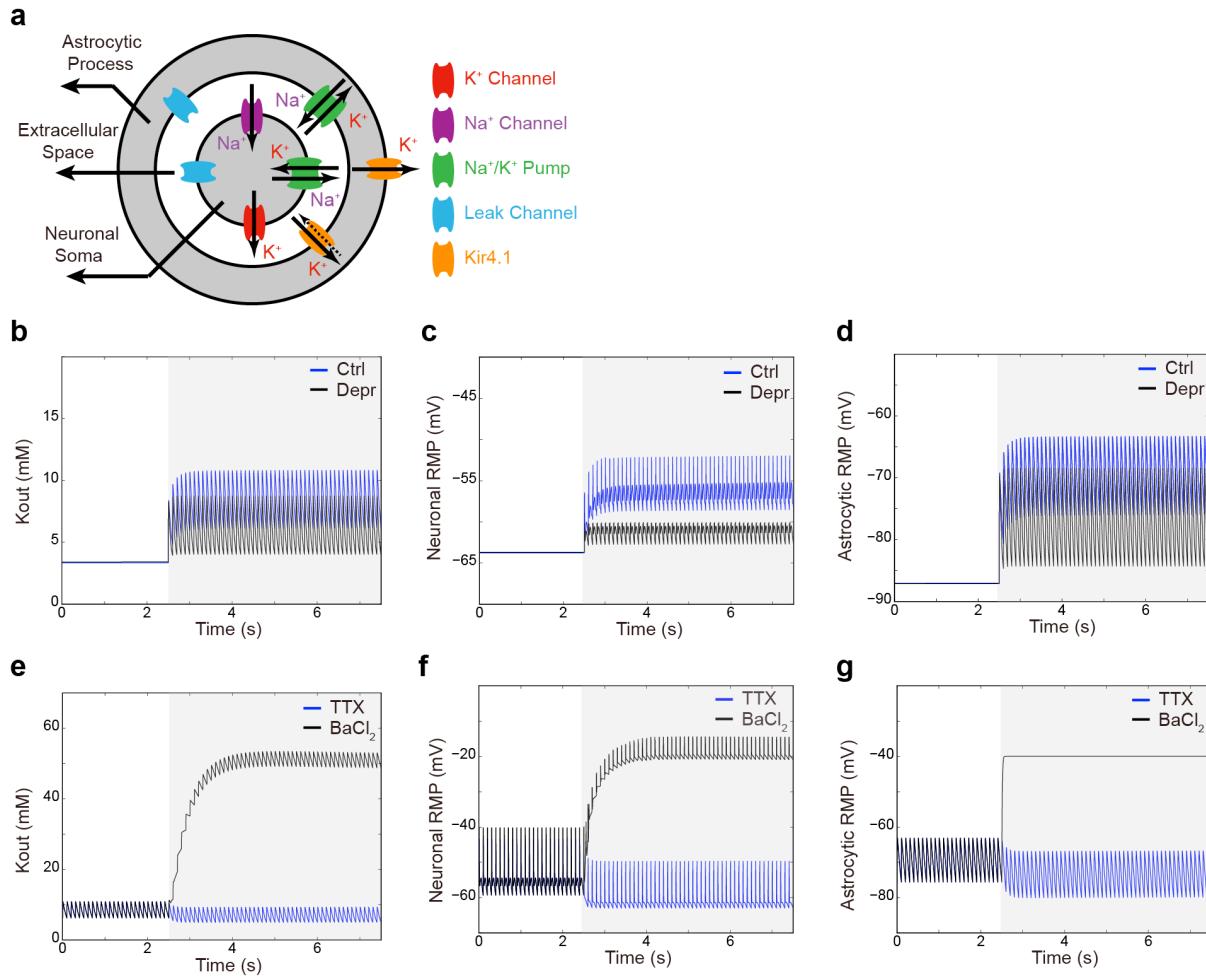
Extended Data Figure 8 | Overexpression of Kir4.1 or Kir4.1-shRNA in Lhb does not affect locomotion in open field test.

897 a-b, Overexpression of Kir4.1 in Lhb does not affect locomotion activities.

898 c-d, Overexpression of Kir4.1-shRNA in Lhb does not affect locomotion activities.

899 Data are means \pm SEM. n.s., not significant (See Supplementary Table 1 for statistical analysis and n
900 numbers).





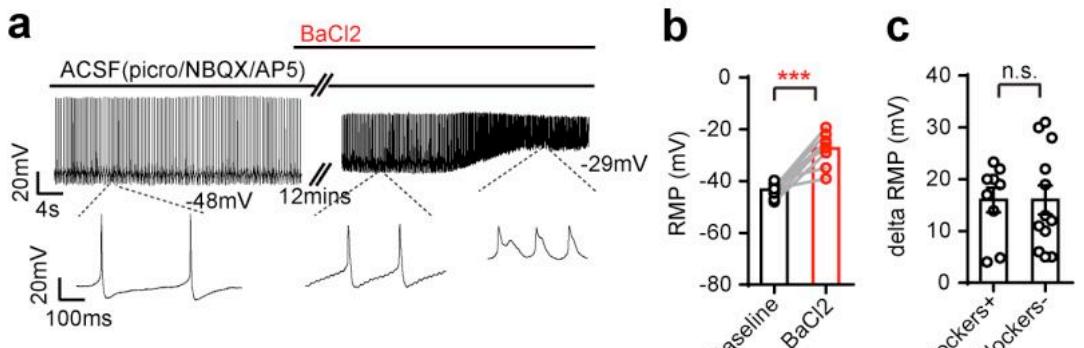
Extended Data Figure 9 | Simulation of the dynamic potassium buffering effect of Kir4.1 in the tri-compartment model.

a, Schematic representation of a tri-compartment model involving neuron, astrocyte and extracellular space in between.

b-d, Effects of increasing Kir4.1 expression level on extracellular K⁺ concentration (**b**), neuronal membrane potential (**c**) and astrocytic membrane potential (**d**). Ctrl: control condition with 1x Kir4.1 conductance; Depr: depression condition with 2x Kir4.1 conductance. Grey shaded areas indicate application of 10 Hz tonic stimulation to neurons. Note that under this neuronal firing condition, Kout is lower, and neuron and astrocyte are more hyperpolarized in Depr than in Ctrl.

e-g, Effects of *in silico* TTX (blocking APs, gNa = 0) or Ba²⁺ (blocking Kir4.1, gKir4.1=0) treatments on extracellular K⁺ concentration (**e**), neuronal membrane potential (**f**) and astrocytic membrane potential (**g**) when neurons are under 10 Hz tonic stimulation. Grey shaded areas indicate *in silico* application of drugs. Note that TTX and Ba²⁺ cause opposite changes to Kout, neuronal membrane potential and astrocytic membrane potential.

Neuronal spikes are not shown for clarity of presentation.



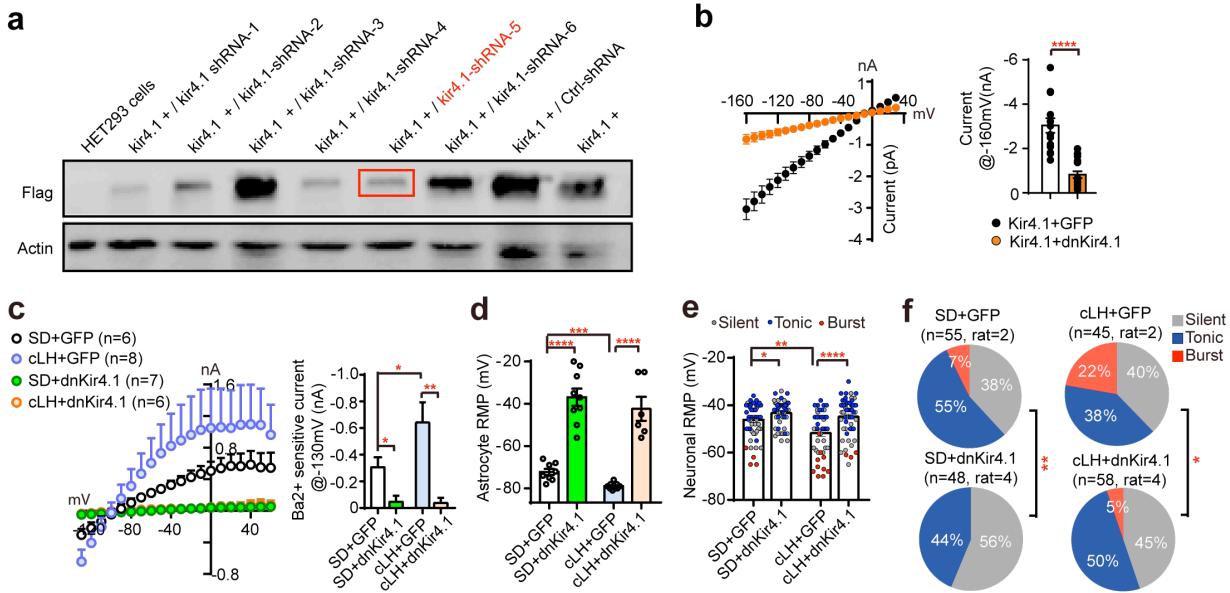
Extended Data Figure 10 | BaCl₂ caused depolarization of neuronal RMP in presence of synaptic transmitter blockers.

a, b, Representative trace (a) and bar graph (b) showing effect of BaCl₂ (100 uM) perfusion onto tonic-firing neurons which have been bathed with transmitter blockers (100 uM picrotoxin, 10 uM NBQX and 100 uM AP5).

c, Bar graph showing the level of RMP depolarization caused by BaCl₂ in presence or absence of transmitter blockers.

Data are means ± SEM. ***P< 0.001, n.s., not significant (See Supplementary Table 1 for statistical analysis and n numbers)

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Extended Data Figure 11 | Characterization of Kir4.1 loss-of-function constructs.

a, Flag-tagged-Kir4.1 plasmid (pAAV-CMV-betaGlobin-Kir4.1-eGFP-3Flag) was co-transfected with pAAV-vector expressing 6 different shRNAs (see Methods) of Kir4.1 or the negative control (shRNA of luciferase) into HEK293TN cell. Based on the knock-down efficiency as shown in the western blot, Kir4.1-shRNA-5 was chosen for viral package.

b, I-V plot showing Kir4.1 currents recorded in HEK293 cells transfected with pAAV-Kir4.1 together with negative control pAAV-GFP or pAAV-dnKir4.1 plasmid. Bars represent the current values recorded at -160mV.

c, I-V plot and bar graph showing Ba²⁺-sensitive currents blocked by AAV-dnKir4.1 in both cLH and SD rats.

d-f, AAV-dnKir4.1 caused a depolarization of RMP in astrocytes (d) and neurons in viral infected area (e), and abolished neuronal bursting (f) in both cLH and SD rats.

Data are means \pm SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (See Supplementary Table 1 for statistical analysis and n numbers).

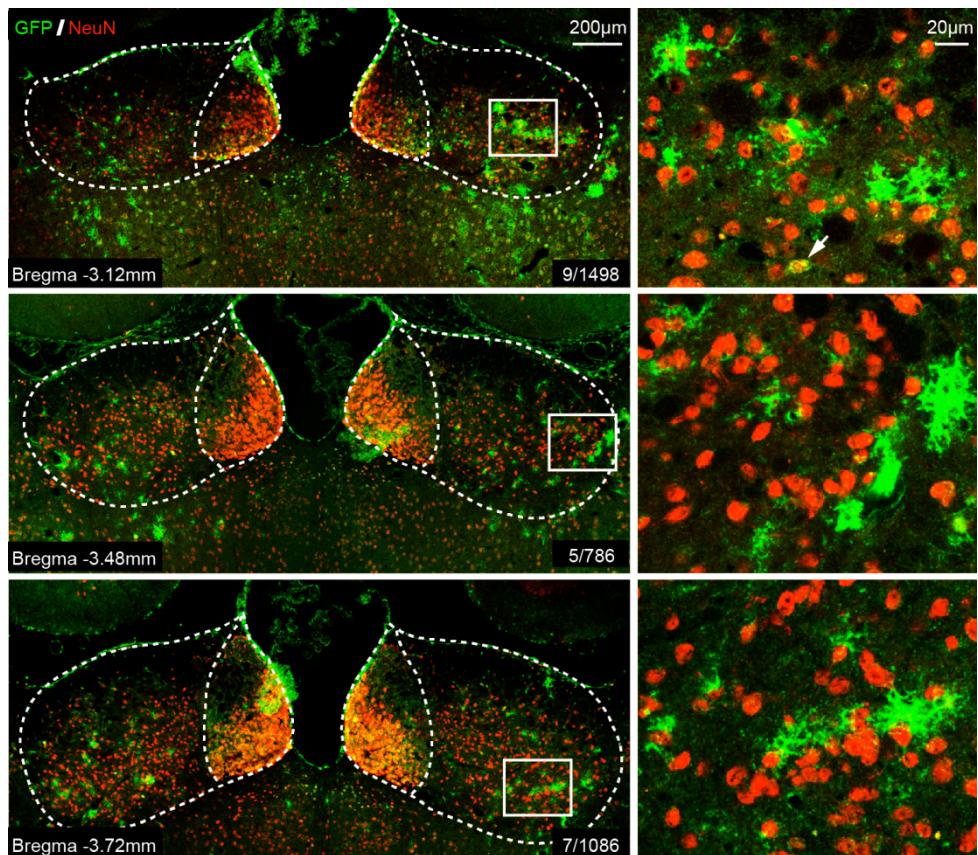
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Extended Data Figure 12 | Characterization of cell-type specificity of GFAP promoter driven expression of dnKir4.1 in rat LHb.

1004 Double immunofluorescence for rat NeuN (red) and GFP (green) in the coronal section of LHb brain
 1005 slices infected with AAV-GFAP::dnKir4.1 (AAV2/5-gfaABC1D-dnKir4.1-2A-eGFP) virus. Left are
 1006 examples of anterior, middle and posterior coronal section of LHb. Numbers in the bottom right corner
 1007 are the number of merged cells/ number of NeuN+ cells in the viral-infected area. Right are zoom-in
 1008 images of the white square area in left. Note that there is only one infected neuron, as indicated by
 1009 the white arrow, in all three fields of view.
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Contents

2 **Supplementary Table 1: Model parameters**

3 **Model description**

4

5 Supplementary Table 2: Model parameters

6

	Value	Unit	Parameter
R	8.31	J · mol ⁻¹ · K ⁻¹	Gas constant
T	308	K	Absolute temperature
F	96485	C · mol ⁻¹	Faraday constant
dt	0.1	ms	Step time
c_N	30	pF	Neuronal capacitance
c_A	1	pF	Astrocytic capacitance
g_K	20	nS	Neuronal K ⁺ channel conductance
g_{Na}	120	nS	Neuronal Na ⁺ channel conductance
g_{Leak,N}	0.5	nS	Neuronal leak conductance
V_{Leak,N}	-50	mV	Neuronal leak potential
i_{max,N}	0.12	mM · ms ⁻¹	Neuronal Na ⁺ /K ⁺ pump rate
g_{Kir}	12	nS	Astrocytic Kir4.1 channel conductance on extracellular side
g_{Kir_vess}	12.5	nS	Astrocytic Kir4.1 channel conductance on vessel side
g_{Leak,A}	0.1	nS	Astrocytic leak conductance
V_{Leak,A}	-40	mV	Astrocytic leak potential
i_{max,A}	0.12	mM · ms ⁻¹	Astrocytic Na ⁺ /K ⁺ pump rate
V_h	178.46	mV	Kir4.1 channel parameter
V_s	119.47	mV	Kir4.1 channel parameter
V_{rest_A}	-10	mV	Kir4.1 channel parameter
km_k	3.0	mM	Na ⁺ /K ⁺ pump parameter
km_{Na}	10.	mM	Na ⁺ /K ⁺ pump parameter
V_N	-63.76	mV	Initial neuronal membrane potential
V_A	-87.08	mV	Initial astrocyte membrane potential
[K ⁺] _N	135.04	mM	Initial neuronal K ⁺ concentration
[K ⁺] _o	3.34	mM	Initial extracellular K ⁺ concentration
[K ⁺] _A	135.00	mM	Initial astrocytic K ⁺ concentration
[Na ⁺] _N	11.16	mM	Initial neuronal Na ⁺ concentration

$[Na^+]_o$	145.95	mM	Initial extracellular Na^+ concentration
$[Na^+]_A$	7.00	mM	Initial astrocytic Na^+ concentration
Vol_o	0.01	pL	Extracellular volume
Vol_N	1.08	pL	Neuronal volume
Vol_A	1.08	pL	Astrocytic volume
d_{K_N}	0.0001	ms ⁻¹	K^+ effective flux rate in the neuron
d_{K_O}	0.001	ms ⁻¹	K^+ effective flux rate in the extracellular space
d_{K_A}	0.1	ms ⁻¹	K^+ effective flux rate in the astrocyte
d_{Na_N}	0.0001	ms ⁻¹	Na^+ effective flux rate in the neuron
d_{Na_O}	0.01	ms ⁻¹	Na^+ effective flux rate in the extracellular space
d_{Na_A}	0.01	ms ⁻¹	Na^+ effective flux rate in the astrocyte
$[K^+]_{N_0}$	135	mM	Neuronal potassium concentration of the effective flux
$[K^+]_{O_0}$	3.5	mM	Extracellular potassium concentration of the effective flux
$[K^+]_{A_0}$	135	mM	Astrocytic potassium concentration of the effective flux
$[Na^+]_{N_0}$	12.0	mM	Neuronal sodium concentration of the effective flux
$[Na^+]_{O_0}$	144	mM	Extracellular sodium concentration of the effective flux
$[Na^+]_{A_0}$	7.0	mM	Astrocytic sodium concentration of the effective flux

8 **Model description**

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10 The mathematical description of I_K and I_{Na} were achieved through a Hodgkin-Huxley style
11 derivation of forward and backward rate equations^{1,2}:

$$I_K = g_K \cdot n^4 \cdot (V_n - E_{Kn})$$

$$I_{Na} = g_{Na} \cdot m^3 \cdot h \cdot (V_n - E_{Nan})$$

12 where g_K and g_{Na} are the conductance of fast Na^+ and K^+ channel; E_{Kn} and E_{Nan} are the
13 neuronal equilibrium potential for K^+ and Na^+ . The gate variables m , h , and n are dimensionless
14 activation and inactivation variables, which describe the activation and inactivation processes of
15 the sodium and potassium channels, each of which is governed by the following differential
16 equations:

$$dn/dt = \alpha_n(V_n) \cdot (1 - n) - \beta_n(V_n) \cdot n$$

$$dm/dt = \alpha_m(V_n) \cdot (1 - m) - \beta_m(V_n) \cdot m$$

$$dh/dt = \alpha_h(V_n) \cdot (1 - h) - \beta_h(V_n) \cdot h$$

17 where the forward and the backward rate α and β describe the transition between the closed and
18 open state of gate. The function of α and β are given by:

$$\alpha_n(V_n) = 0.01 \cdot (V_n + 55) / (1 - \exp[(-V_n - 55)/10])$$

$$\beta_n(V_n) = 0.125 \cdot \exp[(-V_n - 65)/80]$$

$$\alpha_m(V_n) = 0.1 \cdot (V_n + 40) / (1 - \exp[(-V_n - 40)/10])$$

$$\beta_m(V_n) = 4 \cdot \exp[(-V_n - 65)/18]$$

$$\alpha_h(V_n) = 0.07 \cdot \exp[(-V_n - 65)/20]$$

$$\beta_h(V_n) = 1 / (\exp[(-V_n - 35)/10] + 1)$$

19 The neuronal membrane potential is highly dependent on the neuronal equilibrium potential for K^+
20 and Na^+ , which are given by the Nernst equations:

$$E_{Kn} = R \cdot T / F \cdot \ln([K^+]_O / [K^+]_N)$$

$$E_{Nan} = R \cdot T / F \cdot \ln([Na^+]_O / [Na^+]_N)$$

21 where $[K^+]_N$ and $[K^+]_O$ are the neuronal and extracellular K^+ concentration; $[Na^+]_N$ and
22 $[Na^+]_O$ are the neuronal and extracellular Na^+ concentration; R , T and F are the gas constant,
23 absolute temperature and faraday constant.

24 Neuronal leak current is described by:

$$I_{\text{Leak},N} = g_{\text{Leak},N} \cdot (V_n - E_{\text{Leak},N})$$

25 where $g_{\text{Leak},N}$ is the neuronal leak conductance and $E_{\text{Leak},N}$ is the neuronal leak potential.

26 The formula of Kir4.1 channel was modified from a previous model³:

$$I_{\text{Kir}} = g_{\text{Kir}} \cdot \sqrt{[K^+]_o} / (1 + \exp((V_A + V_{\text{restA}} - E_{Ka} + V_h)/V_S)) (V_A + V_{\text{restA}} - E_{Ka})$$

$$I_{\text{Kir_vess}} = g_{\text{Kir_vess}} \cdot \sqrt{[K^+]_{o_0}} / (1 + \exp((V_A + V_{\text{restA}} - E_{Ka_vess} + V_h)/V_S)) (V_A + V_{\text{restA}} - E_{Ka_vess})$$

27 where g_{Kir} and $g_{\text{Kir_vess}}$ are the conductance of Kir4.1 channel on the extracellular and vessel

28 side respectively.

29 Astrocytic equilibrium potential for K^+ and Na^+ are given by:

$$E_{Ka} = R \cdot T/F \cdot \ln([K^+]_o/[K^+]_A)$$

$$E_{NaA} = R \cdot T/F \cdot \ln([Na^+]_o/[Na^+]_A)$$

30 where $[K^+]_A$ and $[Na^+]_A$ are the astrocytic K^+ and Na^+ concentration.

31 The astrocyte membrane potential equation also comprises a leak term:

$$I_{\text{Leak},A} = g_{\text{Leak},A} \cdot (V_A - E_{\text{Leak},A})$$

32 The equation of the Na^+/K^+ pump depends on the extracellular K^+ and intracellular Na^+

33 concentrations as:

$$i_{\text{pump},N} = i_{\text{max},N} \cdot (1 + km_k/[K^+]_o)^{-2} \cdot (1 + km_{Na}/[Na^+]_N)^{-3}$$

$$i_{\text{pump},A} = i_{\text{max},A} \cdot (1 + km_k/[K^+]_o)^{-2} \cdot (1 + km_{Na}/[Na^+]_A)^{-3}$$

34 where $i_{\text{max},N}$ and $i_{\text{max},A}$ are the Na^+/K^+ pump rate for neuron and astrocyte respectively.

35 The kinetics of ion concentrations are calculated as:

$$d[K^+]_o/dt = (I_{\text{Kir}} + I_K)/(F \cdot Vol_o) - 2i_{\text{pump},A} - 2i_{\text{pump},N} - d_{K_o} \cdot ([K^+]_o - [K^+]_{o,0})$$

$$d[K^+]_N/dt = -I_K/(F \cdot Vol_N) + 2i_{\text{pump},N} \cdot Vol_o/Vol_N - d_{K_N} \cdot ([K^+]_N - [K^+]_{N,0})$$

$$d[K^+]_A/dt = (-I_{\text{Kir}} - I_{\text{Kir_vess}})/(F \cdot Vol_A) + 2i_{\text{pump},A} \cdot Vol_o/Vol_A - d_{K_A} \cdot ([K^+]_A - [K^+]_{A,0})$$

$$d[Na^+]_o/dt = I_{Na}/(F \cdot Vol_o) + 3i_{\text{pump},A} + 3i_{\text{pump},N} - d_{Na_o} \cdot ([Na^+]_o - [Na^+]_{o,0})$$

$$d[Na^+]_N/dt = -I_{Na}/(F \cdot Vol_N) - 3i_{\text{pump},N} \cdot Vol_o/Vol_N - d_{Na_N} \cdot ([Na^+]_N - [Na^+]_{N,0})$$

$$d[Na^+]_A/dt = -3i_{\text{pump},A} \cdot Vol_o/Vol_A - d_{Na_A} \cdot ([Na^+]_A - [Na^+]_{A,0})$$

36 where Vol_o , Vol_N and Vol_A are the volumes of the extracellular, neuronal and astrocytic

37 compartments. The term $d \cdot ([X] - [X_0])$ accounts for the diffusion effect, where d is the rate of this

38 effective flux, $[X]$ is the ionic concentration and $[X_0]$ is the equilibrium concentration.

39 Simulations were solved numerically with an explicit Runge-Kutta Prince-Dormand 8th-9th order
40 method with the GSL library in cython and python. The parameters used in the model are
41 presented in Supplementary Table 1.

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