Lrp4 in astrocytes modulates glutamatergic transmission

Xiang-Dong Sun1,6, Lei Li1,6, Fang Liu1, Zhi-Hui Huang1, Jonathan C Bean1, Hui-Feng Jiao2, Arnab Barik1, Seon-Myung Kim1, Haitao Wu1, Chengyong Shen1, Yun Tian1, Thiri W Lin1, Ryan Bates1, Anupama Sathyamurthy1, Yong-Jun Chen1, Dong-Min Yin1, Lei Xiong1, Hui-Ping Lin1, Jin-Xia Hu1, Bao-Ming Li2,3, Tian-Ming Gao4, Wen-Cheng Xiong1,5 & Lin Mei1–3,5

Neurotransmission requires precise control of neurotransmitter release from axon terminals. This process is regulated by glial cells; however, the underlying mechanisms are not fully understood. We found that glutamate release in the brain was impaired in mice lacking low-density lipoprotein receptor–related protein 4 (Lrp4), a protein that is critical for neuromuscular junction formation. Electrophysiological studies revealed compromised release probability in astrocyte-specific Lrp4 knockout mice. Lrp4 mutant astrocytes suppressed glutamatergic transmission by enhancing the release of ATP, whose level was elevated in the hippocampus of Lrp4 mutant mice. Consequently, the mutant mice were impaired in locomotor activity and spatial memory and were resistant to seizure induction. These impairments could be ameliorated by blocking the adenosine A1 receptor. The results reveal a critical role for Lrp4, in response to agrin, in modulating astrocytic ATP release and synaptic transmission. Our findings provide insight into the interaction between neurons and astrocytes for synaptic homeostasis and/or plasticity.

In the nervous system, neurons communicate with each other by synapses, a tripartite structure consisting of the axon terminal of one neuron, the postsynaptic membrane of another neuron and the surrounding glial cell processes1–3. Synaptic transmission is critical for perception, thinking, learning and memory, and response to environmental changes. Synaptic dysfunction has been implicated in various neuropsychiatric disorders, including epilepsy, addiction, schizophrenia and autism. Neurotransmission requires precise control of neurotransmitter release from presynaptic terminals and responsiveness of neurotransmitter receptors on postsynaptic membrane. Increasing evidence suggests that this process is tightly regulated by glial cells1–4. Astrocytes, which account for more than half of the cells in the human brain5, ensheath a majority of excitatory synapses in the hippocampus (for example, see ref. 6). These cells regulate neuronal neurotransmission by releasing soluble factors such as ATP7,8. Astrocytic ATP can rapidly degrade to adenosine, which suppresses glutamatergic transmission by activating presynaptic purinergic receptors9,10. However, little is known about the signaling pathways that control the release of gliotransmitters.

Lrp4 is a type I single transmembrane protein of the LDL receptor family11,12. Recent studies indicate that Lrp4 serves as a receptor for agrin13,14, a motor nerve–derived factor15, and is critical for the formation and maintenance of the neuromuscular junction (NMJ)16,17, a peripheral cholinergic synapse between motor neurons and skeletal muscle fibers18. In the current working model, agrin binds to Lrp4 at the post-junctional membrane to form a tetrameric complex19, to activate the receptor-like tyrosine kinase MuSK in muscle fibers and downstream signaling pathways for post-junctional membrane differentiation20. Lrp4 in muscle fibers may also direct a retrograde signal for presynaptic differentiation at the NMJ21,22.

Lrp4 is expressed in the brain23,24 and has been implicated in hippocampal synaptic plasticity24,25. However, little is known about the underlying mechanisms. Previous studies have found that Lrp4 is a protein that is present at the postsynaptic density of pyramidal neurons23,24. We did not observe abnormal neurotransmission in mutant mice in which Lrp4 was ablated in excitatory neurons. Notably, glutamate release was impaired in astrocyte-specific Lrp4 mutant mice, as well as in GFAP-Lrp4−/− mice in which the Lrp4 gene was ablated in both neurons and astrocytes. These results suggest a role for Lrp4 in astrocytes during glutamatergic transmission. We investigated causal mechanisms using a combination of cell biology, electrophysiology and pharmacology techniques. Our results suggest that astrocytic Lrp4, in response to agrin, controls the release of ATP from astrocytes, and thereby maintains glutamatergic transmission.

RESULTS
Reduced sEPSC frequency in brain-specific Lrp4 mutant mice
Lrp4 was expressed in the brain, including the hippocampus23,24, and its expression was regulated developmentally (Supplementary Fig. 1a, b). We generated brain-specific Lrp4 mutant mice by crossing Lrp4loxP/loxP mice with GFAP::Cre mice, in which Cre expression is under control of the human glial fibrillary acidic protein (GFAP) promoter and restricted to neural progenitor cells that give rise to neurons and glial cells in the brain26 (Fig. 1a). GFAP::Cre; Lrp4loxP/loxP

1Department of Neuroscience and Regenerative Medicine, Medical College of Georgia, Augusta University, Georgia, USA. 2Center for Neuropsychiatric Diseases, Institute of Life Science, Nanchang University, Nanchang, China. 3Jiangxi Medical School, Nanchang University, Nanchang, China. 4State Key Laboratory of Organ Failure Research, Key Laboratory of Psychiatric Disorders of Guangdong Province, Department of Neurobiology, Southern Medical University, Guangzhou, China. 5Charlie Norwood Virginia Medical Center, Augusta, Georgia, USA. 6These authors contributed equally to this work. Correspondence should be addressed to X.-D.S. (xisun@augusta.edu) or L.M. (lmei@augusta.edu).

Received 24 September 2015; accepted 8 May 2016; published online 13 June 2016; doi:10.1038/nn.4326
Reduced EPSC frequency and impaired synaptic plasticity in GFAP-Lrp4−/− mice. (a) Lrp4loxP/loxP mice were crossed with GFAP::Cre mice; the resulting GFAP::Cre;Lrp4loxP/loxP mice were crossed with Lrp4loxP/loxP mice to generate GFAP::Cre;Lrp4loxP/loxP (GFAP-Lrp4−/−) and Lrp4loxP/loxP (control) mice. (b) Lrp4 was not detectable in hippocampus of a 1-month-old GFAP-Lrp4−/− mouse. Shown are representative blots of three independent experiments. Full-length blots/gels are presented in Supplementary Figure 12. (c) Depressed I/O curves in the hippocampus of GFAP-Lrp4−/− mice. (d) Recording diagrams. Pyramidal neurons were recorded in whole-cell configuration. Blue color denotes control (GFAP-Lrp4−/−) mice; red color denotes GFAP-Lrp4−/− mice. Scale bars represent 2 s, 10 pA. (e,f) Cumulative probability plots of sEPSC inter-event intervals and histograms of sEPSC frequency (f) and amplitude (g) (n = 15 neurons of 4 mice for both genotypes; f: Student’s t test, t28 = 2.538, P = 0.017; g: Student’s t test, t28 = 0.9343, P = 0.3581). (h) Representative traces of mEPSCs in CA1 pyramidal neurons from control and GFAP-Lrp4−/− mice. Scale bars represent 2 s, 10 pA. (i,j) Cumulative probability plots of mEPSC inter-event intervals and histograms of mEPSC frequency (i) and amplitude (j) (n = 29 neurons, 5 control mice; n = 22 neurons, 5 GFAP-Lrp4−/− mice; i: Student’s t test, t49 = 2.12, P = 0.0391; j: Student’s t test, t49 = 0.6891, P = 0.494). (k) Depressed I/O curves in the hippocampus of GFAP-Lrp4−/− mice. iEPSPs were recorded by stimulating SC-CA1 pathway with gradual increasing intensities (n = 9 slices from 4 mice for both genotypes; two-way ANOVA, F11,112 = 20.35, P < 0.0001). (l) Impaired LTD at SC-CA1 synapses in the hippocampus of GFAP-Lrp4−/− mice. Normalized iEPSP slopes were plotted every 1 min. Arrow denotes LTD induction. Shown on the right were representative traces taken before (1) and 50 min after high-frequency stimulation (2). Scale bars represent 2 ms, 0.2 mV. (m) Quantitative analysis of LTD level in slices (n = 9 slices from 4 mice for both genotypes; Student’s t test, t149 = 2.879, P = 0.0109). Data in c and k are presented as mean ± s.e.m.; data in f, g, i, j and m are presented as median with interquartile range, whiskers are the minimum and maximum; *P < 0.05, **P < 0.01.

(GFAP-Lrp4−/−) mice were viable and showed no difference in body weight than control mice (Lrp4loxP/loxP) (data not shown). Western blot analysis revealed that Lrp4 was barely detectable in the hippocampus of GFAP-Lrp4−/− mice (Fig. 1b,c). We recorded, in whole-cell configuration, spontaneous and miniature excitatory postsynaptic currents (sEPSCs and mEPSCs, respectively) of pyramidal neurons in the CA1 region of the hippocampus. sEPSC and mEPSC frequency were decreased in GFAP-Lrp4−/− hippocampus compared with control (Fig. 1d–f,h,i). No change was observed in sEPSC and mEPSC amplitude (Fig. 1g,j). In addition, there was a downward shift of fEPSP slope (% collapse) (Fig. 1j). These results are suggestive of hypofunction of glutamatergic transmission in Lrp4 mutant mice. Consistent with this, long-term potentiation in the hippocampus was impaired (Fig. 1l,m)24,25. These observations indicate that Lrp4 is involved in proper glutamatergic transmission and synaptic plasticity in the brain.

To investigate underlying mechanisms, we first characterized the morphology of mutant mouse brains. They showed no detectable defects in the laminar structure of the cortex and hippocampus or in the number of neurons (Supplementary Fig. 1c–f). Similar levels of synaptic proteins were found in homogenates of mutant and control hippocampus (Supplementary Fig. 2a,b). Dentritic arborization of CA1 neurons in control and Lrp4 mutant mice was comparable, and quantitative Sholl analysis revealed no difference in the number of dendritic intersections between genotypes (Supplementary Fig. 2c,d). In addition, Lrp4 mutation had no effect on the intrinsic excitability of pyramidal neurons, I/V curves of AMPA and NMDA receptor (AMPA and NMDAR)-mediated currents, or AMPA/NMDA ratio (Supplementary Fig. 3), consistent with our observations of little change in sEPSC and mEPSC amplitude in GFAP-Lrp4−/− mice (Fig. 1g,j).

CA1 neurons of GFAP-Lrp4−/− mice displayed fewer spines than control mice (Supplementary Fig. 4a,b), consistent with a recent report24. To determine whether Lrp4 mutation alters glutamate release, we performed three sets of experiments. First, we characterized the paired-pulse discharge of sEPSCs and mEPSCs.
and maximum; * given in the presence of MK-801 was considered as 100%. (e) Representative traces of NMDAR currents plotted against stimulus number. The first pulse in the presence of MK-801 was considered as 100%. (f) Representative traces of NMDAR currents plotted against stimulus number. The first pulse in the presence of MK-801 was considered as 100%. (g) Reduced release probability, reduced synaptic efficacy and no change in synaptic potency (n = 13 neurons, 4 control mice; n = 14 neurons, 4 GFAP-Lrp4−/− mice; g; Student’s t test, t(25) = 2.682, P = 0.0126; h; Student’s t test, t(25) = 2.615, P = 0.0149; i; Student’s t test, t(23) = 1.1254, P = 0.9012). Data in b are presented as mean ± s.e.m.; data in e and g–i are presented as median with interquartile range, whiskers are the minimum and maximum; *P < 0.05, **P < 0.01.

Figure 2 Reduced excitatory vesicle release probability in the absence of Lrp4. (a) Representative superimposed sweeps with three different inter-stimulus intervals of pair pulse stimulations from control and GFAP-Lrp4−/− mice. Scale bars represent 50 ms, 100 pA. (b) PPRs plotted against inter-stimulus intervals (n = 11 neurons, 3 mice for both control and GFAP-Lrp4−/− genotypes; two-way ANOVA, F(1,60) = 13.55, P = 0.0005). (c) Representative traces of NMDAR currents in the presence of MK-801 (40 μM). Scale bars represent 20 ms, 40 pA. (d) Normalized NMDAR currents plotted against stimulus number. The first pulse in the presence of MK-801 was considered as 100%. (e) Increased τ values in GFAP-Lrp4−/− mice (n = 7 neurons, 3 mice for both genotypes; Student’s t test, t(123) = 3.594, P = 0.0037). (f) Representative traces of EPSCs evoked by minimal stimulation. Scale bars represent 5 ms, 10 pA. (g–i) Reduced release probability, reduced synaptic efficacy and no change in synaptic potency (n = 13 neurons, 4 control mice; n = 14 neurons, 4 GFAP-Lrp4−/− mice; g; Student’s t test, t(25) = 2.682, P = 0.0126; h; Student’s t test, t(25) = 2.615, P = 0.0149; i; Student’s t test, t(23) = 1.1254, P = 0.9012). Data in b are presented as mean ± s.e.m.; data in e and g–i are presented as median with interquartile range, whiskers are the minimum and maximum; *P < 0.05, **P < 0.01.

Modulation of glutamatergic transmission by Lrp4 in astrocytes

Because of the lack of a reliable Lrp4 antibody for immunostaining, we generated Lrp4−/− mice, in which the Lrp4 gene was replaced with a cassette encoding a β-galactosidase (β-gal) fusion protein, to determine where Lrp4 is expressed. Under the control of the endogenous promoter, β-gal expression is thought to faithfully indicate where Lrp4 is expressed. X-gal staining revealed that β-gal activity was enriched in stratum lacunosum-molecuare layer (SLM) and molecular layer (ML) of the hippocampus (Fig. 3a), areas in which astrocytes were abundant. The staining was weak in stratum pyramidale layer (SP), where somas of pyramidal neurons were located. Moreover, β-gal-labeled cells expressed the astrocyte marker GFAP, whereas colocalization of it with the neuronal marker Neun was hardly detectable (Fig. 3b), suggesting that Lrp4 is abundantly expressed in astrocytes. This notion was confirmed by western blotting of astrocyte cultures from hippocampus (Supplementary Fig. 5a).

To determine whether astrocytic Lrp4 is critical for glutamatergic transmission, we used GFAP::CreER mice in which the expression of CreER is driven by a 2-kb minimal promoter of the human GFAP gene31,32. The Cre recombinase is inactive, but can be induced by tamoxifen (Supplementary Fig. 5b). To verify the specificity of this line, we crossed GFAP::CreER mice with Rosa::LSL-tdTomato reporter mice to generate GFAP::CreER;tdTomato mice. tdTomato co-stained with the astrocytic marker GFAP, but not the neuronal marker Neun, in hippocampal slices of tamoxifen-treated GFAP::CreER;tdTomato mice (Supplementary Fig. 5c). These results demonstrate that Cre activity was specifically activated in GFAP+ cells in GFAP::CreER mice, in agreement with previous reports31,32. Next, we crossed GFAP::CreER mice with Lrp4loxP/loxP mice to generate GFAP::CreER;Lrp4loxP/loxP mice. Lrp4 level was decreased in these mice 2 weeks after tamoxifen treatment compared with control mice (Fig. 3d). We hereafter refer to tamoxifen-treated GFAP::CreER;Lrp4loxP/loxP mice simply as GFAP::CreER;Lrp4−/− mice. Spine numbers of CA1 neurons were similar between control and GFAP::CreER;Lrp4−/− mice (Supplementary Fig. 5d,e), indicating that spines were not altered by astrocyte-specific knockout of Lrp4. However, CA1 pyramidal neurons displayed a reduction in sEPSC and mEPSC frequency, but not amplitude (Fig. 3e–k); increased PPR (Fig. 3i); decreased τ value in GFAP::CreER;Lrp4−/− mice in MK-801 block assay (Fig. 3m); down shifted I/0 curve of IEPSCs (Fig. 3n); and deficient LTP (Fig. 3o,p) compared with control mice. These results demonstrate that astrocytic ablation of Lrp4 impairs presynaptic glutamate release and synaptic plasticity without altering spines, suggesting that Lrp4 in astrocytes is critical for glutamatergic transmission. Consistent with this, Lrp4 mutation specifically in CA1 pyramidal neurons (by Camk2a::Cre) had no apparent effect on Lrp4 level, sEPSC frequency, amplitude or PPR (Supplementary Fig. 6).

Increased astrocytic ATP release by Lrp4 mutation

Lrp4 mutation did not change the number of astrocytes or their morphology, including the distance between astrocytic and synaptic
membranes (Supplementary Figs. 4g and 7a–f). To investigate how Lrp4 in astrocytes modulates glutamatergic transmission, we cultured hippocampal neurons of E18 embryos and astrocytes from P2–3 hippocampus and grew them in separate dishes. Lrp4 mutation had little effect on the number of cultured neurons and astrocytes (Supplementary Fig. 7g–j). At 10 d in vitro (DIV10), we co-cultured one coverslip of neurons and three coverslips of confluent astrocytes, without contact, in a 35-mm dish for 24 h before recording. The sEPSC frequency was decreased in control neurons that were co-cultured with Lrp4 mutant astrocytes compared with that of control neurons co-cultured with control astrocytes (Fig. 4a–c), indicating that Lrp4 in astrocytes is critical for glutamatergic transmission.

sEPSC frequency of Lrp4 mutant neurons that were co-cultured with control astrocytes was similar to that of control neuron–control astrocyte co-culture (Fig. 4c). This suggests a dispensable role of the Lrp4 gene in neurons, consistent with a previous study of neuron–specific mutant mice. Moreover, mutant neurons co-cultured with mutant astrocytes showed similar reduction in sEPSC frequency (Fig. 4c). Notice that sEPSC amplitudes were similar in all combinations of co-culture, suggesting that Lrp4 mutation has no effect on sEPSC amplitudes (Fig. 4d). These findings suggest that Lrp4 in astrocytes modulate glutamatergic transmission through a secretable factor. Factors released from astrocytes that regulate neuronal transmission include D-serine and ATP4,7,8. D-serine activates NMDAR.

Figure 3 Astrocytic Lrp4 is critical for glutamatergic transmission. (a) Enriched β-gal activity in SLm and ML layers. X-gal staining was carried out with coronal brain sections of muscle-rescued Lrp4-LacZ homozygous mice. Left, whole-brain section; right, enlarged dotted area of the left image. SO: stratum oriens; SP: stratum pyramidale; SR: stratum radiatum; SLm: stratum lacunosum-moleculare; ML: molecular layer. Scale bars represent 2 mm (left), 0.2 mm (right). Shown are representative images of more than three independent experiments. (b) Colocalization of β-gal with astrocytic marker GFAP. Sections were stained with antibodies to β-gal and GFAP or neuronal marker NeuN. Arrowheads, cells expressing both β-gal and GFAP; arrows, cells expressing NeuN, but not β-gal. Scale bars represent 20 μm (left), 5 μm (right). Shown are representative images of three independent experiments. (c) Reduced Lrp4 level in the hippocampus in GFAP-CreER;Lrp4−/− mice, 2 weeks after tamoxifen treatment. Shown are representative blots of three independent experiments. Full-length blots/gels are presented in Supplementary Figure 12. (d) Quantitative analysis of data in c (n = 4 pairs of mice). Lrp4 band density was normalized by the loading control β-actin; values of control mice were taken as 100% (paired Student’s t-test, t(23) = 3.727, P = 0.0337). (e) Recording diagrams. Pyramidal neurons were recorded in whole-cell configuration. Blue denotes Lrp4 mutant and red denotes control. (f) Representative sEPSC traces of CA1 pyramidal neurons of control and GFAP-CreER;Lrp4−/− mice. Scale bars represent 1 s, 10 pA. (g) Reduced sEPSC frequency in CA1 pyramidal neurons of GFAP-CreER;Lrp4−/− mice (n = 13 neurons, 4 control mice; n = 14 neurons, 4 GFAP-CreER;Lrp4−/− mice; Student’s t-test, t(23) = 2.145, P = 0.0419). (h) sEPSC amplitude was comparable between control and GFAP-CreER;Lrp4−/− hippocampus (n = 13 neurons, 4 control mice; n = 14 neurons, 4 GFAP-CreER;Lrp4−/− mice; Student’s t-test, t(23) = 0.3502, P = 0.7291). (i) Representative traces of mEPSCs in CA1 pyramidal neurons from control and GFAP-CreER;Lrp4−/− mice. Scale bars represent 2 s, 10 pA. (j) Reduction in mEPSC frequency (j), but not amplitude (k) (n = 13 neurons, 3 control mice; n = 12 neurons, 3 GFAP-CreER;Lrp4−/− mice; j, Student’s t-test, t(23) = 2.368, P = 0.0266; k: t(23) = 0.2528, P = 0.8027). (l) PPRs plotted against inter-stimulus intervals (n = 10 neurons, 4 control mice; n = 11 neurons, 4 GFAP-CreER;Lrp4−/− mice; two-way ANOVA, F(2,27) = 16.15, P = 0.0002). (m) Increased τ values in GFAP-CreER;Lrp4−/− mice (n = 7 neurons, 4 control mice; n = 8 neurons, 4 GFAP-CreER;Lrp4−/− mice; Student’s t-test, t(13) = 2.32, P = 0.0373). (n) Depressed I/O curves in the hippocampus of GFAP-CreER;Lrp4−/− mice. fEPSPs were recorded by stimulating SC-CA1 pathway with gradual increasing intensities (n = 8 slices from 4 control mice; n = 7 from 4 GFAP-CreER;Lrp4−/− mice; two-way ANOVA, F(1,91) = 14.52, P = 0.0003). (o) Impaired LTP at SC-CA1 synapses in the hippocampus of GFAP-CreER;Lrp4−/− mice. Normalized fEPSP slopes were plotted every 1 min. Arrow denotes LTP induction. Shown on the right are representative traces taken before (1) and 50 min after high frequency stimulation (2). Scale bars represent 4 ms, 0.4 mV. (p) Quantitative analysis of LTP level in o (n = 8 slices from 4 control mice; n = 7 from 4 GFAP-CreER;Lrp4−/− mice; Student’s t-test, t(13) = 2.354, P = 0.035). Data in d, g, h, j, k, m and p are presented as median with interquartile range, whiskers are the minimum and maximum; data in i and n are presented as mean ± s.e.m.; *P < 0.05, **P < 0.01.
on presynaptic terminal to promote glutamate release and that on postsynaptic membrane to potentiate NMDA response. We treated hippocampal slices with the NMDAR antagonist dl-AP5 (100 µM), which caused a dose-dependent reduction in sEPSC frequency (Supplementary Fig. 8b), consistent with previous reports. Notably, this effect was diminished in Lrp4−/− slices (Fig. 5a). These results suggest that N-serine's role as an Lrp4-modulated factor in the regulation of glutamate release is limited.

On the other hand, ATP can directly or indirectly (by converting to adenosine) regulate glutamate release. To investigate whether ATP is regulated by Lrp4, we determined whether its response is altered by Lrp4 mutation. Treatment with ATP (10 µM) reduced sEPSC frequency in hippocampal slices of control mice (Supplementary Fig. 8b), consistent with previous reports. Notably, this effect was diminished in GFAPlrp4−/− slices (Fig. 5a). The inability of exogenous ATP to reduce sEPSC frequency suggests that endogenous ATP might have been elevated by Lrp4 mutation. To test this hypothesis, we measured ATP levels in the hippocampus by microdialysis. Indeed, ATP level in GFAPlrp4−/− dialysates were higher than those in control mice (Fig. 5b), in support of the hypothesis. To identify the source of elevated ATP, we cultured hippocampal neurons and astrocytes of different genotypes and measured ATP in the condition medium. Higher ATP levels were observed in the condition medium of hippocampal mutant astrocytes than in that of controls. However, ATP levels were similar between the condition media of mutant and control neurons (Fig. 5b).

To further test this in vivo, we collected microdialysates from the hippocampus of GFAPlrp4−/− mice. Compared with controls, ATP level was higher in the hippocampus when Lrp4 was specifically knocked out in astrocytes (Fig. 5b). These results suggest that increased ATP in the brain came from Lrp4 mutant astrocytes, and not neurons. It should be noted that Lrp4 mutation had no effect on the protein level of APT5a (a major ATP synthetase) or total ATP concentration in astrocytes (Fig. 5c). These results support a working model in which Lrp4 may inhibit ATP release from astrocytes and, in the absence of Lrp4, ATP release from astrocytes is increased. In addition to ATP, the level of its metabolite adenosine was also elevated in hippocampal dialysates of mutant mice and condition medium of mutant astrocytes (Fig. 5f).

To determine whether ATP or adenosine is involved, we treated hippocampal slices with ARL67156, an inhibitor of ecto-ATPase that catalyzes the hydrolysis of ATP to ADP, or adenosine 5′-(α, β-methylene) diphosphate (AOPCP), a blocker of AMP dephosphorylation. Both chemicals increased sEPSC frequency in control slices, suggesting that adenosine is involved in regulating glutamate release (Fig. 5a and Supplementary Fig. 8c). This effect was increased in GFAPlrp4−/− slices (Fig. 5a). These results indicate that inhibition of the ATP-to-adenosine reaction ameliorates the suppression effect of Lrp4 mutation on glutamate release and suggest the involvement of adenosine. To further test this hypothesis, we treated hippocampal slices with Suramin (an antagonist of P2 receptor), DPCPX (a blocker of A1 receptor) and SCH58261 (an A2A antagonist). As observed previously, sEPSC frequency was increased by Suramin and DPCPX, but reduced by SCH58261 (Supplementary Fig. 8d–f), and Lrp4 mutation had no effect on the effects of Suramin or SCH58261 (Fig. 5a). In contrast, the effect of DPCPX was substantially increased in GFAPlrp4−/− slices (Fig. 5a). These data are consistent with the idea that the adenosine A1 receptor is the primary means by which elevated adenosine levels suppress glutamate release.

Modulation of astrocytic ATP release by agrin signaling

Lrp4 is a receptor of neuronal agrin for NMJ formation and maintenance. As a surface protein in astrocytes (Supplementary Fig. 9a), Lrp4 may serve as an agrin receptor for regulating ATP release. Indeed, agrin was expressed in various regions in the brain (Fig. 6a). The concentration of neuronal agrin (that is, the isoform with B′z′ insert) was more than 1,000-fold higher in cultured hippocampal neurons than in cultured astrocytes, suggesting that neurons are a major source of neuronal agrin in the brain (Fig. 6a). To determine whether agrin regulates ATP release, we treated astrocytes with agrin at a concentration that stimulates AChR clustering in muscle cells (Supplementary Fig. 9b). Agrin had no effect on astrocyte proliferation (Supplementary Fig. 9f). However, agrin stimulation caused an increase in MusK phosphorylation in astrocytes (Fig. 6d). Concomitantly, ATP level in astrocytic condition medium was reduced (Fig. 6f), suggesting that agrin may inhibit ATP release by activating MusK. These effects...
of agrin (to reduce ATP release and to activate MuSK) required Lrp4 (Fig. 6d–f). These observations demonstrate a role of agrin signaling in modulating ATP release from astrocytes.

Consistent with these findings, sEPSC frequency was enhanced in agrin-treated hippocampal slices (Fig. 6g), indicating that agrin is involved in modulating glutamatergic transmission. This effect was not observed in GFAP-CreER;Lrp4−/− slices (Fig. 6g), suggesting that it is dependent on astrocytic Lrp4. It should be noted that, in the presence of the AI receptor antagonist DPCPX, agrin was unable to further increase sEPSC frequency (Fig. 6g), indicating that the effect of agrin is dependent on AI receptor. Together, these observations support a working model in which agrin-Lrp4 signaling controls astrocytic ATP release that maintains homeostasis of glutamatergic activity.

**Behavioral deficits in GFAP-Lrp4−/− mice**

Synapse dysfunction has been implicated in various neurological and psychiatric disorders. To further investigate the function of Lrp4 in the CNS, we tested locomotor activity of GFAP-Lrp4−/− mice in an open field and found that they displayed a pronounced decrease in total travel distance compared with control mice (Fig. 7a,b), indicating that mutant mice were hypactive. In addition, the mutant mice had fewer entries into the central area and spent less time there (Fig. 7c,d).

However, no difference was observed between control and mutant mice in elevated plus maze and light and dark box tests, suggesting that the mutation had little anxiogenic effect (Supplementary Fig. 10a–h).

To investigate whether Lrp4 mutation results in deficits in learning and memory, we examined mutant mice in the Morris water maze test9. The mutant mice exhibited similar swimming velocity as control mice (Supplementary Fig. 10i). During the training phase, the escape latency for GFAP-Lrp4−/− mice to locate the hidden platform was also similar to that of control mice (Fig. 7e,f), suggesting that their learning ability may be normal. However, during the probe test, when the platform had been removed, GFAP-Lrp4−/− mice exhibited fewer crosses over the absent platform and spent less time in the 30-cm area (N30) surrounding the absent platform than control mice (Fig. 7g,h).

To determine whether the Lrp4 mutant mice are ‘smarter’ at reversal learning when the platform is absent, we analyzed the number of crosses over the absent platform and duration in the N30 area every 10 s for 60 s. We found no difference in the interaction between genotype and time (Supplementary Fig. 10j,k), which suggests that Lrp4 mutant mice may not adapt faster than control mice after the platform removal. Together, these results suggest that Lrp4 mutant mice may be impaired in spatial memory consolidation. To determine whether the behavioral impairment was mediated by A1 receptor activation, we injected mice with DPCPX 30 min before tests. DPCPX-treated GFAP-Lrp4−/− and control mice showed no difference in locomotor activity (Fig. 7b–d) and spatial memory (Fig. 7g,h). These observations demonstrate a role of Lrp4 for proper behavior and cognitive function.

Reduced glutamate release by Lrp4 mutation may alter the vulnerability to epilepsy. To test this hypothesis, we injected mice with pilocarpine to generate status epilepsy40. Most control mice developed status epilepticus after the fifth injection (Fig. 7i). In contrast, a majority of GFAP-Lrp4−/− mice did not develop status epilepticus even after the eighth injection (Fig. 7j). These data indicate that

---

**Figure 5** Modulation of astrocytic ATP release by Lrp4. (a) Effects of chemicals on sEPSCs of CA1 pyramidal neurons in control and GFAP-Lrp4−/− mice. sEPSC frequency was calculated by (f2 − f1)/f1, where f1 and f2 were the frequencies of sEPSCs recorded before and after drug treatment, respectively. The number of recorded neurons and mice were as follows: eight neurons of three control mice and nine neurons of three GFAP-Lrp4−/− mice for DL-AP5 (100 µM) (Student’s t test, t(15) = 0.2202, P = 0.8287), seven neurons of three mice of each genotype for ATP (10 µM) (Student’s t test, t(15) = 2.944, P = 0.0123), eight neurons of four mice of each genotype for ARL67156 (100 µM) (Student’s t test, t(15) = 2.675, P = 0.0181), eight neurons of three mice of each genotype for AOPCP (300 µM) (Student’s t test, t(14) = 2.483, P = 0.0263), nine neurons of three mice of each genotype for Suramin (10 µM) (Student’s t test, t(16) = 0.4528, P = 0.6567), seven neurons of three control mice and eight neurons of three GFAP-Lrp4−/− mice for DPCPX (800 nM) (Student’s t test, t(13) = 2.451, P = 0.0291), and eight neurons of three control mice and seven neurons of three GFAP-Lrp4−/− mice for SCH58261 (5 µM) (Student’s t test, t(13) = 0.8303, P = 0.4213). (b) Increased ATP levels in the dialysate from GFAP-Lrp4−/− and GFAP-CreER;Lrp4−/− hippocampus and condition medium of mutant astrocytes, but not neurons. Cells were cultured from control and GFAP-Lrp4−/− (mutant) mice (n = 5 samples of dialysate from 5 control and 5 GFAP-Lrp4−/− mice, Student’s t test, t(9) = 3.33, P = 0.0104; n = 4 samples of dialysate from 4 control and 4 GFAP-CreER;Lrp4−/− mice; Student’s t test, t(9) = 3.565, P = 0.0119; n = 8 dishes (4 mice) for astrocytes, Student’s t test, t(14) = 2.276, P = 0.0391; n = 5 dishes (4 mice) for neurons, Student’s t test, t(9) = 0.7401, P = 0.4804). (c) Western blotting showing similar level of ATP5a in cultured astrocytes of control and mutant genotype. Shown are representative blots of two independent experiments with similar results. Full-length blots and gels are presented in Supplementary Figure 12. (d) Quantitative analysis of data in c (n = 4 dishes from 2 times of astrocyte culture for each genotype). ATP5a band density was normalized by the loading control β-actin; values of control mice were taken as 100% (paired Student’s t test, t(6) = 2.923, P = 0.0613). (e) Similar levels of total ATP in mutant hippocampal astrocytes. Cultured astrocytes were homogenized. Supernatants after centrifugation were subjected to ATP measurement (n = 8 dishes from 2 times of culture for both genotypes; paired Student’s t test, t(6) = 0.4237, P = 0.6845). (f) Increased adenosine levels in the dialysate from GFAP-Lrp4−/− and GFAP-CreER;Lrp4−/− hippocampus and condition medium of mutant astrocytes, but not neurons (n = 5 samples of dialysate from 5 control and 5 GFAP-Lrp4−/− mice, Student’s t test, t(9) = 3.394, P = 0.0094; n = 4 samples of dialysate from 4 control and 4 GFAP-CreER;Lrp4−/− mice, Student’s t test, t(9) = 2.816, P = 0.0305; n = 6 dishes (4 mice) for astrocytes, Student’s t test, t(10) = 2.562, P = 0.0283; n = 4 dishes (4 mice) for neurons, Student’s t test, t(9) = 0.4766, P = 0.6505). Data in a, b, e and f are presented as median with interquartile range, whiskers are the minimum and maximum; data in d are presented as mean ± s.e.m.; P ≥ 0.05 was considered not significant (n.s.); ∗P < 0.05.
Astrocytes are intimately involved in many aspects of brain development, function and disease\(^1\)–\(^3\). Our results identify a previous unknown function of Lrp4 in the modulation of glutamatergic transmission. When Lrp4 was mutated in all cells in developing brain (that is, by GFAP-Cre), mutant mice displayed a reduction in both spine density and mEPSC frequency. However, ablation of Lrp4 specifically in astrocytes (that is, in tomaxifen-treated GFAP::Cre;Lrp4\(^{-/-}\)) mice had a raised threshold to seizure. To further test this notion, we injected mice with pentylenetetrazol (PTZ), a GABAA receptor antagonist that induces seizure via different mechanisms\(^4\). The latency to the onset of generalized convulsive seizures was higher in GFAP-Lrp4\(^{-/-}\) mice than in control mice (Fig. 7k). The increased vulnerability in Lrp4 mutant mice was diminished by DPCPX treatment (Fig. 7k), once again suggesting the involvement of the A1 receptor.

**DISCUSSION**

Astrocytes are intimately involved in many aspects of brain development, function and disease\(^1\)–\(^3\). Our results identify a previous unknown function of Lrp4 in the modulation of glutamatergic transmission. When Lrp4 was mutated in all cells in developing brain (that is, by GFAP-Cre), mutant mice displayed a reduction in both spine density and mEPSC frequency. However, ablation of Lrp4 specifically in astrocytes (that is, in tomaxifen-treated GFAP-CreER;Lrp4\(^{-/-}\)) mice had no effect on spine numbers of hippocampal CA1 neurons, but instead reduced mEPSC frequency and glutamate release probability (Fig. 3j,m and Supplementary Fig. 5d,e). These observations suggest that Lrp4 mutation may alter glutamate release. Consistent with this, electrophysiological studies revealed increased PPR, decreased decay of NMDAR currents in the presence of MK-801 and reduced successful rate of EPSCs by minimal stimulation in these mutant mice, suggesting compromised probability of glutamate release. Together, our results indicate that astrocytic Lrp4 modulates glutamate release.

The experiments with non-contact neuron-astrocyte co-culture provide evidence that Lrp4 mutant astrocytes impair glutamatergic transmission via a secretory factor. Such factors include glutamate and the co-agonist \(\delta\)-serine\(^4\)–\(^8\),\(^42\), which have been shown to promote glutamate release by activating presynaptic NMDARs\(^33\),\(^43\). Postsynaptically, activation of NMDARs is expected to change the amplitude of NMDAR-mediated EPSCs and AMPA/NMDA ratio\(^33\),\(^44\). However, neither was changed in Lrp4 mutant mice (Supplementary Fig. 3h and data not shown), suggesting that there is similar NMDAR level at synapses. In addition, GFAP-Lrp4\(^{-/-}\) CA1 neurons displayed similar \(\alpha\)-IV curves (Supplementary Fig. 3g). Thus, the factor regulated by Lrp4 did not appear to be glutamate or \(\delta\)-serine. The following evidence suggests that this factor may be ATP. First, levels of ATP and its derivative adenosine were higher in the dialysates from Lrp4 mutant hippocampus and in the condition medium of culture Lrp4 mutant astrocytes, as compared with controls. Second, Lrp4 mutation–mediated reduction of sEPSC frequency was not inhibited by Suramin, an antagonist of P2 receptor for ATP, but was inhibited by ARL67156 or AOPCP (to inhibit ATP hydrolysis) or by DPCPX (to block adenosine A1 receptor). These observations suggest that A1 receptor is involved in suppressing glutamatergic transmission. Third, DPCPX also diminished behavioral deficits in GFAP-Lrp4\(^{-/-}\) mice, including decreased locomotor activity, impaired spatial memory and increased threshold to seizure induction. These observations suggest that glutamatergic transmission in Lrp4 mutant mice is likely suppressed by A1 receptor activation by adenosine, a product of ATP hydrolysis.

Despite ATP having an established role in regulating glutamatergic transmission, the mechanisms of astrocytic ATP release have not yet been elucidated\(^7\),\(^8\),\(^42\). Astrocytic ATP release could be mediated by activation of metabotropic and ionotropic receptors\(^45\). It could also be modulated by anion channels, ATP-binding cassette proteins, gap junctions, P2X7 receptors and lysosome\(^46\)–\(^49\). Our results provide evidence that ATP release from astrocytes is regulated by agrin signaling, a pathway critical for NMJ formation and maintenance\(^13\),\(^14\),\(^17\),\(^21\),\(^22\). Notably, MuSK expression in astrocytes is more...
than fivefold higher than in skeletal muscles (Fig. 6c). Thus, our findings reveal a previously unknown function of the agrin signaling in the brain. How agrin regulates ATP release is unclear. Previous studies have suggested that astrocytic ATP release involves SNARE-dependent exocytotic pathways. However, Lrp4 did not appear to associate with SNARE proteins in co-precipitation experiments (data not shown). Agrin signaling is likely to regulate ATP release via a unique mechanism. The possible involvement of MuSK provides new leads for future investigation.

The spine deficits in GFAP-Lrp4<sup>−/−</sup> mice (Supplementary Fig. 4a,b) and Lrp4<sup>mutt</sup> (muscle rescued) mice<sup>24</sup> suggest a role for Lrp4 in spine development. Astrocytes have been implicated in synaptogenesis<sup>3</sup>. Given the Lrp4 astrocytic enrichment in brain regions, particularly in the hippocampus (Fig. 3), the spine deficits may result from loss of secretable factors from astrocytes, such as thrombospondins (TSPs)<sup>50</sup>, during development. Lrp4 regulation of spinogenesis may differ, as there were no apparent spine deficits resulting from astrocytic ablation of Lrp4 in young adult (GFAP-CreER;Lrp4<sup>−/−</sup>) mice (Supplementary Fig. 5d,e). We noticed that PPR was found not changed in the hippocampus of Lrp4<sup>mutt</sup> mice<sup>24</sup>, however, could express a truncated N-terminal fragment of Lrp4 containing eight LDLA repeats<sup>16</sup>. The Lrp4 mutant mice that we examined, however, could express a truncated N-terminal fragment of Lrp4 containing eight LDLA repeats<sup>16</sup>. The Lrp4 mutant mice that we examined, however, could express a truncated N-terminal fragment of Lrp4 containing eight LDLA repeats<sup>16</sup>. The Lrp4 mutant mice that we examined, however, could express a truncated N-terminal fragment of Lrp4 containing eight LDLA repeats<sup>16</sup>. The Lrp4 mutant mice that we examined, however, could express a truncated N-terminal fragment of Lrp4 containing eight LDLA repeats<sup>16</sup>. The Lrp4 mutant mice that we examined, however, could express a truncated N-terminal fragment of Lrp4 containing eight LDLA repeats<sup>16</sup>. The Lrp4 mutant mice that we examined, however, could express a truncated N-terminal fragment of Lrp4 containing eight LDLA repeats<sup>16</sup>. The Lrp4 mutant mice that we examined, however, could express a truncated N-terminal fragment of Lrp4 containing eight LDLA repeats<sup>16</sup>. The Lrp4 mutant mice that we examined, however, could express a truncated N-terminal fragment of Lrp4 containing eight LDLA repeats<sup>16</sup>. The Lrp4 mutant mice that we examined, however, could express a truncated N-terminal fragment of Lrp4 containing eight LDLA repeats<sup>16</sup>. The Lrp4 mutant mice that we examined, however, could express a truncated N-terminal fragment of Lrp4 containing eight LDLA repeats<sup>16</sup>. The Lrp4 mutant mice that we examined, however, could express a truncated N-terminal fragment of Lrp4 containing eight LDLA repeats<sup>16</sup>. The Lrp4 mutant mice that we examined, however, could express a truncated N-terminal fragment of Lrp4 containing eight LDLA repeats<sup>16</sup>. The Lrp4 mutant mice that we examined, however, could express a truncated N-terminal fragment of Lrp4 containing eight LDLA repeats<sup>16</sup>. The Lrp4 mutant mice that we examined, however, could express a truncated N-terminal fragment of Lrp4 containing eight LDLA repeats<sup>16</sup>. The Lrp4 mutant mice that we examined, however, could express a truncated N-terminal fragment of Lrp4 containing eight LDLA repeats<sup>16</sup>. The Lrp4 mutant mice that we examined, however, could express a truncated N-terminal fragment of Lrp4 containing eight LDLA repeats<sup>16</sup>.
is >1000-fold greater than in astrocytes and Lrp4 is enriched in astrocytes, future studies are warranted to determine whether other functions of astrocytes are regulated by the agrin-Lrp4 signaling. Such studies may provide insight into the interaction between neurons and astrocytes for synaptic homeostasis and/or plasticity and pathological mechanisms of neurological and psychiatric disorders.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

We are grateful to W.-P. Ge (UT Southwestern Medical Center) and K.D. McCarthy (University of North Carolina) for GFAP::CreER mice. We thank members of the Mei and Xiong laboratories for helpful discussions. This work was supported in part by grants from the US National Institutes of Health (L.M., W.-C.X.) and Veterans Affairs (L.M., W.-C.X.), “Thousand Talents” Innovation Project from Jiangxi Province (L.M.), National Natural Science Foundation of China (NSFC, 81471116, B.-M.L), NSFC (81329003; U1201225; 31430032, T.-M.G.), Guangzhou Science and Technology Project (201300000093, T.-M.G.) and Specialized Research Fund for the Doctoral Program of Higher Education of China (2013443130002, T.-M.G.).

AUTHOR CONTRIBUTIONS

X.-D.S. and L.M. conceived, designed and directed the project, and wrote the manuscript. X.-D.S. performed electrophysiological recordings and analysis. L.L. conducted immunoblots, quantitative reverse-transcription PCR (qRT-PCR) and co-immunoprecipitation. F.L., R.B., A.B. and A.S. conducted Golgi staining, X-gal staining, immunofluorescence staining and analysis. Z.-H.H. conducted behavioral tests and microdialysis analysis, with the assistance of J.-C.Y. and D.-M.Y. H.-F.J., S.-M.K. and Y.T. conducted cell culture experiments and analysis. H.W. and C.S., provided and assisted with characterization of Lrp4 conditional knockouts. T.W.L. conducted spine and synapse analysis. L.X., H.-P.L., J.-X.H. assisted with breeding and genotyping Lrp4 mutant lines. B.-M.L., T.-M.G. and W.-C.X. helped with data interpretation and provided instruction. L.M. supervised the project.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.
Cultured astrocytes were incubated with Sulfo-NHS-biotin (ThermoFischer Scientific) in phosphate-buffered saline (PBS) for 45 min at 4 °C, and treated with 10 mM glycine to quench all unreacted biotin. After rinse with PBS, cells were lysed in RIPA buffer. Biotinylated proteins were precipitated overnight with Avidin Agarose Beads (Pierce).

**Cell surface biotinylation.** Cultured astrocytes were incubated with Sulfo-NHS-biotin (ThermoFischer Scientific) in phosphate-buffered saline (PBS) for 45 min at 4 °C, and treated with 10 mM glycine to quench all unreacted biotin. After rinse with PBS, cells were lysed in RIPA buffer. Biotinylated proteins were precipitated overnight with Avidin Agarose Beads (Pierce).

**Electrophysiological recording.** Hippocampal slices were prepared as described previously57. Mice (5–7 weeks old, male) were anesthetized with ketamine/xylazine (Sigma, 100/20 mg/kg, respectively, intraperitoneal), brains were quickly removed and chilled in ice-cold modified artificial cerebrospinal fluid (ACSF) containing (in mM): 250 glucose, 2 KCl, 10 MgSO4, 0.2 CaCl2, 1.3 NaH2PO4, 26 NaHCO3, and 10 glucose. Coronal hippocampal slices (300 µm) were cut in ice-cold modified ACSF using a VT-1000S vibratome (Leica) and transferred to a storage chamber containing regular ACSF (in mM) (126 NaCl, 3 KCl, 1 MgSO4, 2 CaCl2, 1.25 NaH2PO4, 26 NaHCO3, and 10 glucose) at 34 °C for 30 min and at 25 ± 1 °C for additional 1 h before recording. All solutions were saturated with 95% O2 and 5% CO2 (vol/vol).

Slices were placed in the recording chamber, which was superfused (2 ml/min) with ACSF at 32–34 °C. Whole-cell patch-clamp recording from CA1 pyramidal neurons were visualized with infrared optics using an upright microscope equipped with a 40× water-immersion lens (Axioskop 2 Plus, Zeiss) and infrared-sensitive CCD camera (C2400-75, Hamamatsu). The Pipettes were pulled by a micropipette puller (P-97, Sutter instrument) with a resistance of 3–5 MΩ. Recordings were made with MultiClamp 700B amplifier and 1440A digitizer (Molecular Device). For sEPSC recording, pyramidal neurons were held at −70 mV in the presence of 20 µM BMI, with the pipette solution containing (in mM): 125 cesium methanesulfonate, 5 CsCl, 10 HEPES, 0.2 EGTA, 1 MgCl2, 4 Mg-ATP, 0.3 Na-GTP, 10 phosphocreatine and 5 QX314 (pH 7.4, 285 mosm). In co-culture experiments, neurons on the Cover slips were recorded for sEPSCs with bath solution containing (in mM): 140 NaCl, 5 KCl, 1 MgSO4, 2 CaCl2, 1.25 NaH2PO4, 26 NaHCO3, and 10 glucose (pH 7.4, 290 mosm). In the presence of co-culture medium (20% of total solution), mEPSCs were recorded in the presence of 1 µM TTX.

To detect electric property of CA1 excitatory neurons, they were current-clamped and measured by injecting a series of depolarizing pulses (from 50 to 140 pA at a step of 10 pA) in the presence of 20 µM CNQX, 100 µM m-AP5 and 20 µM BMI, with the pipette solution containing (in mM): 125 K-glutamate, 5 KCl, 10 HEPES, 0.2 EGTA, 1 MgCl2, 4 Mg-ATP, 0.3 Na-GTP, 10 phosphocreatine and 5 QX314 (pH 7.4, 285 mosm). The spike threshold was calculated in response to 50-µA current injection. Membrane input resistance was calculated in response to a series of hyperpolarizing pulses.

To investigate property of AMPAR and NMDAR currents, EPSCs were evoked by stimulating SC-CA1 pathway at holding potentials ranging from −60 to +60 mV at a step of 20 mV in the presence of 20 µM BMI. AMPAR and NMDAR current were measured as the peak amplitudes and 50 ms after the peak amplitude, respectively.

For PPRs recording, EPSCs were evoked by stimulating SC-CA1 pathway at holding potential of −70 mV in the presence of 20 µM BMI. Interval of paired stimulations was set 25, 50 and 100 ms respectively. Value of ratios was defined as [p2/p1] × 100, where p1 and p2 are the amplitude of the EPSCs evoked by the first and second pulse, respectively.

For MK-801 block assay, pyramidal neurons in the CA1 of hippocampus were clamped at +40 mV. Evoked NMDAR-mediated EPSCs were recorded in response to 0.1 Hz presynaptic stimulation in the presence of 20 µM CNQX and 20 µM BMI. Slices were treated with 40 µM MK801 for 5 min before evoking.
and recording EPSCs for 20 min. EPSC amplitudes were normalized to the first EPSC, and fitted with single-exponential functions to calculate decay constants (τ, in number of stimuli).

For minimal stimulation recording, CA1 pyramidal neurons were clamped at −70 mV. EPSCs were evoked by stimulating the SC-CA1 pathway (0.067 Hz) with ACSF-filled glass pipettes. Stimulus intensity was adjusted to fulfill the following four criteria: 1) all or none synaptic events were generated; 2) little or no variation in EPSC latency; 3) no change in mean size or shape of EPSCs by a small change in stimulus intensity, and 4) complete failure to evoke EPSCs by 10–20% reduction in stimulus intensity.56 Responses that did not meet these criteria were rejected.

LTP was recorded as described previously.55 Briefly, S.C-CA1 pathway was stimulated with a concentric bipolar electrode (FHC), and field EPSCs were recorded in CA1 neurons in current-clamp with ACSF-filled glass pipettes (1–5 MΩ). Stimuli consisted of monophasic 100-μs pulses of constant currents (with intensity adjusted to produce −30% of maximal amplitudes) at a frequency of 0.03 Hz. The strength of synaptic transmission was determined by measuring the initial (10–60% rising phase) slope of EPSCs. LTP was induced by a train of 100 pulses at same intensity in 1 s. The level of LTP was determined at an average of 50–60 min after tetanus stimulation.

In some experiments, after 10-min baseline recording, DL-AP5, ATP, ARL67156, AOPCP, suramin, SCH58261 or DPCPX was bath applied for 30–40 min. In all experiments, series resistance was controlled below 20 MΩ and not compensated. Cells would be rejected if membrane potentials were more positive than −80 mV; or if series resistance fluctuated more than 20% of initial values. All recordings were done at 32–34 °C. Data were filtered at 1 kHz and sampled at 10 kHz.

Brain morphological analysis. Anesthetized mice were perfused transcardially with 4% paraformaldehyde (PFA) in PBS and tissues were fixed in 4% PFA at 4 °C for 8 h. After dehydration by 30% sucrose, brain blocks were frozen and cut into 30-μm-thick sections on cryostat (HM550; Thermo Scientific). Sections were permeabilized with 0.3% Triton X-100 and 5% BSA in PBS and incubated with primary antibodies at 4 °C overnight. After washing with PBS for 3 times, samples were incubated with Alexa Fluor-conjugated secondary antibodies (1:1,000, Invitrogen) for 1 h at 22–25 °C. Samples were mounted with VectaShield mounting medium (Vector Lab) and images were taken by Zeiss LSM510 confocal microscope.

For in situ X-gal assay, brains were quickly isolated and embedded in OCT (Tissue-Tek). Coronal sections were cut at 20-μm interval and every fourth section was collected and mounted on slides. Sections were fixed for 2 min in PBS containing (in mM): 2 MgCl₂, 5 EGTA with 0.2% glutaraldehyde. Sections were washed in ice-cold PBS and stained in X-gal solution (1 mg/ml X-gal, 5 mM K₃Fe( CN)₆, 5 mM K₄Fe( CN)₆, 0.02% NP-40, 0.01% deoxycholate, and 2 mM MgCl₂ in PBS) at 37 °C overnight. After washing with PBS, they were counterstained with nuclear Fast Red (Vector Labs, H-3403).

Golgi staining. Golgi staining was performed by using a kit (FD Neuro-Technologies). Images were randomly taken from dorsal hippocampus. Spines were counted on primary, secondary and tertiary branches of apical dendrites in the stratum radiatum of CA1 hippocampal region by using ImageJ. To measure dendritic morphology, CA1 pyramidal neurons were traced with Reconstruct (SynapseWeb). Neurons with clear dendritic branches without breaks and staining artifacts/precipitates were subjected to Sholl Analysis. The investigator who performed spine analysis was blinded to genotypes.

Electron microscopy analysis. Electron microscopy studies were carried as described previously.55 Briefly, anesthetized mice were perfused transcardially with 0.1 M phosphate buffer containing 2% glutaraldehyde and 2% paraformaldehyde. Brains were post-fixed for 1 h at 25 °C and 4 °C overnight. Ultrathin sections of the CA1 region were examined with a JEM 1230 transmission electron microscope (JEOL USA) at 110 kV. Images were collected with an UltraScan 4000 CCD camera and First Light Digital Camera Controller (Gatan). Synapses were identified by ultrastructural specializations, including alignment of presynaptic and postsynaptic membranes, presynaptic and postsynaptic thickenings, and clusters of synaptic vesicles. The length of postsynaptic density (PSD), density of synaptic vesicles within 0.04 μm² areas surrounding active zones or adjacent to terminals, synaptic vesicles diameter and distance between astrocytes and synaptic membrane were quantified by investigators unaware of genotypes using ImageJ.

qRTP-PCR analysis. qRTP-PCR was performed as described previously.22 Briefly, total RNA was isolated by using TRIzol reagent (15596-026, Invitrogen). After DNaseI (18068-015, Invitrogen) digestion, RNA (1 μg) was reverse-transcribed with oligo dT-primers using Maxima reverse transcriptase (EP 0742, Fermentas), 5% of resulting cDNA was used for q-PCR by using SYBR Green detection (K 0222, Fermentas). Samples were assayed in triplicates, with each plate having loading standards in duplicate. mRNA levels of agrin and MuSK were normalized to those of GAPDH. Primer sequences were: agrin, 5′- CAC TGG GAG GGG ATA GTT G-3′ and 5′-GGC TGG GAT CTC ATG GAT C-3′; Musk, 5′- ACC GTC ATC ATC TCC ATC ATG TGT T-3′ and 5′- CAA GTT ATT CCT CGG ATC CTC C-3′; GAPDH, 5′- GCA CAG TCA AGG CCG AGA AT-3′ and 5′- GCC TTC TCC ATG GTG GTG AA-3′.

In vivo microdialysis. Mice were anesthetized with ketamine/xylazine (Sigma, 100/20 mg/kg, intraperitoneal), and implanted stereotactically with guide cannula (CMA-7) into the right hippocampus (AP: −2.0 mm, ML: ± 1.5 mm, DV: −1.0 mm). After surgery, mice were individually housed for 5 d for recovery. On the sixth day, microdialysis was performed with a probe (CMA/P000082, Harvard Apparatus) at the flowrate of 1.0 μl/min with ACSF containing (in mM): 147 NaCl, 2.7 KCl, 1.2 CaCl₂ and 0.85 MgCl₂ (pH, 7.4). Samples were collected into a polypropylene tube in a refrigerated fraction collector (Harvard Apparatus) and stored at −80 °C before assay. After microdialysis, brain sections were examined to confirm probe placement in the hippocampus.

ATP and adenosine test. ATP is measured by a luciferase reaction where light (560 nm) was emitted when α-luciferin is converted to oxyluciferin using the ENLITEN ATP Assay System (EF200, Promega)54. To inhibit ATP hydrolysis, condition media or microdialysis samples were incubated with the ectonucleotidase inhibitor ARL67156 (6-N,N-diethyl-β-γ-dibromo-methylene-d-adenosine-5-triphosphate trisodium salt hydrate, FPL 67156). Luminescence was measured using a luminometer (PE Applied Biosystems, TR717). ATP in samples was calculated based on a calibration curve with standard ATP samples.

Adenosine is measured by using the Adenosine Assay Kit (K327-100, BioVision). To inhibit adenosine degradation, samples were incubated with adenosine deminase inhibitor EHNA hydrochloride (E114, Sigma). Fluorescence was measured using a luminometer (PE Applied Biosystems, TR717). Adenosine in samples was calculated based on a calibration curve from standard adenosine samples.

Behavioral analysis. Mice were handled by investigators for three days before any behavioral test. Locomotor activity was measured as described previously.58 Mice were placed in a chamber (50 × 50 × 10 cm) and movement was monitored for 30 min using an overhead camera and tracking software (EthoVision, Noldus). The center 25 × 25 cm region was artificially defined as the center region and frequency and duration spent in the center region was recorded.

Elevated plus maze (EPM) test was performed as described previously59, in an EPM that has two opposing wall–closed arms and two open arms, each at 5 × 66 cm. The EPM was placed −50 cm above the floor. Each mouse was placed in one of the closed arms and was recorded for 15 min using an overhead camera and tracking software (EthoVision, Noldus). The time mice spent in the open arms, the number of entries and the latency to first entry were quantified autonomously.

In the light/dark box test, mice were placed in the dark chamber (20 × 15 × 23 cm) that was connected with the light chamber (30 × 20 × 23 cm) through an open door (7 × 5 cm), and recorded for 10 min. The time mice spent in the light box, number of entries and latency to first entry to the light box were quantified by the computational software.

Morris water maze test was performed as described previously60. A 120-cm pool and 10-cm platform were used for water maze studies. The day before training, mice were placed in a pool and scored for ability to find the visible platform within 60 s. Mice that failed to locate and climb onto the platform twice were eliminated from further testing. The platform was then moved to a new location and submerged 1 cm beneath surface of colored water. Mice were trained for 5 d with 4 trials per day and 60 s per trial to locate the hidden platform. Seven start positions were used to ensure that visual spatial memory was used by mice to find
the hidden platform. On the sixth day the platform was removed and mice were placed into the pool at a new start position and scored for time spent in platform area (N30: 30 cm as diameter) and number of platform crossings within 60 s.

For the pilocarpine model, mice were injected with scopolamine (2 mg kg\(^{-1}\), intraperitoneal) 30 min before pilocarpine treatment to block peripheral side effects. Mice were then injected with pilocarpine in 0.9% saline (wt/vol, intraperitoneal, 200 mg kg\(^{-1}\)), followed by injections every 30 min at a dosage of 100 mg kg\(^{-1}\). Behavioral seizures were scored based on the criteria by Racine\(^6\): stage 0, no seizure; stage 1, head nodding; stage 2, sporadic full-body shaking, spasms; stage 3, chronic full-body spasms; stage 4, jumping, shrieking, falling over; stage 5, violent convulsions, falling over, death. Some mice were injected with PTZ (intraperitoneal, 50 mg kg\(^{-1}\)) as previously described\(^4\) and observed for the latency till the onset of generalized convulsive seizures (GS). If GS was not observed in 20 min, 20 min were scored.

In some experiments, mice were injected with DPCPX (1 mg/kg, intraperitoneal) 30 min before behavioral tests. Genotypes of mice in all behavior tests were blinded to investigators. Except Golgi staining, EM and behavioral analysis described above, data collection and analysis were not performed blind to the conditions of the experiments.

**Statistical analysis.** Statistical analysis was done by the GraphPad Prism version 5.0 (GraphPad Software). Sample size choice was based on previous studies\(^22,59,62\), not predetermined by a statistical method. No randomization method was used. Data distribution was assumed to be normal, but this was not formally tested. Two-way ANOVA was used in morphological and electrophysiological studies that analyze more than two parameters. One-way ANOVA was used for analysis of data from three or more groups. Student’s t test or paired Student’s t test was used to compare data from two groups. All tests were two-sided. \(P < 0.05\) was considered to be statistically significant.

**Data availability.** The data that support the findings of this study are available from the corresponding author upon reasonable request.

A Supplementary Methods Checklist is available.

---

51. Tsien, J.Z. et al. Subregion- and cell type–restricted gene knockout in mouse brain. *Cell* **87**, 1317–1326 (1996).
52. Shen, C. et al. Antibodies against low-density lipoprotein receptor-related protein 4 induce myasthenia gravis. *J. Clin. Invest.* **123**, 5190–5202 (2013).
53. Ting, A.K. et al. Neuregulin 1 promotes excitatory synapse development and function in GABAergic interneurons. *J. Neurosci.* **31**, 15–25 (2011).
54. Cao, X. et al. Astrocyte-derived ATP modulates depressive-like behaviors. *Nat. Med.* **19**, 773–777 (2013).
55. Chen, Y.J. et al. ErbB4 in parvalbumin-positive interneurons is critical for neuregulin 1 regulation of long-term potentiation. *Proc. Natl. Acad. Sci. USA* **107**, 21818–21823 (2010).
56. Gil, Z., Connors, B.W. & Amitai, Y. Efficacy of thalamocortical and intracortical synaptic connections: quantas, innervation, and reliability. *Neuron* **23**, 385–397 (1999).
57. Yin, D.M. et al. Regulation of spine formation by ErbB4 in PV-positive interneurons. *J. Neurosci.* **33**, 19295–19303 (2013).
58. Wen, L. et al. Neuregulin 1 regulates pyramidal neuron activity via ErbB4 in parvalbumin-positive interneurons. *Proc. Natl. Acad. Sci. USA* **107**, 1211–1216 (2010).
59. Bi, L.L. et al. Amygdala NRG1-ErbB4 is critical for the modulation of anxiety-like behaviors. *Neuropsychopharmacology* **40**, 974–986 (2015).
60. Yin, D.M. et al. Reversal of behavioral deficits and synaptic dysfunction in mice overexpressing neuregulin 1. *Neuron* **78**, 644–657 (2013).
61. Racine, R.J. Modification of seizure activity by electrical stimulation. II. Motor seizure. *Electroencephalography Clin. Neurophysiol.* **32**, 281–294 (1972).
62. Lu, Y. et al. Maintenance of GABAergic activity by neuregulin 1-ErbB4 in amygdala for fear memory. *Neuron* **84**, 835–846 (2014).