FMRP acts as a key messenger for visceral pain modulation

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Abstract
Visceral pain is a common clinical symptom, which is caused by mechanical stretch, spasm, ischemia and inflammation. Fragile X syndrome (FXS) with lack of fragile X mental retardation protein (FMRP) protein is an inherited disorder that is characterized by moderate or severe intellectual and developmental disabilities. Previous studies reported that FXS patients have self-injurious behavior, which may be associated with deficits in nociceptive sensitization. However, the role of FMRP in visceral pain is still unclear. In this study, the FMR1 knock out (KO) mice and SH-SY5Y cell line were employed to demonstrate the role of FMRP in the regulation of visceral pain. The data showed that FMR1 KO mice were insensitive to zymosan treatment. Recording in the anterior cingulate cortex (ACC), a structure involved in pain process, showed less presynaptic glutamate release and postsynaptic responses in the FMR1 KO mice as compared to the wild type (WT) mice after zymosan injection. Zymosan treatment caused enhancements of adenylyl cyclase 1 (AC1), a pain-related enzyme, and NMDA GluN2B receptor in the ACC. However, these up-regulations were attenuated in the ACC of FMR1 KO mice. Last, we found that zymosan treatment led to increase of FMRP levels in the ACC. These results were further confirmed in SH-SY5Y cells in vitro. Our findings demonstrate that FMRP is required for NMDA GluN2B and AC1 upregulation, and GluN2B/AC1/FMRP forms a positive feedback loop to modulate visceral pain.

Keywords
FMRP, GluN2B, AC1, visceral pain

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Introduction
Visceral pain is a common clinical symptom, which can be caused by mechanical stretch, spasm, ischemia and inflammation.¹ Hypersensitivity mechanisms of chronic visceral pain are closely related to peripheral and central sensitization.² The increased excitability of primary sensory neurons and neurotransmitter release are contributed to peripheral sensitization.³ Anterior cingulate cortex (ACC) and cingulate cortex play key roles in regulation of central sensitization.⁴ Our previous study has found that adenylyl cyclase 1 (AC1), a pain-related enzyme, is increased in the ACC of mice with visceral pain, and AC1 is required for GluN2B upregulation.⁵ Our data suggest that GluN2B and AC1 may form a positive feedback for visceral pain processing in the ACC.⁵

Fragile X syndrome (FXS) with lack or deficiency of FMRP protein is an inherited disorder that is...
characterized by moderate or severe intellectual and developmental disabilities. FMRP, a neuronal RNA-binding protein, interacts with the coding region of transcripts that encode pre- and postsynaptic proteins, whose deficit leads to many neuronal dysfunctions such as language barrier, autism and epilepsy. The FMR1 knockout (KO) mice, a kind of FXS animal model, show aberrant synaptic plasticity in the hippocampus. FXS patients have self-injurious behavior, which may be associated with deficits in nociceptive sensitization. In addition, FMR1 KO mice also showed the decreased nociceptive sensitization after formalin and (RS)-3,5-dihydroxyphenylglycine (DHPG) treatments.

Chronic visceral pain conditions are typically difficult to manage because visceral sensory mechanisms are poorly understood and anatomical organization of the visceral sensory innervation that distinguishes the viscera from innervation of all other tissues in the body. Clinic studies from irritable bowel syndrome patients found greater activation bilaterally in the anterior insula, SII, and anterior cingulate cortex (ACC). However, the role of FMRP in the cortex is not well known in chronic visceral pain.

In this study, zymosan was injected into colons to induce the chronic visceral pain in mice. However, pain-related behaviors were significantly reduced in FMR1 KO mice. The results of whole-cell patch clamp recording in the ACC showed that zymosan injection did not induce the enhancements of presynaptic glutamate release and postsynaptic responses in the FMR1 KO mice. SH-SY5Y cells were treated with L-glutamate to mimic the increased presynaptic release (Figure 2(a)). We found that application of L-glutamic acid (100, 300 and 500 µM) for 1 h significantly increased the levels of NMDA receptors in a dose-dependent manner including GluN1 (F(3,20) = 20.232, p < 0.001, LSD, Figure 2(b) and (c)), GluN2A (F(3,20) = 7.174, p = 0.002, LSD, Figure 2(b) and (d)), GluN2B (F(3,20) = 18.602, p < 0.001, LSD, Figure 2(b) and (e)), and phosphorylated GluN2B at Try1472 (F(3,20) = 20.861, p < 0.001, LSD, Figure 2(b) and (f)) sites, which were in tune with our previous results. Our previous study has testified that AC1 expression is upregulated in the ACC of mice with visceral pain. Therefore, we want to know if AC1 will be upregulated when exogenous L-glutamic acid was added into SH-SY5Y cells. As expected, AC1 level was dose-dependently enhanced in SH-SY5Y cells after L-glutamic acid (100, 300 and 500 µM) treatment (F(3,20) = 10.088, p < 0.001, LSD, Figure 3(a) and (b)). Meanwhile, phosphorylated CREB (p-CREB) at Ser133 site (F(3,20) = 25.612, p < 0.001, LSD, Figure 3(a) and (c)), but not CREB level (F(3,20) = 1.082, p = 0.379, LSD, Figure 3(a) and (d)) was increased in SH-SY5Y cells treated with L-glutamic acid. These data suggest that enhanced presynaptic glutamate release can activate the NMDAR/AC1/CREB signaling pathway.

**Results**

**Insensitivity to visceral pain and decreased presynaptic release in FMR1 KO mice**

Zymosan was rectally injected into the colons of mice to induce sterile inflammation according to the timeline of the experiments (Figure 1(a)). Zymosan injection produced visceral pain-related behaviors in wild type (WT) mice as shown by increased pain numbers (F(3,20) = 13.460, p = 0.002, Bonferroni test, Figure 1(b)) and decreased vertical counts (F(3,20) = 4.507, p = 0.046, Bonferroni test, Figure 1(c)). However, pain-related behaviors were abolished in FMR1 KO mice. Next, the whole-cell patch clamp recording was employed to reveal the reasons of FMRP deficit-induced insensitivity to visceral pain. Miniature excitatory postsynaptic current (mEPSC) of pyramidal neurons in the second and third layer of ACC was recorded. Consistent with the behavioral results, zymosan injection induced the increased frequency and amplitude of mEPSC in WT mice, which were abolished in FMR1 KO mice (Figure 1(d) to (h); frequency: F(3,42) = 16.283, p < 0.001, Bonferroni test, Figure 1(e) and (g); amplitude: F(3,42) = 3.841, p = 0.057, Bonferroni test, Figure 1(f) and (h)). The results indicate that visceral pain-induced increases of presynaptic release and postsynaptic response are attenuated in the ACC of FMR1 KO mice.

**Exogenous glutamate upregulates the levels of NMDA receptors, AC1 and phosphorylated CREB**

To investigate the role of FMRP in visceral pain modulation, human neuronal cell line was employed in the flowing experiment. The SH-SY5Y, a human derived cell line, is often used as a model of neuronal function in vitro. The SH-SY5Y cells were treated with L-glutamic acid to mimic the increased presynaptic release (Figure 2(a)). We found that application of L-glutamic acid (100, 300 and 500 µM) for 1 h significantly increased the levels of NMDA receptors in a dose-dependent manner including GluN1 (F(3,20) = 20.232, p < 0.001, LSD, Figure 2(b) and (c)), GluN2A (F(3,20) = 7.174, p = 0.002, LSD, Figure 2(b) and (d)), GluN2B (F(3,20) = 18.602, p < 0.001, LSD, Figure 2(b) and (e)), and phosphorylated GluN2B at Try1472 (F(3,20) = 20.861, p < 0.001, LSD, Figure 2(b) and (f)) sites, which were in tune with our previous results. Our previous study has testified that AC1 expression is upregulated in the ACC of mice with visceral pain. Therefore, we want to know if AC1 will be upregulated when exogenous L-glutamic acid was added into SH-SY5Y cells. As expected, AC1 level was dose-dependently enhanced in SH-SY5Y cells after L-glutamic acid (100, 300 and 500 µM) treatment (F(3,20) = 10.088, p < 0.001, LSD, Figure 3(a) and (b)). Meanwhile, phosphorylated CREB (p-CREB) at Ser133 site (F(3,20) = 25.612, p < 0.001, LSD, Figure 3(a) and (c)), but not CREB level (F(3,20) = 1.082, p = 0.379, LSD, Figure 3(a) and (d)) was increased in SH-SY5Y cells treated with L-glutamic acid. These data suggest that enhanced presynaptic glutamate release can activate the NMDAR/AC1/CREB signaling pathway.
Ro25-6981 (GluN2B-selective antagonist), RS-MCPG (a non–selective group I/group II metabotropic glutamate receptor antagonist), nimodipine (L-type calcium channel blocker) and NB001 (AC1 antagonist) in SH-SY5Y cells (Figure 4(a)). The increases of GluN2B, AC1 and P-CREB induced by L-glutamic acid (300 μM, 1h) were inhibited by pretreatment of D-AP5 (100 μM), Ro25-6981 (3 μM), RS-MCPG (500 μM), nimodipine (10 μM) and NB001 (100 μM) for 30 min in SH-SY5Y cells (Figure 4(b)). The enhanced GluN2B level was totally blocked by pretreatment of NB001, and enhanced AC1 level was totally blocked by pretreatment with Ro25-6981, suggesting positive feedback modulation between NMDA GluN2B and AC1 (Figure 4(b) to (d)). To further confirm the role of GluN2B in regulating AC1 activity, GluN2B was overexpressed in SH-SY5Y cells by transfection of GluN2B plasmid (F(3,20) = 4.995, p = 0.037, Bonferroni test, Figure 4(g) and (h)). GluN2B overexpression induced the upregulation of AC1, which was comparable with that of glutamate treatment in SH-SY5Y cells (F(3,20) = 4.951, p = 0.038, Bonferroni test, Figure 4(g) and (i)). P-CREB and CREB levels were also increased after GluN2B overexpression (F(3,12) = 2.226, p = 0.162, Bonferroni test, Figure 4(g) and (j); F(3,12) = 0.020, p = 0.890, Bonferroni test, Figure 4(g) and (k)). However, the levels of AC1 and CREB did not further enhanced in GluN2B plasmid-transfected SH-SY5Y cells after L-glutamic acid treatment (Figure 4(g), (i), and (k)). We want to know if AC1 overexpression could in turn influence GluN2B level. As shown in Figure 5, AC1 overexpression also enhanced P-CREB, CREB and GluN2B levels in SH-SY5Y cells. (F(3,20) = 0.041, p = 0.842, Bonferroni test, Figure 5(a) and (b); F(3,20) = 3.702, p = 0.069, Bonferroni test, Figure 5(a) and (c); F(3,16) = 1.654, p = 0.217, Bonferroni test, Figure 5(a) and (d); F(3,20) = 1.564, p = 0.226, Bonferroni test, Figure 5(a) and (e)).

**GluN2B and AC1 upregulate the level of FMRP**

LTP triggered by activation of NMDAR in the ACC contributes to chronic pain states. FMRP (encoded...
by FMR1), commonly found in the brain, is crucial for LTP induction and is regulated by CREB.\textsuperscript{16,17} Price et al. reported that FMR1 KO mice exhibited lower nociceptive sensitization, suggesting FMRP is involved in pain processing.\textsuperscript{9} We found that FMRP level was evidently elevated in SH-SY5Y cells after treated with glutamate and overexpressed GluN2B (F(3,20) = 0.730, p = 0.403, Bonferroni test, Figure 6(a) and (b)) or overexpressed AC1 (F(3,20) = 1.210, p = 0.284, Bonferroni test, Figure 6(e) and (h)) and CREB levels (F (3,16) = 0.038, p = 0.849, Bonferroni test, Figure 6(e) and (j)) were enhanced. P-CREB level had a rising trend, but there was no statistical difference (F(3,12) = 0.012, p = 0.916, Figure 2).

FMRP acts as a key messenger for GluN2B and AC1

Next, FMRP was overexpressed in SH-SY5Y cells (F(3,20) = 3.732, p = 0.068, Bonferroni test, Figure 6(e) and (f)), and GluN2B (F(3,20) = 3.269, p = 0.086, Bonferroni test, Figure 6(e) and (g)), AC1 (F(3,20) = 1.210, p = 0.284, Bonferroni test, Figure 6(e) and (h)) and CREB levels (F(3,16) = 0.038, p = 0.849, Bonferroni test, Figure 6(e) and (j)) were enhanced. P-CREB level had a rising trend, but there was no statistical difference (F(3,12) = 0.012, p = 0.916, Figure 2).
Bonferroni test, Figure 6(e) and (i)). On the contrary, GluN2B (F(3,20) = 0.041, p = 0.841, Bonferroni test, Figure 6(k) and (m)), AC1 (F(3,12) = 0.008, p = 0.928, Bonferroni test, Figure 6(k) and (n)), P-CREB (F(3,20) = 0.392, p = 0.538, Bonferroni test, Figure 6(k) and (o)) and CREB (F(3,20) = 7.165, p = 0.014, Bonferroni test, Figure 6(k) and (p)) levels were reduced when FMRP were knocked down (F(3,28) = 3.110, p = 0.089, Bonferroni test, Figure 6(k) and (l)) in SH-SY5Y cells. These data indicate that FMRP is a key regulator for GluN2B and AC1 in chronic visceral pain.

FMRP deficit abolishes visceral pain-induced GluN2B and AC1 upregulation

FMR1 KO mice were employed to further confirm the key role of FMRP in GluN2B and AC1 modulation under chronic visceral pain condition. We had found that there were less pain-related behaviors in FMR1 KO mice than those of WT mice (Figure 1(a) and (b)) after zymosan injection. FMRP expression was lack in the ACC of FMR1 KO mice, but it significantly elevated in the ACC of WT mice with chronic visceral pain (p < 0.01, T-test, Figure 7(a) and (b)). Furthermore, the increased levels of GluN2B, phosphorylated GluN2B at Try1472 and Ser1303 sites induced by visceral pain were abolished in the ACC of FMR1 KO model mice (F(3,20) = 21.173, p < 0.001, Bonferroni test, Figure 7(a) and (c)); F(3,20) = 13.328, p = 0.002, Bonferroni test, Figure 7(a) and (d); F(3,20) = 22.770, p < 0.001, Bonferroni test, Figure 7(a) and (e)); F(3,20) = 14.065, p = 0.001, Bonferroni test, Figure 7(f) and (g)), P-CREB (F(3,20) = 10.157, p < 0.005, Bonferroni test, Figure 7(f) and (i)) induced by zymosan injection was also blocked in the ACC of FMR1 KO model mice. These data indicate that GluN2B/AC1/FMRP signaling pathway forms a positive loop to regulate the synaptic strength in the ACC of mice with chronic visceral pain.

Discussion

In present study, the FMR1 KO mice and SH-SY5Y cell line were employed and electrophysiological and pharmacological methods, plasmid transfection and shRNA were used to demonstrate the key role of FMRP in regulation of NMDA GluN2B and AC1, which are very important in the ACC of mice with zymosan injection. Our previous study has shown that the presynaptic release and postsynaptic NMDA receptor mediated responses were enhanced in the ACC of visceral pain model mice.5 In this study, we found that pain-related
Figure 4. Inhibition or overexpression of GluN2B positively modulated the level of AC1. (a) The diagram displays the timeline in SH-SY5Y cells pretreated with different chemicals and plasmids. (b) In SH-SY5Y cells, the levels of GluN2B, AC1 and CREB were detected by western blot after pretreatment with D-AP5 (NMDAR antagonist, 100 μM), Ro25-6981 (GluN2B-selective antagonist, 3 μM), RS-MCPG (a non-selective group I/group II metabotropic glutamate receptor antagonist, 500 μM), nimodipine (L-type calcium channel blocker, 10 μM) and NB001 (AC1 antagonist, 100 μM) for 30 min before glutamate exposure. The increased expressions of GluN2B (c), AC1 (d) and P-CREB (e) after glutamate exposure were markedly blocked by Ro25-6981, RS-MCPG, nimodipine and NB001. (f) CREB level did not change among these groups. *p < 0.05, **p < 0.01 vs. control. #p < 0.05, ##p < 0.01 vs. glu (glutamate). (g) The levels of GluN2B, AC1 and CREB were detected by western blot after transfection with GluN2B plasmid. (h) GluN2B plasmid indeed increased the expression of GluN2B. The overexpression of GluN2B induced the enhancements of AC1 (i), P-CREB (j) and CREB (k). n = 6 for each group; ***p < 0.01, ###p < 0.01, two-way ANOVA by Bonferroni post hoc test.
behaviors and enhanced presynaptic release were attenuated in FMR1 KO mice. In order to explore the mechanism, SH-SY5Y cells were pretreated with L-glutamate to mimic the increase of presynaptic release in vitro. NMDA receptors, AC1 and P-CREB-ser133 levels were enhanced in L-glutamate-treated SH-SY5Y cells, which were consistent with the results in vivo. However, the total CREB did not change in vitro, which was different from the upregulated results in vivo. We infer that the short time (1h) exposure to L-glutamate is not enough to enhance the total CREB expression in vitro; but the upregulated CREB expression in vivo was detected in a few days later after visceral pain.

AC1 is required for GluN2B upregulation in the ACC of mice with zymosan treatment in our previous data. The results from SH-SY5Y cells treated with Ro25-6981 and NB001 before L-glutamate exposure and overexpression of GluN2B and AC1 further confirmed that GluN2B and AC1 indeed form positive feedback. At the same time, we found that not only P-CREB but also total CREB level were enhanced after GluN2B and AC1 overexpression in SH-SY5Y cells. The overexpression of GluN2B and AC1 were detected at 72h after plasmid transfection, which had enough time inducing total CREB upregulation.

FMRP has lots of function in the nervous system besides its role in intellectual disability. FMRP modulates mRNA translation by interacting post-transcriptional factors or mRNA itself. Many researchers have found that FMRP interacts with the mRNAs of PSD-95 and Shank1. PSD-95-dependent GluN2B activation is involved in spinal nerve ligation-induced neuropathic pain. Shank proteins are a constituent family for post-synaptic density (PSD), and Shank1 is linked to PSD-95 (PSD marker) and GluN2B. Shank1 siRNA not only abolished the accumulation of Shank1 but also alleviated pain-related behaviors. These researches have indicated that FMRP has close links with pain. Furthermore, there is a low cAMP level in FXS fly and mouse models and FXS patients. Phosphodiesterases (PDEs) have the function of hydrolyzing cAMP and ending its effects, and PDE-4 inhibition could be a potential intervention for FXS treatment. The level of cAMP can also be modulated by AC1. Our previous studies have verified that AC1 expression is enhanced in the ACC of mice with visceral pain, and AC1 inhibitor NB001 relieves the pain-related behaviors in model mice. These data indirectly indicate that FMRP may be involved in the pain modulation. In present study, we found that zymosan injection could cause visceral pain-related behaviors in WT mice but not in FMR1 KO mice, which further prove the role of FMRP in pain regulation. Insensitivity to visceral pain in FMR1 KO mice might be attributed to the absence of AC1.
of FMRP in central nervous system, because FMRP is highly expressed in the brain and the tubules of the testes, but less expressed in the colon. Zymosan was injected into the colon to induce visceral pain in WT mice. Therefore, FMR1 KO mice probably exhibited an inflammatory response in the colon similar to that of WT mice.

Our previous in vivo study has shown that NMDA GluN2B and AC1 play key roles in visceral pain. In this study, we found that FMRP regulated GluN2B and AC1 in vitro, and FMRP was required for GluN2B and AC1 upregulation in the ACC of mice, suggesting that GluN2B/AC1/FMRP may form a positive feedback loop in visceral pain processing. However, this study has some limitations. In future experiments, we will introduce the conditional FMR1 knock in or KO mice to explore more mechanisms of FMRP on visceral pain regulation.

Figure 6. FMRP positively modulated GluN2B and AC1 levels. (a–b) The FMRP level was upregulated after glutamate exposure and GluN2B overexpression in SH-SY5Y cells. (c–d) AC1 overexpression increased the FMRP level. (e) The levels of GluN2B, AC1, P-CREB and CREB were tested by western blot in SH-SY5Y cells after transfected with FMRP plasmid. (f) The FMRP level was enhanced after FMRP plasmid transfection. The FMRP overexpression increased the expressions of GluN2B (g), AC1 (h) and CREB (i). There was a rising trend but not significant difference on P-CREB (j) level. (k) The levels of GluN2B, AC1, P-CREB and CREB were tested by western blot after FMRP knockdown. FMRP shRNA downregulated the levels of FMRP (l), GluN2B (m), AC1 (n), P-CREB (o) and CREB (p). n = 6 for each group; *p < 0.05, **p < 0.01, #p < 0.05, ##p < 0.01, two-way ANOVA by Bonferroni post hoc test.
Methods

Animals and visceral pain model

Adult WT (n = 36) and FMR1 KO (n = 36) male mice (8–12 weeks old) were purchased from Laboratory Animal Center of the Fourth Military Medical University and Charles River Laboratories (St. Constant, Quebec, Canada). The animals were housed under standard laboratory conditions (12 h light and 12h dark, temperature 22–26°C, humidity 55–60%) with water and mice chow available ad libitum. All experiments were performed in accordance with protocols approved by the Animal Care and Use Committee of the Fourth Military Medical University and the Jiaotong University. Zymosan (a glucan from yeast cell wall) was employed to induce visceral sterile inflammation. In brief, mice were anesthetized by 1.5% isoflurane inhalation, and then 0.1 ml zymosan suspension (30mg/ml in saline, Sigma, St. Louis, MO) or saline was rectally injected into the colons of mice once daily for three consecutive days.26.

Whole-cell patch-clamp recording

Whole-cell patch-clamp recording was performed as previously described. Briefly, mice were anesthetized with 4% isoflurane in air and then decapitated. Brains were removed and placed for 2–3 min in an ice-cold and oxygenated artificial cerebrospinal fluid (ACSF, in mM) containing 124NaCl, 25NaHCO3, 2.5KCl, 1KH2PO4, 2CaCl2, 2MgSO4 and 10 glucose, and continuously gassed with 95% O2/5% CO2. Coronal slices (300 μm) containing the ACC were prepared on a vibratome (Vibratome) in ice-cold ACSF. Slices were then incubated in a room temperature-submerged recovery chamber with oxygenated (95% O2 and 5% CO2) ACSF. After 1 h of recovery, slices were placed in a recording chamber on the stage of an Olympus microscope with infrared digital interference contrast optics for visualization of whole-cell patch-clamp recordings. Recordings were performed at room temperature (23–25°C), with continuous perfusion of ACSF at a rate of 3–5ml/min. For mEPSC recording, recording pipettes (3–5 MΩ) were filled with 1.5M KCl.
solution containing 145 mM K-glutonate, 5 mM NaCl, 1 mM MgCl₂, 0.2 mM EGTA, 10 mM HEPES, 2 mM Mg-ATP and 0.1 mM Na₃-GTP, adjusted to pH 7.2 with KOH (280–300 mOsmol). mEPSCs were collected in the neurons clamped at −70 mV with the picrotoxin (100 μM) in the ACSF. Access resistance (15–30 MΩ) was monitored throughout the experiment. Data were discarded if access resistance changed >15% during an experiment.

Visceral pain-related behaviors and open field tests

For visceral pain-related behaviors test, the mice were put into a transparent plexiglass box (20 × 20 × 40 cm) and allowed to move freely. Visceral pain-related behaviors included licking of the abdomen, whole body stretching, flattening abdomen against floor, or an arched posture adopted for 1–2 sec (abdominal retractions). The behaviors were recorded by the above camera and the numbers were calculated by the experimenter for 10 min. The open field is a square isolation chamber with a fan and dim illumination (43.2 × 43.2 × 30.5 cm; Med Associates, St. Albans, Vermont). Mice were placed in the center and allowed to freely explore 30 min. Vertical counts (one of the spontaneous activities) were recorded with an activity monitoring system (Activity Monitor, Med Associates, St. Albans, Vermont). All behavioral experiments were performed by two experimenters, one conducted the behavioral tests, and the other repeated the above results. Both of them were blinded to the treatment and genotype.

SH-SY5Y cell culture and drug treatment

SH-SY5Y cell line was purchased from American Type Culture Collection. SH-SY5Y cells were cultured with 1:1 mixture of Dulbecco’s modified Eagle’s medium (DMEM) and F12 medium supplemented with 10% fetal bovine serum from Gibco (Invitrogen, CA, USA). The SH-SY5Y cells were maintained at 37°C in a humidified (5% CO₂) incubator. SH-SY5Y cells were cultured in 6cm culture dishes at a density of 1 × 10⁶ per plate for 2 days. In order to eliminate the interference of serum, the complete medium was replaced to serum-free medium 2 hours before drug treatment. The cultures were pretreated with D-AP5 and Ro25-6981 for 30 min, and then L-glutamic acid was added to the same medium for another 1h. The medium was discarded, and cells were washed with ice-cold 1×PBS for 3 times and were cleaved by RIPA buffer. Samples were stored at −80°C until use.

Overexpression of GluN2B, AC1 and FMRP in SH-SY5Y cells

Rat GluN2B plasmid (a gift from Prof. Stefano Vicini), human AC1 plasmid (TaKaRa Biotechnology Co., Dalian, China), or mouse FMRP plasmid (prepared in our lab) was transfected into SH-SY5Y cells with K2 Transfection System according to the instruction (Biontex, Munich, Germany). GluN2B receptor subunits in rat and human have over 95% homology. The transfection reagents and DNA were added into SH-SY5Y cells and cultured for 24 h, medium was replaced to DMEM/F12 with 10% fetal bovine serum and cultured for another 48 h for subsequent experiments.

Knockdown of FMRP in SH-SY5Y cells

Human FMRP shRNA plasmid was purchased from Santa Cruz Biotechnology (CA, USA), which was transfected into SH-SY5Y cells and transfection method was similar with above. For selection of stably transfected cell, the transfected SH-SY5Y cells were cultured with fresh growth medium containing puromycin (2 g/ml) for 5 days after transfection for 48 h. Non-transfected cells were killed by puromycin, and the FMRP of survival cells were knocked down in SH-SY5Y cells.

Western blot analysis

The 300 μm brain slices including ACC were cut by vibratome, and the ACC tissues were dissected into cold ACSF under anatomical microscope. The samples were put into RIPA buffer (Tris 10mM pH7.4, NaCl 150 mM, EDTA 1mM, SDS 0.1%, TritonX-100 1%, Sodium deoxycholate 1%) including a protease inhibitor cocktail and phosphatase inhibitor cocktails 2 and 3 (Sigma, USA), homogenized and quantified by Bradford assay. SDS-polyacrylamide gels were used to separate the protein (30 μg) of each group, and then they were electro-transferred onto PVDF membranes (Roche, USA). The membranes were blocked with 5% milk in TBST at room temperature for 2h and probed with primary antibodies overnight at 4°C. The following primary antibodies were used: rabbit anti-FMRP (#ab27455; 1:1000; 85 kD; Abcam), rabbit anti-GluN1 (#05-432; 1:500; 120 kD; Millipore), rabbit anti-GluN2A (#ab1555, 1:5000; 180 kD; Millipore), rabbit anti-GluN2B (#ab1557; 1:1000; 180 kD; Millipore), mouse anti-β-actin (#A5316; 1:50000; 43 kD; Sigma), rabbit anti-phosphorylated GluN2B at the S1303 site (p-GluN2B-S1303; #07-398; 1:1000; 180 kD; Millipore), rabbit anti-phosphorylated GluN2B at the T1472 site (p-GluN2B-T1472; #ab5403; 1:1000; 180 kD; Millipore), rabbit anti-AC1 (#ab69597; 1:1000; 130 kD; Abcam), rabbit anti-P-cAMP-response element binding protein (p-CREB; #ab32096; 1:1000; 43 kD; Abcam) and...
rabbit anti-CREB (#ab32515, 1:2000; 43 kD; Abcam). The membranes were washed in TBST for 3 times and incubated with HRP-coupled anti-rabbit/mouse IgG secondary antibody for 1h at room temperature. Finally, the proteins of membranes were detected with the Western Lightning Chemiluminescence Reagent Plus according to the instructions of the manufacturer. The density of proteins was analyzed with Quantity One version 4.6.2 (Bio-Rad) and calculated as ratio relative to β-actin. The band intensity of control was set as 100%, and the band intensity of other groups were expressed as percentage to the control group.

Statistical methods

Data were expressed as mean ± SEM. Comparison between two groups was analyzed by independent sample two-tailed T-tests. The differences among multiple groups were evaluated by one-way or two-way analysis of variance (ANOVA, IBM SPSS 21). The data were analyzed by one-way ANOVA Least Significant Difference (LSD) test if they passed the homogeneity test, or else they were analyzed by one-way ANOVA Dunnett’s T3 test. Two-way ANOVA Tukey test was used to analyze data if two fixed factors were involved in experiments. In all cases, p < 0.05 was considered statistically significant.

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Authors Contributions

S.B. L. and M. Z. planned the study, L. K. Y., L. L., and J.Y. contributed to the experiments on the SH-SY5Y cells and western blot. X.S. W. carried out the visceral pain model and behavioral test. B. F. performed the electrophysiological recordings. L. K. Y., S.B. L. and M. Z. wrote the manuscript.

Declaration of Conflicting Interests

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