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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

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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,[6] whose deficit leads to many neuronal dysfunctions such as language barrier, autism and epilepsy.[7] The *FMR1* knock out (KO) mice, a kind of FXS animal model, show aberrant synaptic plasticity in the hippocampus.[8] FXS patients have self-injurious behavior, which may be associated with deficits in nociceptive sensitization.[9] In addition, *FMR1* KO mice also showed the decreased nociceptive sensitization after formalin and (*RS*)-3,5-dihydroxyphenylglycine (DHPG) treatments.[10]

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.[2] Clinic studies from irritable bowel syndrome patients found greater activation bilaterally in the anterior insula, SII, and anterior cingulate cortex (ACC).[11–13] 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 increase of presynaptic release and explore the role of FMRP in regulation of NMDA GluN2B receptor and AC1. We found that FMRP was required for GluN2B and AC1 upregulation, and GluN2B/AC1/FMRP was a positive feedback loop to modulate the visceral pain.

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-SY5Ycells 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)) and Ser1303 ($F_{(3,20)} = 21.061$, $p < 0.001$, LSD, Figure 2(b) and (g)) 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.

Mutual regulation between NMDA GluN2B and AC1

Previous study has shown that NMDAR antagonist and GluN2B-selective antagonist produce antinociception in acute and neuropathic pain.[14] Therefore, we want to know the responses of GluN2B, AC1 and CREB to the pretreatment with D-AP5 (NMDAR antagonist),

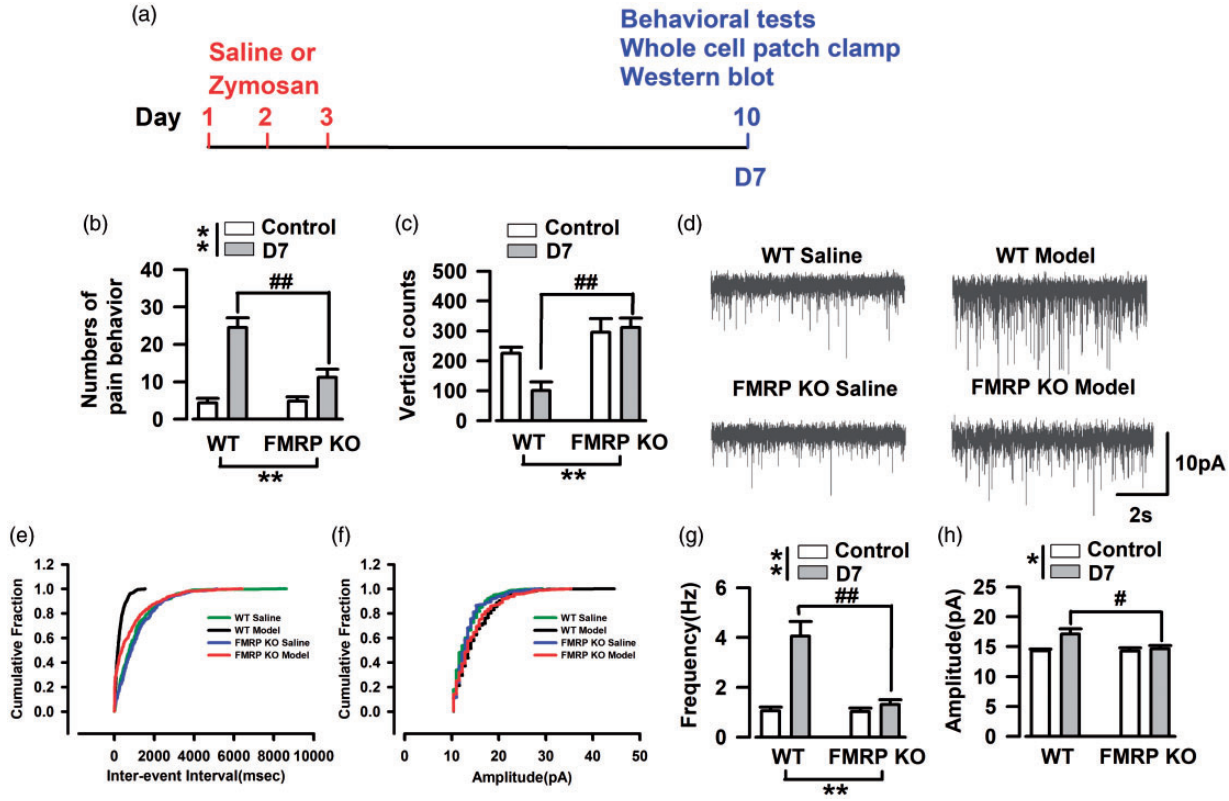

Figure 1. The increased pain-related behaviors and presynaptic release were abolished in *FMR1* KO mice. (a) The diagram displays the timeline in vivo experiments. After zymosan injection, the increased pain-related behaviors (b) and the decreased vertical counts (c) in WT mice were abolished in *FMR1* KO mice. Superimposed samples (d) and cumulative fraction results (e–f) showed that the mEPSC frequency (g) and amplitude (h) were increased in WT model mice, which was reversed in FMR1 KO model mice. n = 6 mice for each group; $^{**}p < 0.01$, $^{\#\#}p < 0.01$, two-way ANOVA by Bonferroni *post hoc test*.

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 ($F_{(6,21)} = 11.866$, $p < 0.001$, LSD, Figure 4(b) and (c); $F_{(6,21)} = 5.968$, $p = 0.001$, LSD, Figure 4(b) and (d); $F_{(6,21)} = 40.867$, $p < 0.001$, LSD, Figure 4(b) and (e); $F_{(6,21)} = 2.549$, $p = 0.052$, LSD, Figure 4(b) and (f)). 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.[15] FMRP (encoded

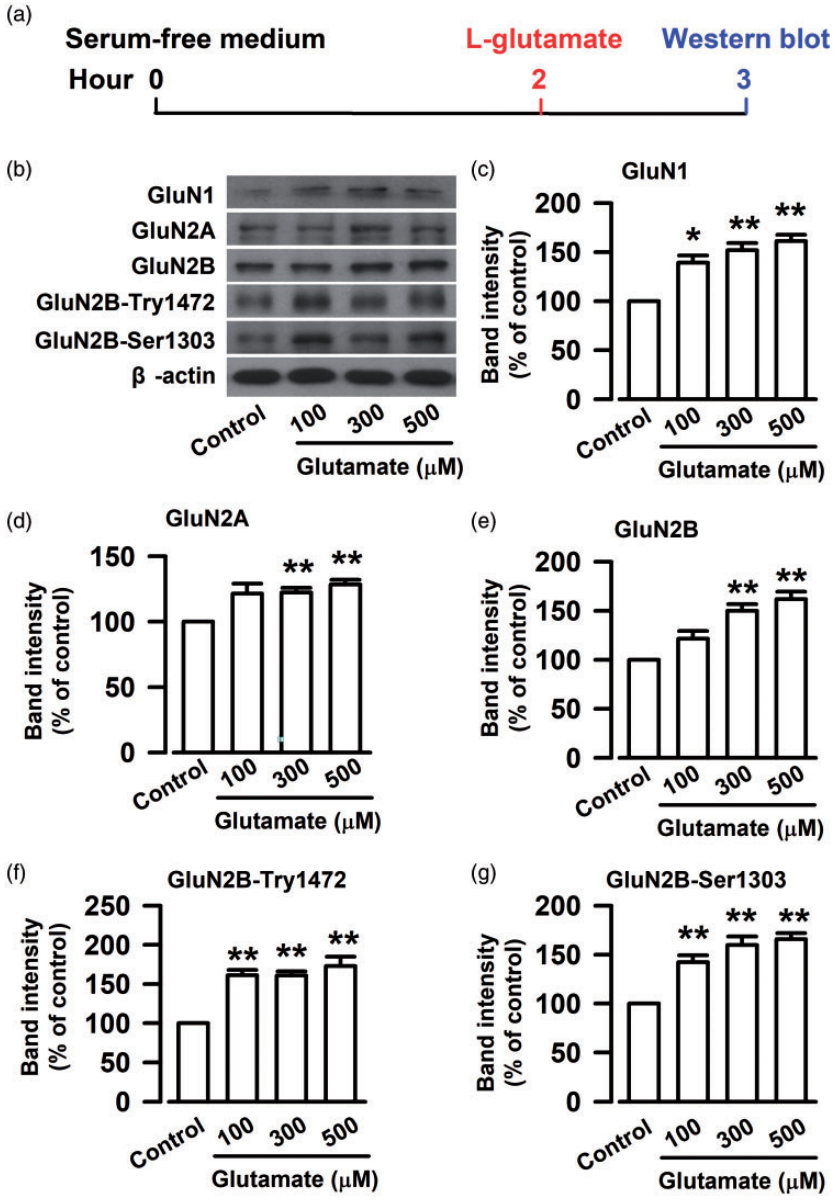

Figure 2. The enhanced levels of NMDA receptors after glutamate exposure in SH-SY5Y cells. (a) The diagram displays the timeline in vitro experiments. (b) Western blot was employed to detect the expression of NMDA receptors after treatment with different concentrations of glutamate. The levels of NMDA receptors including GluN1 (c), GluN2A (d), GluN2B (e), phosphorylated GluN2B at Try1472 (f) and Ser1303 (g) sites were significantly increased in a dose-dependent manner. n=6 for each group; *$p<$ 0.05, **$p<$ 0.01vs. control, one-way ANOVA for multiple group comparison.

by FMR1), commonly found in the brain, is crucial for LTP induction and is regulated by CREB.[16,17] Price et al. reported that *FMR1* KO mice exhibited lower nociceptive sensitization, suggesting FMRP is involved in pain processing.[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 ($F_{(3,20)}=1.038$, $p=0.321$, Bonferroni test, Figure 6(c) and (d)), suggesting that FMRP level was modulated by glutamate, GluN2B and AC1.

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 ($F_{(3,12)}=0.012$, $p=0.916$,

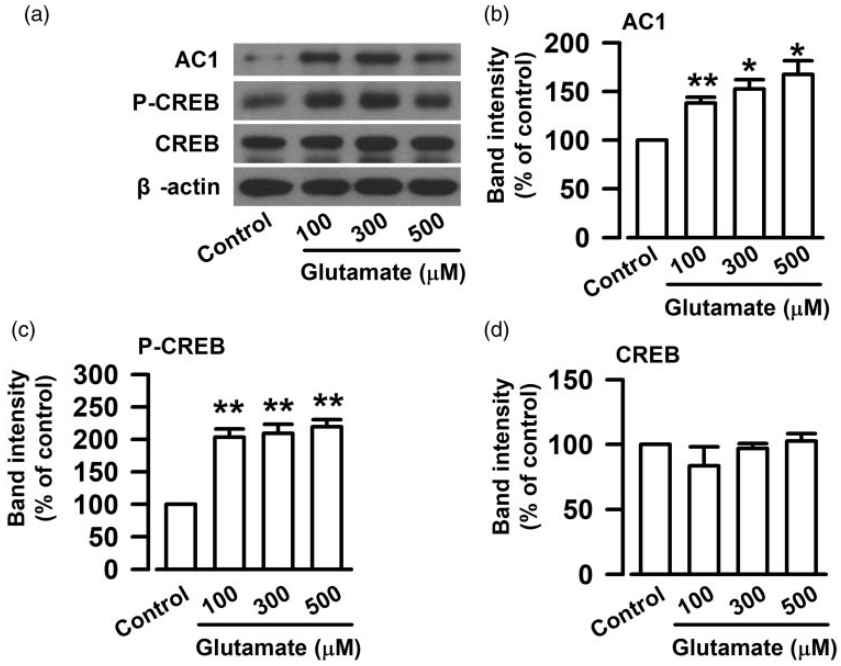

Figure 3. Exogenous glutamate increased the levels of AC1 and phosphorylated CREB in SH-SY5Y cells. (a) AC1 and CREB levels were detected by western blot after glutamate exposure. The levels of AC1 (b) and Phosphorylated CREB (P-CREB) at Ser133 site (c) but not CREB level (d) were dose-dependently enhanced in SH-SY5Y cells after glutamate treatment. n=6 for each group; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control, one-way ANOVA for multiple group comparison.

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 6(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)). These results strongly support the role of FMRP in the regulation of GluN2B functions and chronic visceral pain processing. Similarly, the increased levels of AC1 ($F_{(3,20)} = 15.470$, $p = 0.001$, Bonferroni test, Figure 7(f) and (g)), P-CREB ($F_{(3,20)} = 14.065$, $p = 0.001$, Bonferroni test, Figure 7(f) and (h)) and 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

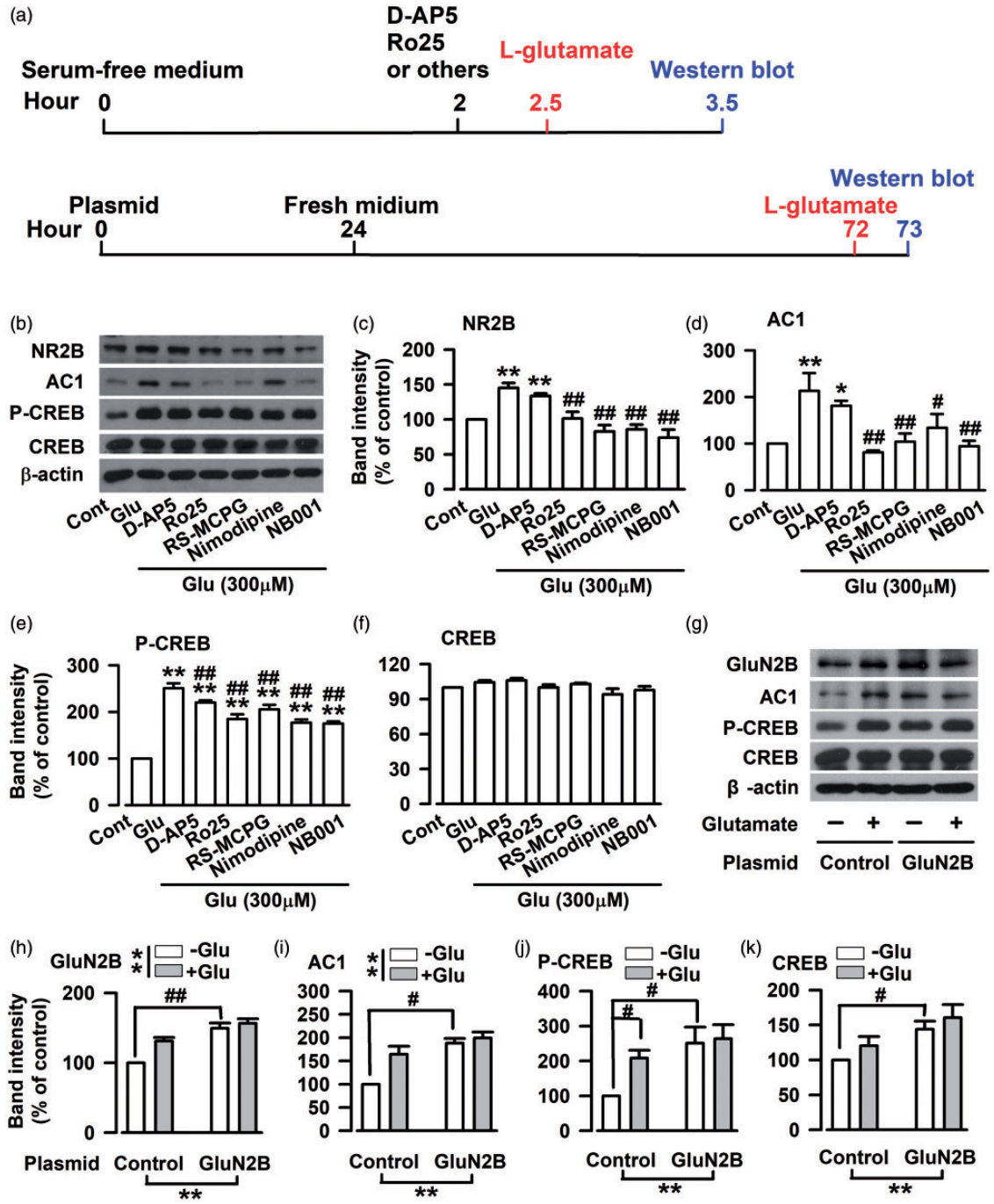

Figure 4. Inhibition or overexpression of GluN2B positively modulated the level of AC1. (a) The diagram displays the timeline in SH-SY5Y cells pretreated with different chemicals and plasmids. (b) In SH-SY5Y cells, the levels of GluN2B, AC1 and CREB were detected by western blot after pretreatment with 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; $^{**}p< 0.01$, $^{\#}p < 0.05$, $^{\#\#}p< 0.01$, two-way ANOVA by Bonferroni *post hoc test.*

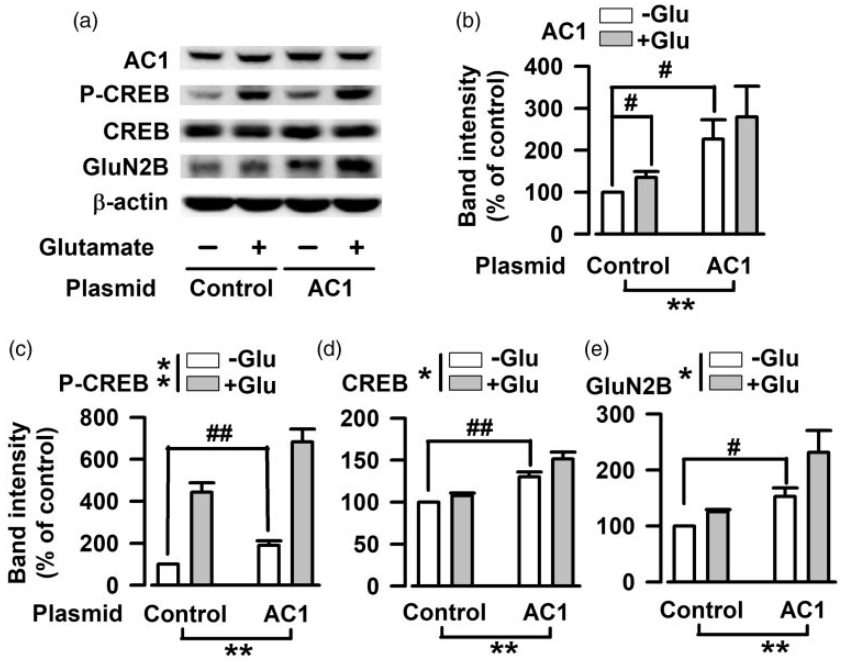

Figure 5. AC1 overexpression upregulated the GluN2B level. (a) The levels of GluN2B, AC1, P-CREB and CREB were detected by western blot after transfection with AC1 plasmid in SH-SY5Y cells. (b) The level of AC1 was increased after transfection with AC1 plasmid. AC1 overexpression upregulated P-CREB (c), CREB (d) and GluN2B (e) levels. 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*.

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.[5] However, the total CREB did not change in vitro, which was different from the upregulated results in vivo.[5] 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.[5]

AC1 is required for GluN2B upregulation in the ACC of mice with zymosan treatment in our previous data.[5] 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.[18,19] PSD-95-dependent GluN2B activation is involved in spinal nerve ligation-induced neuropathic pain.[20] 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.[21] 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.[22] Phosphodiesterases (PDEs) have the function of hydrolyzing cAMP and ending its effects, and PDE-$_4$ inhibition could be a potential intervention for FXS treatment.[22,23] 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.[5,24] 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

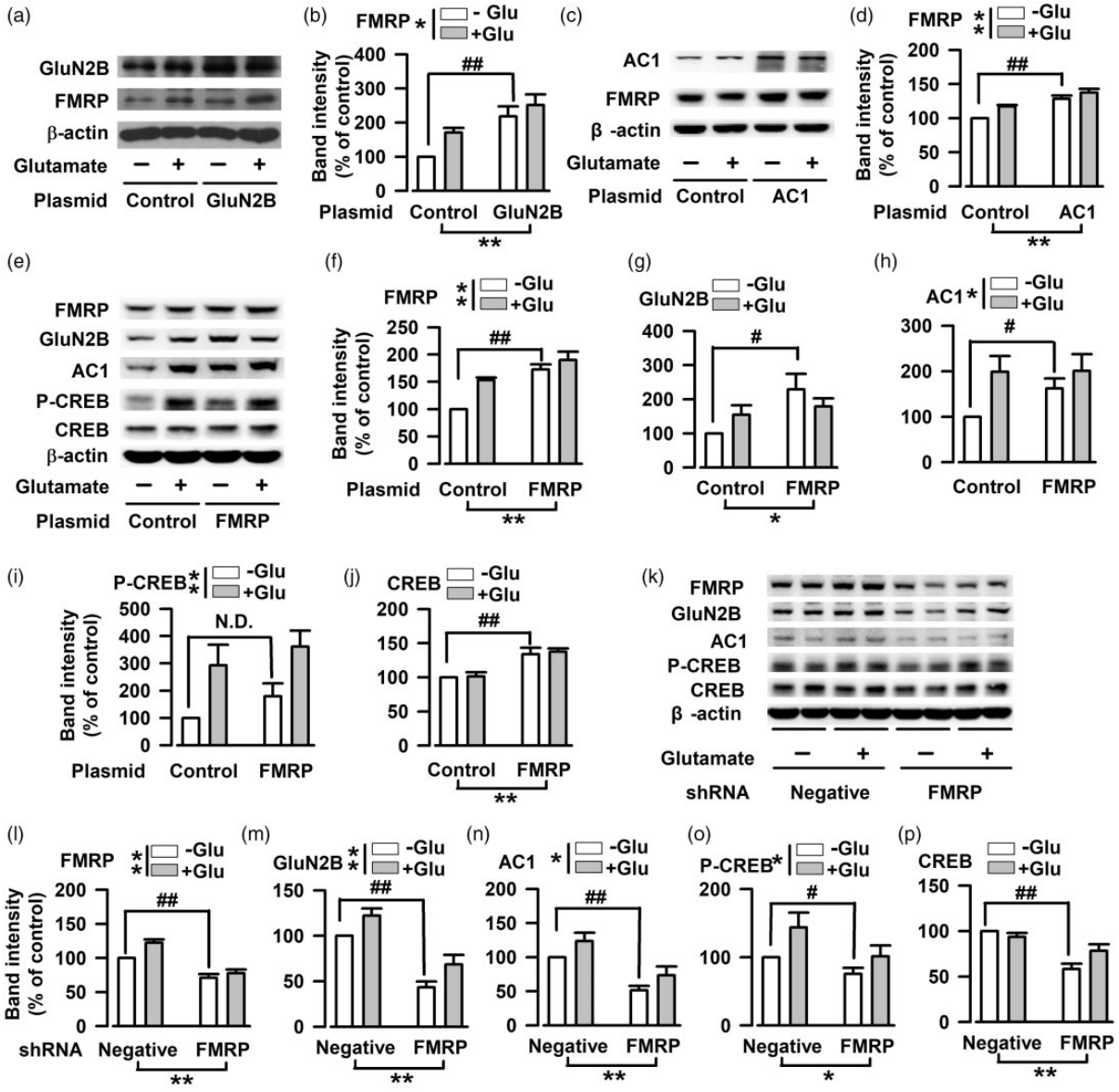

Figure 6. FMRP positively modulated GluN2B and AC1 levels. (a–b) The FMRP level was upregulated after glutamate exposure and GluN2B overexpression in SH-SY5Y cells. (c–d) AC1 overexpression increased the FMRP level. (e) the levels of GluN2B, AC1, P-CREB and CREB were tested by western blot in SH-SY5Y 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 (j). There was a rising trend but not significant difference on P-CREB (i) 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*.

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.[25] 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 that of WT mice.

Our previous in vivo study has shown that NMDA GluN2B and AC1 play key roles in visceral pain.[5] 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.

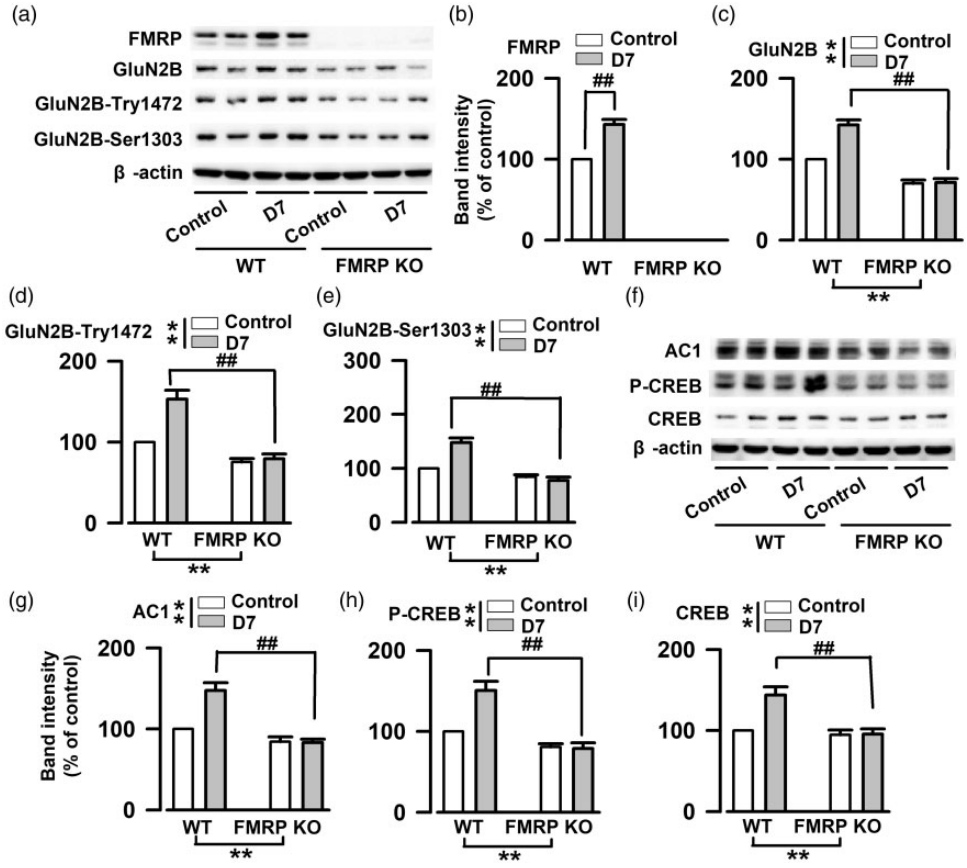

Figure 7. Visceral pain-induced GluN2B and AC1 upregulations were abolished in *FMR1* KO mice. (a) The levels of FMRP and GluN2B was detected by western blot in the ACC of WT and *FMR1* KO mice. (b) The FMRP level was significantly enhanced in the ACC of WT mice with zymosan injection for 7 days. The increased expression of GluN2B (c), phosphorylated GluN2B at Try1472 (d) and Ser1303 sites (e) were abolished in the ACC of *FMR1* KO mice with zymosan injection. (f) The levels of AC1 and CREB were detected by western blot in the ACC of WT and *FMR1* KO mice. The elevated expressions of AC1 (g), P-CREB (h) and CREB (i) were eliminated in the ACC of *FMR1* KO mice. 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 124 NaCl, 25 $NaHCO_3$, 2.5 KCl, 1 KH_2PO_4, 2 $CaCl_2$, 2 $MgSO_4$ and 10 glucose, and continuously gassed with 95% O_2/5% CO_2. 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% O_2 and 5% CO_2) 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 3–5 ml/min. For mEPSC recording, recording pipettes (3–5 MΩ) were filled with

solution containing 145 mM K-gluconate, 5 mM NaCl, 1 mM $MgCl_2$, 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).[5,24] 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% CO_2) incubator.

SH-SY5Y cells were cultured in 6cm culture dishes at a density of 1×10^6 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)[27] 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), mouse 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; 1:500000; 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 lgG 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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References

1. Grundy L, Erickson A, Brierley SM. Visceral pain. *Annu Rev Physiol* 2019; 81: 261–284.
2. Gebhart GF, Bielefeldt K. Physiology of visceral pain. *Compr Physiol* 2016; 6: 1609–1633.
3. Petho G, Reeh PW. Sensory and signaling mechanisms of bradykinin, eicosanoids, platelet-activating factor, and nitric oxide in peripheral nociceptors. *Physiol Rev* 2012; 92: 1699–1775.
4. Weng RX, Chen W, Tang JN, Sun Q, Li M, Xu X, Zhang PA, Zhang Y, Hu CY, Xu GY. Targeting spinal TRAF6 expression attenuates chronic visceral pain in adult rats with neonatal colonic inflammation. *Mol Pain* 2020; 16: 1744806920918059.
5. Liu SB, Wang XS, Yue J, Yang L, Li XH, Hu LN, Lu JS, Song Q, Zhang K, Yang Q, Zhang MM, Bernabucci M, Zhao MG, Zhuo M. Cyclic AMP-dependent positive feedback signaling pathways in the cortex contributes to visceral pain. *J Neurochem* 2020; 153: 252–263.
6. Darnell JC, Van Driesche SJ, Zhang C, Hung KY, Mele A, Fraser CE, Stone EF, Chen C, Fak JJ, Chi SW, Licatalosi DD, Richter JD, Darnell RB. FMRP stalls ribosomal translocation on mRNAs linked to synaptic function and autism. *Cell* 2011; 146: 247–261.
7. Liu B, Li Y, Stackpole EE, Novak A, Gao Y, Zhao Y, Zhao X, Richter JD. Regulatory discrimination of mRNAs by FMRP controls mouse adult neural stem cell differentiation. *Proc Natl Acad Sci U S A* 2018; 115: E11397–E11405.
8. Sharma A, Hoeffer CA, Takayasu Y, Miyawaki T, McBride SM, Klann E, Zukin RS. Dysregulation of mTOR signaling in fragile X syndrome. *J Neurosci* 2010; 30: 694–702.
9. Price TJ, Rashid MH, Millecamps M, Sanoja R, Entrena JM, Cervero F. Decreased nociceptive sensitization in mice lacking the fragile X mental retardation protein: role of mGluR1/5 and mTOR. *J Neurosci* 2007; 27: 13958–13967.
10. Albayram O, Alferink J, Pitsch J, Piyanova A, Neitzert K, Poppensieker K, Mauer D, Michel K, Legler A, Becker A, Monory K, Lutz B, Zimmer A, Bilkei-Gorzo A. Role of CB1 cannabinoid receptors on GABAergic neurons in brain aging. *Proc Natl Acad Sci USA* 2011; 108: 11256–11261.
11. Song GH, Venkatraman V, Ho KY, Chee MW, Yeoh KG, Wilder-Smith CH. Cortical effects of anticipation and endogenous modulation of visceral pain assessed by functional brain MRI in irritable bowel syndrome patients and healthy controls. *Pain* 2006; 126: 79–90.
12. Smith JK, Humes DJ, Head KE, Bush D, White TP, Stevenson CM, Brookes MJ, Marciani L, Spiller RC, Gowland PA, Francis ST. fMRI and MEG analysis of visceral pain in healthy volunteers. *Neurogastroenterol Motil* 2011; 23: 648–e260.
13. Mertz H, Morgan V, Tanner G, Pickens D, Price R, Shyr Y, Kessler R. Regional cerebral activation in irritable bowel syndrome and control subjects with painful and nonpainful rectal distention. *Gastroenterology* 2000; 118: 842–848.

14. Swartjes M, Morariu A, Niesters M, Aarts L, Dahan A. Nonselective and NR2B-selective N-methyl-D-aspartic acid receptor antagonists produce antinociception and long-term relief of allodynia in acute and neuropathic pain. *Anesthesiology* 2011; 115: 165–174.
15. Bliss TV, Collingridge GL, Kaang BK, Zhuo M. Synaptic plasticity in the anterior cingulate cortex in acute and chronic pain. *Nat Rev Neurosci* 2016; 17: 485–496.
16. Wang H, Wu LJ, Kim SS, Lee FJ, Gong B, Toyoda H, Ren M, Shang YZ, Xu H, Liu F, Zhao MG, Zhuo M. FMRP acts as a key messenger for dopamine modulation in the forebrain. *Neuron* 2008; 59: 634–647.
17. Wang H, Morishita Y, Miura D, Naranjo JR, Kida S, Zhuo M. Roles of CREB in the regulation of FMRP by group I metabotropic glutamate receptors in cingulate cortex. *Mol Brain* 2012; 5: 27.
18. DeMarco B, Stefanovic S, Williams A, Moss KR, Anderson BR, Bassell GJ, Mihailescu MR. FMRP – G-quadruplex mRNA - miR-125a interactions: Implications for miR-125a mediated translation regulation of PSD-95 mRNA. *PLoS One* 2019; 14: e0217275.
19. Imperatore JA, McAninch DS, Valdez-Sinon AN, Bassell GJ, Mihailescu MR. FUS recognizes G quadruplex structures within neuronal mRNAs. *Front Mol Biosci* 2020; 7: 6.
20. Peng HY, Chen GD, Lai CY, Hsieh MC, Lin TB. Spinal SIRPalpha1-SHP2 interaction regulates spinal nerve ligation-induced neuropathic pain via PSD-95-dependent NR2B activation in rats. *Pain* 2012; 153: 1042–1053.
21. Miletic G, Dumitrascu CI, Honstad CE, Micic D, Miletic V. Loose ligation of the rat sciatic nerve elicits early accumulation of Shank1 protein in the post-synaptic density of spinal dorsal horn neurons. *Pain* 2010; 149: 152–159.
22. Choi CH, Schoenfeld BP, Weisz ED, Bell AJ, Chambers DB, Hinchey J, Choi RJ, Hinchey P, Kollaros M, Gertner MJ, Ferrick NJ, Terlizzi AM, Yohn N, Koenigsberg E, Liebelt DA, Zukin RS, Woo NH, Tranfaglia MR, Louneva N, Arnold SE, Siegel SJ, Bolduc FV, McDonald TV, Jongens TA, McBride SM. PDE-4 inhibition rescues aberrant synaptic plasticity in Drosophila and mouse models of fragile X syndrome. *J Neuroscience* 2015; 35: 396–408.
23. Gurney ME, Cogram P, Deacon RM, Rex C, Tranfaglia M. Multiple behavior phenotypes of the fragile-X syndrome mouse model respond to chronic inhibition of phosphodiesterase-4D (PDE4D). *Sci Rep* 2017; 7: 14653.
24. Zhang MM, Liu SB, Chen T, Koga K, Zhang T, Li YQ, Zhuo M. Effects of NB001 and gabapentin on irritable bowel syndrome-induced behavioral anxiety and spontaneous pain. *Mol Brain* 2014; 7: 47.
25. Hinds HL, Ashley CT, Sutcliffe JS, Nelson DL, Warren ST, Housman DE, Schalling M. Tissue specific expression of FMR-1 provides evidence for a functional role in fragile X syndrome. *Nat Genet* 1993; 3: 36–43.
26. Liu SB, Zhang MM, Cheng LF, Shi J, Lu JS, Zhuo M. Long-term upregulation of cortical glutamatergic AMPA receptors in a mouse model of chronic visceral pain. *Mol Brain* 2015; 8: 76.
27. Yang Q, Feng B, Zhang K, Guo YY, Liu SB, Wu YM, Li XQ, Zhao MG. Excessive astrocyte-derived neurotrophin-3 contributes to the abnormal neuronal dendritic development in a mouse model of fragile X syndrome. *PLoS Genet* 2012; 8: e1003172.