Brain Research Bulletin 79 (2009) 219–223

Contents lists available at ScienceDirect

Brain Research Bulletin

journal homepage: www.elsevier.com/locate/brainresbull

Research report

# Antinociceptive synergistic effect of spinal mGluR2/3 antagonist and glial cells inhibitor on peripheral inflammation-induced mechanical hypersensitivity

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ARTICLE INFO

*Article history:*
Received 20 January 2009
Accepted 20 January 2009
Available online 29 January 2009

*Keywords:*
Metabotropic glutamate receptor 2/3
Glial cell
Mechanical allodynia
Spinal cord
Complete Freund's adjuvant
Synergistic interaction

ABSTRACT

Metabotropic glutamate receptor (mGluR) 2/3 is distributed in neurons and glial cells in many regions of the nervous system, but its role in nociceptive processing is unclear. In this study, we examined the mRNA expressions of *mGluR2* and *mGluR3*, by real-time RT-PCR, in the spinal cord. We further investigated the possible involvement of mGluR2/3 and mechanisms underlying peripheral inflammatory pain induced by subcutaneous complete Freund's adjuvant (CFA) injection. We demonstrate that compared to the controls, the mRNA expression levels of *mGluR2* and *mGluR3* were significantly higher 4 h after CFA injection. Functionally, blocking mGluR2/3 by their antagonist (2S)-2-amino-2-[(1S, 2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY341495) alleviated the CFA-induced mechanical allodynia and the inhibitory effects were reversed by mGluR2/3 agonist (2R, 4R)-4-aminopyrrolidine-2,4-dicarboxylate ((2R, 4R)-APDC). In addition, a glial metabolism inhibitor DL-fluorocitric acid barium salt (fluorocitric acid) also inhibited the CFA-induced mechanical allodynia in a dose-dependent manner. Remarkably, simultaneous inhibition of mGluR2/3 and glial metabolism had synergistic effects. The co-administration of LY341495 and fluorocitric acid with minimal dosages produced significant more inhibition than the additive effects by the individual inhibitor alone. In summary, our data suggest that spinal mGluR2/3 contributes to the generation of mechanical allodynia induced by peripheral inflammation. We also suggest that involvement of mGluR2/3 in the communication between glial cells and neurons takes part in the processing of nociceptive information.

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## 1. Introduction

Inflammatory pain is a common clinical symptom and usually leads to prominent allodynia that markedly affects the quality of life of patients. In the generation of inflammatory pain glutamate and its receptors exert profound influences. According to the previous studies, mGluR2/3 played dual roles (both nociceptive and antinociceptive) in pain information processing in certain experimental pain model such as the formalin model and the capsaicin model [9,15,23]. However, it remains unclear whether the activation of mGluR2/3 is nociceptive or antinociceptive in the spinal cord during peripheral inflammatory pain.

Several lines of evidence indicated that mGluR2/3 was preferentially located in pre- and post-synaptic membrane in neurons of lamina $II_i$, lamina III of spinal dorsal horn [3,8,17] and as well as in glial cells [1,14,17]. Because of the recent findings on the involvement of glial cells in pathological pain [10,18–20], it hereby is conceivable that mGluR2/3 is also likely to exert function on glial cells, or plays a role in communication between glial cells and neurons in nociceptive processing. Therefore, the purpose of the present studies aimed to examine the role of mGluR2/3 and glial cells in complete Freund's adjuvant (CFA)-induced mechanical allodynia and thermal hyperalgesia. For this purpose, we first investigated the expression levels of spinal *mGluR2* and *mGluR3* by quantitative real-time PCR. Further, we observed the behavioral alterations after pharmacological blockade or activation of mGluR2/3. Significantly, the potential function of mGluR2/3 on communication between neurons and glial cells was observed in the behavioral tests.

## 2. Materials and methods

### 2.1. Animals

Adult male Sprague–Dawley rats (190–220 g) were used in all experiments. All procedures followed the guidelines outlined in the *Principles of Laboratory Animal Care* (NIH Publications No. 86-23 revised in 1985). The animals were housed separately with free access to standard rat diet and tap water in a room with 12 h light/12 h dark cycle.

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doi:10.1016/j.brainresbull.2009.01.009

### 2.2. Drugs

To evaluate the functions of mGluR2/3 and its interaction with glial cells in nociceptive processing in the spinal cord, we used the well characterized mGluR2/3 antagonist (2S)-2-amino-2-[(1S, 2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY341495) (Tocris Cookson Ltd., Avonmouth, BS118TA, UK). According to previous reports [23], 1, 10 or 100 nmol drug was dissolved in 10 μl 70% DMSO followed as the supplier's instructions. Additionally, the agonist of mGluR2/3 (2R, 4R)-4-aminopyrrolidine-2,4-dicarboxylate ((2R, 4R)-APDC) (0.5 μmol in 10 μl saline; Tocris cookson Ltd.), and DL-fluorocitric acid barium salt (fluorocitric acid) (0.01, 0.1, 1 nmol, in 10 μl saline; Sigma–Aldrich Co., St. Louis, MO 63178, USA), the widely used glial inhibitor, were intrathecally (i.t.) administered respectively. 50 μl CFA (Sigma–Aldrich Co.) was subcutaneously (s.c.) injected into the plantar of left hind-paw to induce mechanical allodynia and thermal hyperalgesia. To observe drugs' efficacy on the process of development of CFA-induced mechanical and thermal hypersensitivity, each dosage of LY341495 and fluorocitric acid or the vehicle controls were separately i.t. administered 10 min prior to CFA injection. To observe whether (2R, 4R)-APDC could reverse the effect of LY341495, 0.5 μmol (2R, 4R)-APDC or its vehicle was i.t. delivered 5 min before or after administration of LY341495 or its vehicle. To observe the possible interaction of LY341495 and fluorocitric acid, co-administration of either drugs or their vehicles was performed 10 min prior to CFA injection. Except for the baseline which was attained when rats were naïve, other behavioral pharmacological data were obtained at 4 h after CFA injection because at the time-point CFA-induced mechanical allodynia and thermal hyperalgesia are well-established. If the development of hypersensitivity was blockaded by drugs' pre-treatment, the behavioral variations would be attenuated at 4 h after CFA injection.

### 2.3. Real-time quantitative RT-PCR technique

The left and right side of L3–L5 dorsal horn spinal cord segments of sacrificed rats were dissected and collected from naïve rats or 2 or 4 h after CFA injection. After being isolated and purified from individual tissue, RNA was pooled with three rats and was reverse-transcripted to cDNA using the PCR cDNA Synthesis Kit (Promega, USA) according to the manufacturer's instructions. Real-time PCR was performed using the Bio-Rad Laboratories DNA Engine OPTICON 2 system (USA) with SYBR Green detection. Validation of the real-time RT-PCR was made through two biological replicates. Results of the real-time RT-PCR analysis were expressed as $C_T$ values, which were used to determine the amount of target gene mRNA in relation to the amount of reference gene (GAPDH) mRNA. $\Delta C_T$ indicates the difference between the number of cycles necessary to detect the PCR products for each gene and reference gene. $\Delta\Delta C_T$ stands for the difference between the $\Delta C_T$ of the different pooling samples. Data were expressed as $2^{-\Delta\Delta C_T}$ to give an estimation of the amount of target gene mRNA relative to the reference gene. PCR primers for each gene were used as follow: *rmGluR2* FW: GTGGTGACATTGCGCTGTAA, RV: GCGATGAGGAGCACATTGTA; *rmGluR3* FW: CTGGTGATCCTATGCACTGT, RV: GAGGAATGCCAACCAGATGA; *GAPDH* FW: GTCTCCTGTGACTTCAACAG, RV: AGTTGTCATTGAGAGCAATGC.

### 2.4. Implantation of intrathecal catheter

For intrathecal drug delivery, animals were first anesthetized with intraperitoneal ketamine (100 mg/kg), then a polyethylene 10 tubing (I.D., 0.28 mm; O.D., 0.61 mm; BECTON DICKINSON, USA) was inserted into the subarachnoid space through a slit made between T2–T3. The catheter was advanced caudally up to 3 cm to reach the lumbar enlargement. Saline was injected into the catheter to avoid jam. At least five days after surgical operation the rats without dyskinesia were used in behavioral tests.

### 2.5. Behavioral tests

Quantitative measurements of mechanical and thermal hypersensitivities were performed as reported in previous studies [5,7]. Rats were arranged in a Plexiglas box (25 cm × 25 cm × 30 cm), beneath of which was a metal mesh for mechanical threshold test or a piece of glass (thickness: 2 mm) for thermal withdrawal latency test. A series of von Frey filaments (from 8 mg to 300 g) were used to test the mechanical threshold of the plantar of one hind-paw. A von frey filament was employed ten times with an interval of 2 s and duration of 1 s. The bending force value of the von Frey filament that caused an appropriate 50% occurrence of paw withdrawal was expressed as the paw withdrawal mechanical threshold (g). Additionally, by using a radiant heat stimulator (TC-1, BoBang Laboratory, Xi'an, PR China), the paw withdrawal latency to thermal stimulus was determined as the duration from start of the thermal stimulation to the occurrence of the hind-paw withdrawal reflex and was averaged from four heat stimuli (interval for the same site was longer than 10 min). If the latency exceeded 40 s, the stimulus was manually cut off to avoid excessive tissue injury and the region was considered completely unresponsive.

### 2.6. Statistical analysis

All the data were expressed as mean ± SEM after one-way analysis of variance (ANOVA) with Fisher's PLSD post hoc. The $p$-value less than 0.05 was considered as statistical significance. According to Yaksh and his colleagues' report [4], responses to drug delivery were transformed to percentage maximal effect (%MPE) by designating the vehicle pre-treatment value as 0% effect and the value of baseline as 100% effect, using the formula:

$$\%\mathrm{MPE} = \frac{\text{mechanical threshold}_{\text{drug pre-treatment}} - \text{mechanical threshold}_{\text{vehicle pre-treatment}}}{\text{mechanical threshold}_{\text{baseline}} - \text{mechanical threshold}_{\text{CFA}}} \times 100\%$$

## 3. Results

### 3.1. CFA-induced increase in mRNA expression levels of mGluR2 and mGluR3

Our results showed that under the state of naïve, the level of mRNA expression of *mGluR3* was more abundant than that of *mGluR2* (57.4 folds) in the spinal cord (Fig. 1A), suggesting that mGluR3 may be the major player for group II mGluR-mediated signalings. Furthermore, at the injected side the expression levels of both genes were dramatically increased 4 h after subcutaneous injection of CFA compared with naïve state; while 2 h after CFA injection, the expression level of *mGluR2* were slightly but no significantly increased, although the expression level of *mGluR3* was significantly increased (Fig. 1A–C). Interestingly, the time-course of mRNA expression levels correlated well with behavioral variations. Both mechanical allodynia and thermal hyperalgesia were well-established at 4 h but not 2 h after CFA injection. Additionally, at the control side, the expression level of *mGluR3* was also increased at both 2 and 4 h after CFA injection (Fig. 1A and C). These results indicate that spinal cord is probably the mainly location of mGluR3 but not mGluR2, however, peripheral inflammatory pain activates up-regulations of both mRNAs.

### 3.2. Inhibitory effects of mGluR2/3 antagonist on CFA-induced mechanical hypersensitivity

In behavioral tests, blocking mGluR2/3 by LY341495 alleviated CFA-induced mechanical allodynia in a dose-dependent manner, in which the minimal and maximal effects separately appeared in 1 and 100 nmol dosages (Fig. 2A; Table 1). i.t. administration of 0.5 μmol (2R, 4R)-APDC alone did not produce any discernible changes in paw withdrawal mechanical threshold (Fig. 2B), however, when administered prior to LY341495, (2R, 4R)-APDC effectively eliminated the inhibitory effect of 100 nmol LY341495 on mechanical allodynia from 35.5 ± 7.94 to 14.83 ± 3.75 g (Fig. 2B; Table 1), in which the %MPE decreased from 66% to 13%. Furthermore, when it was administered after LY341495, (2R, 4R)-APDC did not affect the inhibitory effect of LY341495 on mechanical allodynia (Fig. 2B; Table 1). These results indicate that spinal mGluR2/3, especially mGluR3, is essentially involved in the generation of mechanical allodynia induced by peripheral inflammation.

### 3.3. No effects of mGluR2/3 antagonist on CFA-induced thermal hypersensitivity

Different from the effects on mechanical allodynia, LY341495 or (2R, 4R)-APDC was i.t. administered 10 min prior to CFA injection and it did not affect the decreased thermal latency of rats induced by CFA injection (Data not shown). Therefore, we postulate that spinal mGluR2/3 is not involved in the generation of thermal hyperalgesia induced by peripheral inflammation.

### 3.4. Synergistic inhibitory effects of mGluR2/3 antagonist with glial cells inhibitor on mechanical hypersensitivity

To examine whether glial cells participate in the CFA-induced mechanical allodynia, varied concentrations of glial inhibitor fluorocitric acid were i.t. delivered to the spinal cord. The results showed

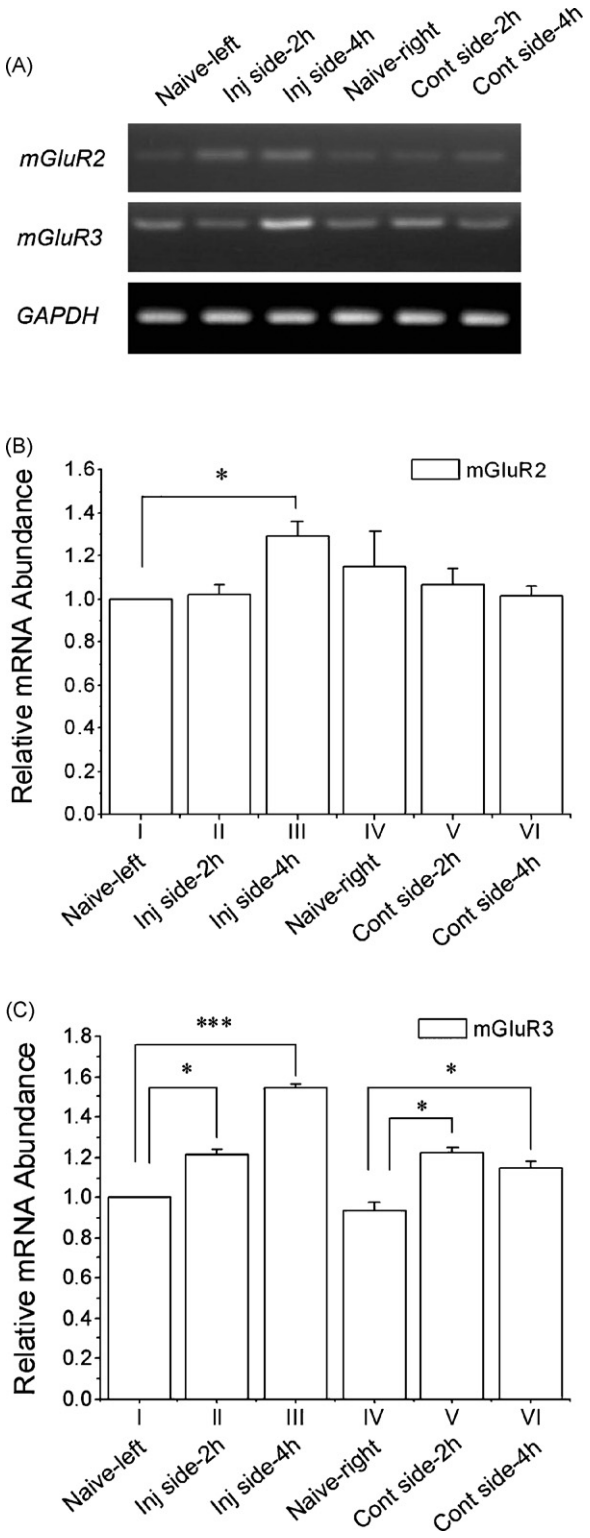


**Fig. 1.** Relative mRNA expression of *rmGluR2* and *rmGluR3* in the spinal cord. (A) Electropherasis images shown *rmGluR2* and *rmGluR3* expression in the spinal cord under naïve, 2 and 4 h after CFA treatment (left side hind-paw) states based on semi-quantitative real-time PCR. In the spinal cord, quantitative real-time PCR displays that mRNA expression level of *rmGlu2* (B) is up-regulated 4 h after s.c. CFA injection; but that of *rmGluR3* (C) is up-regulated 2 and 4 h after CFA treatment at the injected side and control side. The *y*-axis represents the relative intensity of mRNA expression. The value of each gene mRNA expression in the left side of naïve is designated 1, and the levels of mRNA expression in other samples are calibrated to this value. $^{*}p < 0.05$; $^{***}p < 0.0001$.

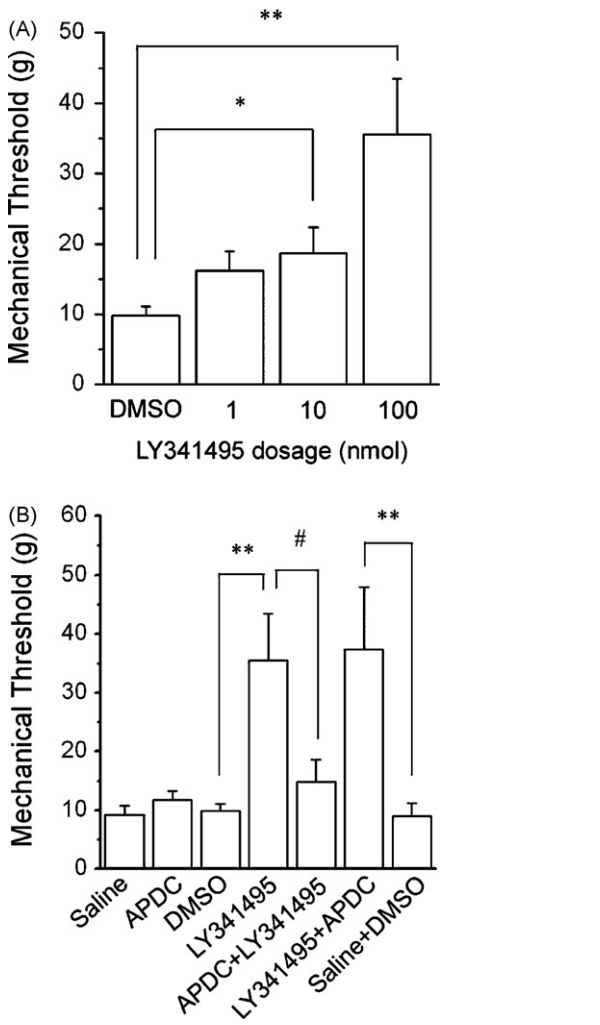


**Fig. 2.** Role of spinal mGluR2/3 on generation of mechanical allodynia induced by peripheral CFA injection.
Columns show mechanical threshold after different treatments. (A) LY341495 inhibits mechanical allodynia in a dose-dependent manner. LY341495 vs. DMSO: $^{*}p < 0.05$; $^{**}p < 0.01$. (B) i.t. 0.5 μmol (2R, 4R)-APDC does not affect mechanical allodynia; however, it can reverse the inhibitory effect of LY341495 on mechanical allodynia when it is i.t. administered 5 min before (column: APDC + LY341495) but not after (column: LY341495 + APDC) 100 nmol LY341495. APDC: (2R, 4R)-APDC. LY341495 vs. DMSO; LY341495 + APDC vs. Saline + DMSO: $^{**}p < 0.01$. APDC + LY341495 vs. LY341495: $^{\#}p < 0.05$.

that fluorocitric acid dose-dependently inhibited the generation of mechanical allodynia, in which the minimal and maximal effect appeared at 0.01 and 1 nmol respectively (Fig. 3A; Table 1). More importantly, when 1 nmol LY341495 and 0.01 nmol fluorocitric acid, both the minimal dosages used in the present study, were i.t. co-administered, the mechanical threshold was remarkably increased from 9.50 ± 2.75 g (fluorocitric acid alone), 16.14 ± 2.80 g (LY341495 alone) to 44.20 ± 10.99 g (compared to vehicle: $p < 0.0001$) (Fig. 3B; Table 1) and the resultant %MPE increased from 16.28% (LY341495 alone) and 2.69% (fluorocitric acid alone) to 89.23%. These pharmacological behavioral results suggest the existence of the inhibitory synergistic interaction between LY341495 and fluorocitric acid in the generation of mechanical allodynia induced by inflammatory pain.

## 4. Discussion

In the present study our results indicate that spinal mGluR2/3 facilitates the generation of mechanical allodynia, but not thermal hyperalgesia induced by peripheral inflammation. Furthermore, the

**Table 1**
Mechanical threshold 4 h after CFA injection in different treatments groups.

| | Vehicle 1 | Dose 1 | Dose 2 | Dose 3 | Vehicle 2 | Co-administered with | |
|---|---|---|---|---|---|---|---|
| i.t. LY341495 administration | 9.83 ± 1.22 g ($n$ = 6) | 16.14 ± 2.80 g ($n$ = 7) | 18.71 ± 3.62 g ($n$ = 7)$^{*}$ | 35.5 ± 7.94 g ($n$ = 6)$^{**}$ | 9.01 ± 2.11 g ($n$ = 7) | Pre-treatment with APDC 14.83 ± 3.75 g ($n$ = 6)$^{\#}$ | Post-treatment with APDC 37.4 ± 10.55 g ($n$ = 5)$^{**}$ |
| i.t. Fluorocitric acid administration | 8.45 ± 0.97 g ($n$ = 7) | 9.50 ± 2.75 g ($n$ = 6) | 16.00 ± 2.37 g ($n$ = 7)$^{*}$ | 54.33 ± 6.20 g ($n$ = 7)$^{***}$ | 9.47 ± 2.52 g ($n$ = 5) | LY341495 44.2 ± 10.99 g ($n$ = 7)$^{***}$ | |

In row 2, 10 min prior to CFA injection intratheca 1, 10 and 100 nmol LY341495 could reverse mechanical allodynia in a dose-dependent manner. Pre-, but not post-treatment with 0.5 μmol (2R, 4R)-APDC, the reversal effect of 100 nmol LY341495 could be offset. In row 3, intrathecal fluorocitric acid 0.01, 0.1, 1 nmol administration could reverse mechanical allodynia induced by CFA injection in a dose-dependent manner. Co-administered with 1 nmol LY341495 and 0.01 nmol fluorocitric acid could reverse mechanical allodynia with a synergistic but not summative inhibitory effect. Vehicle 1 refers to vehicle of LY341495 or fluorocitric acid respectively; vehicle 2 refers to vehicles of co-administrations. APDC represents (2R, 4R)-APDC.

$^{*}$ Compared to the corresponding vehicle, $p < 0.05$.
$^{**}$ Compared to the corresponding vehicle, $p < 0.01$.
$^{***}$ Compared to the corresponding vehicle, $p < 0.0001$.
$^{\#}$ Compared to dose 3 of LY341495, $p < 0.05$.

synergistic inhibitory effects of mGluR2/3 antagonist with glial cell inhibitor hints us that signal transmission between neuron and glial cell involving in mGluR2/3 mediate this cooperative effect on nociceptive transmission.

### 4.1. Involvement of spinal mGluR2/3 in nociceptive transmission

We first identified that peripheral inflammation increases the expression level of *mGluR2* and *3* in the spinal cord, these suggesting that mGluR2/3 is probable involved in nociceptive transmission. We then demonstrated that blocking mGluR2/3's function, the mechanical allodynia was alleviated. Several studies with pain behavioral [6,23], molecular biological [2] and electrophysiological measurements [24] were consistent with our results that spinal mGluR2/3 potentially exerted the facilitatory effect on peripheral tissue-damaged induced nociception processing. By contrast, other studies suggested that in the processing of pain, mGluR2/3 may be either pro-nociceptive or antinociceptive effect. These controversial issues reported that the antinociceptive effects of mGluR2/3 tend to function on neuropathic pain model and other action site, such as periphery or supraspinal [9,11,15,21,22]. Despite the controversial views on mGluR2/3 function, our results demonstrate the nociceptive functions of spinal mGluR2/3.

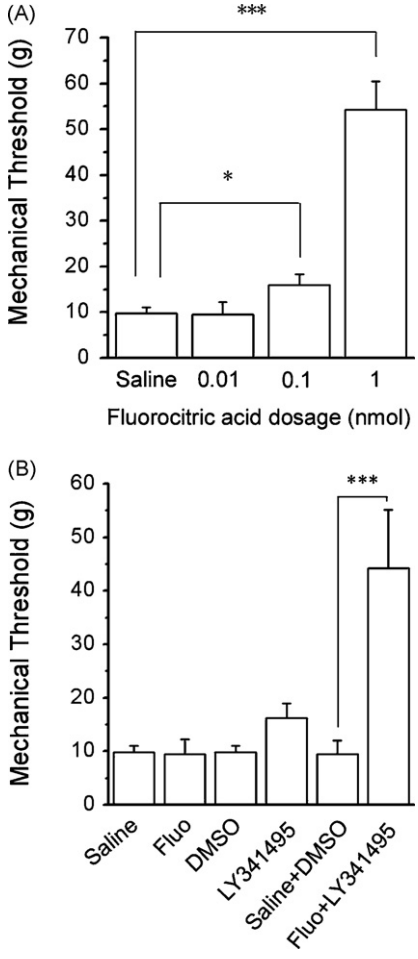


**Fig. 3.** Synergistic effect of mGluR2/3 antagonist and glial cells inhibitor on generation of mechanical allodynia.
Columns show mechanical threshold after different treatments. (A) Fluorocitric acid inhibits mechanical allodynia in a dose-dependent manner. Fluo vs. Saline: $^{*}p < 0.05$; $^{***}p < 0.0001$. (B) The minimize dosages of LY341495 and fluorocitric acid exert synergistic but not cumulative inhibitory effects on generation of mechanical allodynia. Fluo + LY341495 vs. Saline + DMSO: $^{***}p < 0.0001$. Fluo: fluorocitric acid.

The possible mechanism of the facilitatory effect may be mediated by the inhibition of pre-synaptic mGluR2/3 on GABA releasing from the interneuron in the spinal cord dorsal horn [24]. In addition, mGluR2/3 increases transcription factor activator protein-1 (AP1) DNA binding activity in cultured rat cortical neurons through voltage-sensitive $Ca^{2+}$ channel mediating intracellular $Ca^{2+}$ level release, sequentially modulate the transcriptional level of downstream genes [16]. Third, the neuroprotective property of mGluR2/3 in NMDA and AMPA receptor-mediated toxicity should be taken into consideration. Peripheral inflammation promotes excessive glutamate release from the primary afferent terminal [13,16], and the excessive glutamate release enhances risk of cell death.

### 4.2. Roles of mGluR2/3 in communication of glial cells and neurons in proceeding of nociception transmission

In our present results the mGluR2/3 antagonist synergistically attenuated mechanical allodynia with glial cell inhibitor. It is well established that glial cells contribute to pathological nociception processing [18–20]. Further, in morphological studies, some experiments showed that the processes of astrocytes were mGluR2/3 immunopositive and they encircled the nearby neuronal cell bodies and synapses tightly [17]. One of the main functions of astrocytes is modulating the amount of glutamate release depending on intracellular $Ca^{2+}$ concentration to participate in neuron-glial communication [12]. This communication also influences the sequential function of mGluR2/3 in neurons [16]. In fact, when peripheral inflammation is induced, in the spinal cord dorsal horn, neuron and astrocyte are activated, at the same time, $Ca^{2+}$ is released from astrocyte. These extracellular $Ca^{2+}$ source via L-type voltage-sensitive $Ca^{2+}$ channel enter into intracellular, hereby, modulate mGluR2/3 to increase AP1 DNA binding activity, or influence the neuroprotective function of mGluR2/3 [16]. Once the $Ca^{2+}$-dependent signaling transmission is interrupted (such as glial function of present study is inhibited by inhibitor), the function of mGluR2/3 will be attenuated.

In general, the present study indicates that spinal mGluR2/3 plays a significant role in nociception processing. Importantly, the synergistic inhibitory effect of mGluR2/3 antagonist and glial cell inhibitor suggests that the involvement of mGluR2/3 in communication between neurons and glial cells should be taken into account when the detailed mechanism of nociception processing is explored.

## Conflict of interest

The authors declare that they have no any conflict of competing financial interests.

## Acknowledgement

This work was supported by the National Natural Science Foundation of China (Nos. 30500154, 30600175).

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