Supporting Information for

Exploring the Role of Spinal Astrocytes in the Onset of Hyperalgesic Priming Signals in Acid-Induced Chronic Muscle Pain

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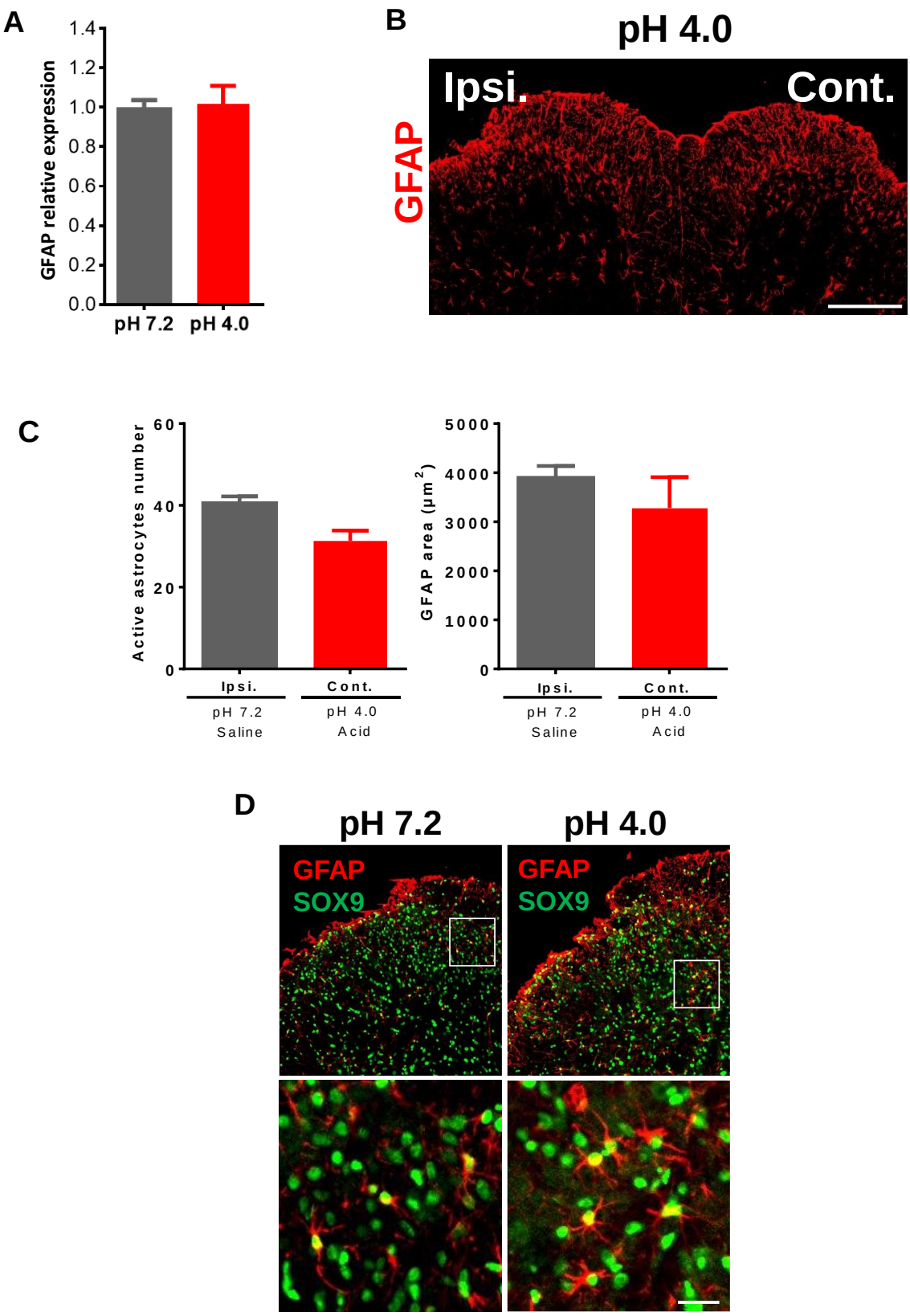
Supplementary figure 1

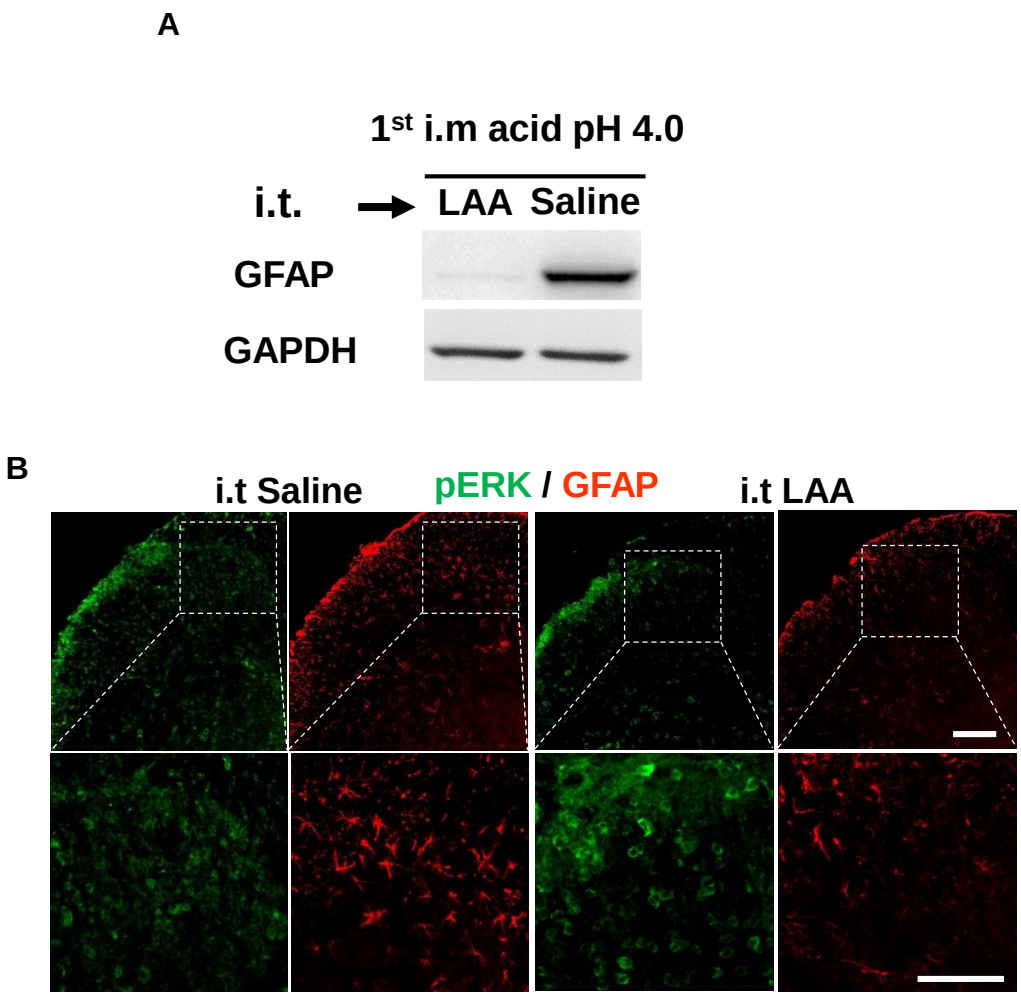


S 1

Bilateral astrocyte activation occurs after the first acid injection, but not at the mRNA level

(A) Spinal GFAP mRNA expression shows no significant changes after first acid injection. (B) Representative confocal images of GFAP in the dorsal horn 4h after the first acid injection, (C) Quantification of bilateral GFAP-expressing cell number and GFAP area at 4 hours after acid pH 4.0 injection. ipsilateral (Ipsi.) and contralateral (Cont.). (D) Representative confocal image of spinal cord 4 hours after acid pH 4.0 injection, Immunofluorescent staining GFAP-positive (Red) and SOX9 (Green). Compared with pH 7.2 saline as control. Data were presented as mean ± SEM (n=3). (Scale bar, 50 μm.).

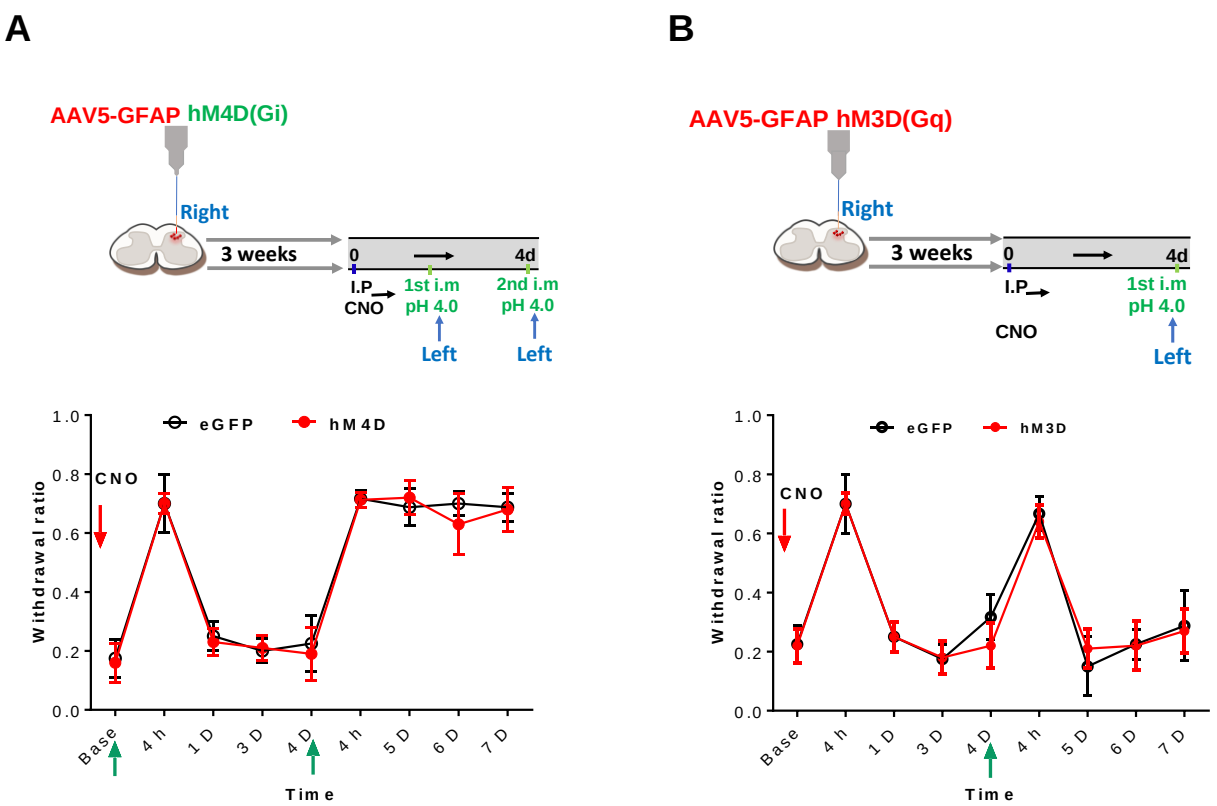
Supplementary figure 2



S2

Astrocyte inhibitor (LAA) attenuates increased GFAP expression but not increased pERK induced by the first acid injection.
(A) Western blot analysis of GFAP after i.t. injection of the astrocyte inhibitor 100nmol LAA or control saline (5µl) 90 minutes prior to first acid pH 4.0 (B) Representative confocal images of Immunofluorescent staining of GFAP (red) and pERK (green) in lumbar spinal cord 4 hours after first acid injection vs i.t saline. n = 3, (Scale bar, 200µm.)

Supplementary figure 3

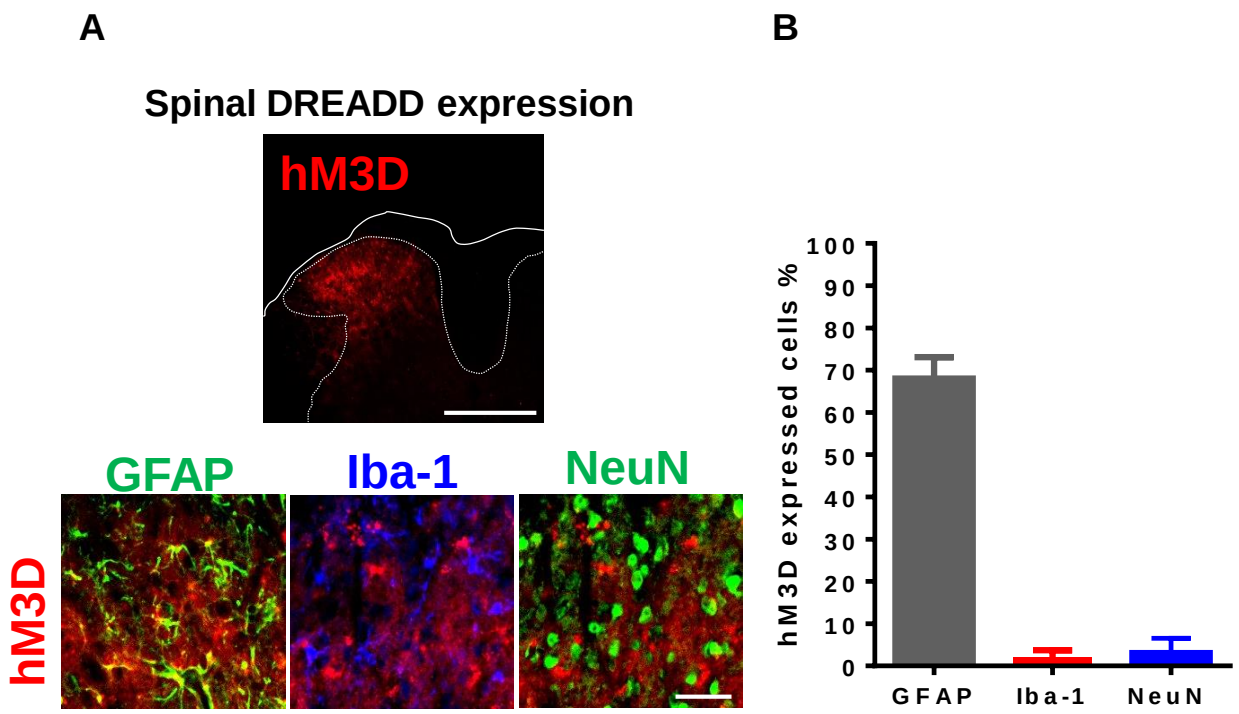


S 3

Contralateral chemogenetic activation of spinal astrocytes induces transient hyperalgesia without priming.

(A & B) Upper panels, schematic diagrams of the experimental procedures, lower panels, (A) bilateral mechanical withdrawal measurement for hM4D group, the first acid was injected 40 min after CNO and the second acid injection was at day 4 (n=5). (B) Mechanical withdrawal measurement of mice received i.p. CNO followed by the first acid injection at day 4 in hM3D group (n=5). Behavior data are presented as mean ± SEM, compared to the eGFP control group. Red arrows indicate CNO i.p. injection, and the green arrows indicate the intramuscular injection of pH 4.0 acid.

Supplementary figure 4

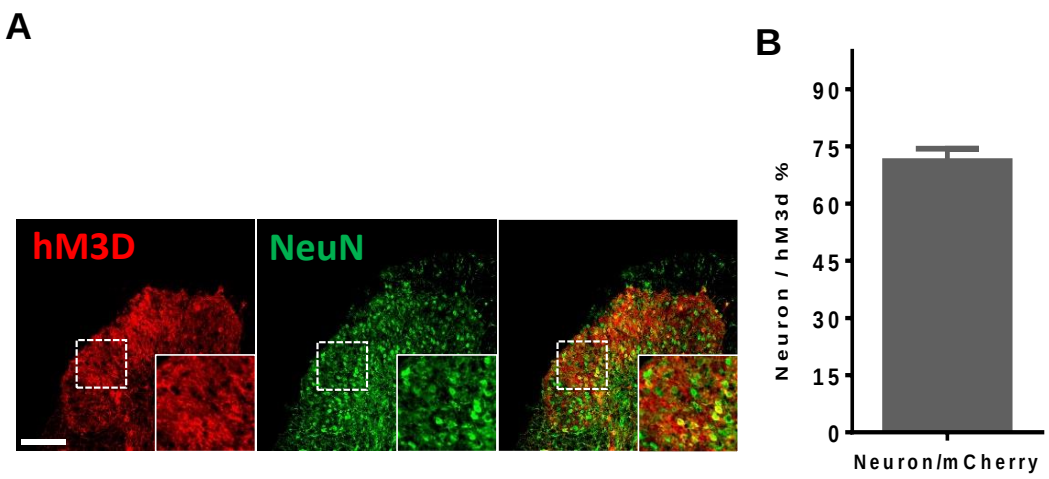


S 4

Spinal DREADD expressed specifically in GFAP positive astrocytes

(A) Upper image, representative confocal image of spinal cord dorsal horn 3 weeks after the (AAV5-GFAP-hM3d(Gq)-mCherry) injection, and lower panel, immunofluorescent staining GFAP-positive cells, Iba1 and NeuN in the SDH express hM3D-mCherry on the injected hM3D DREADD side, and (B) The percentage of colocalization of each marker in SDH hM3D+ expression. Quantification data were presented as mean ± SEM (n = 4). (Scale bars 50, 200 µm.).

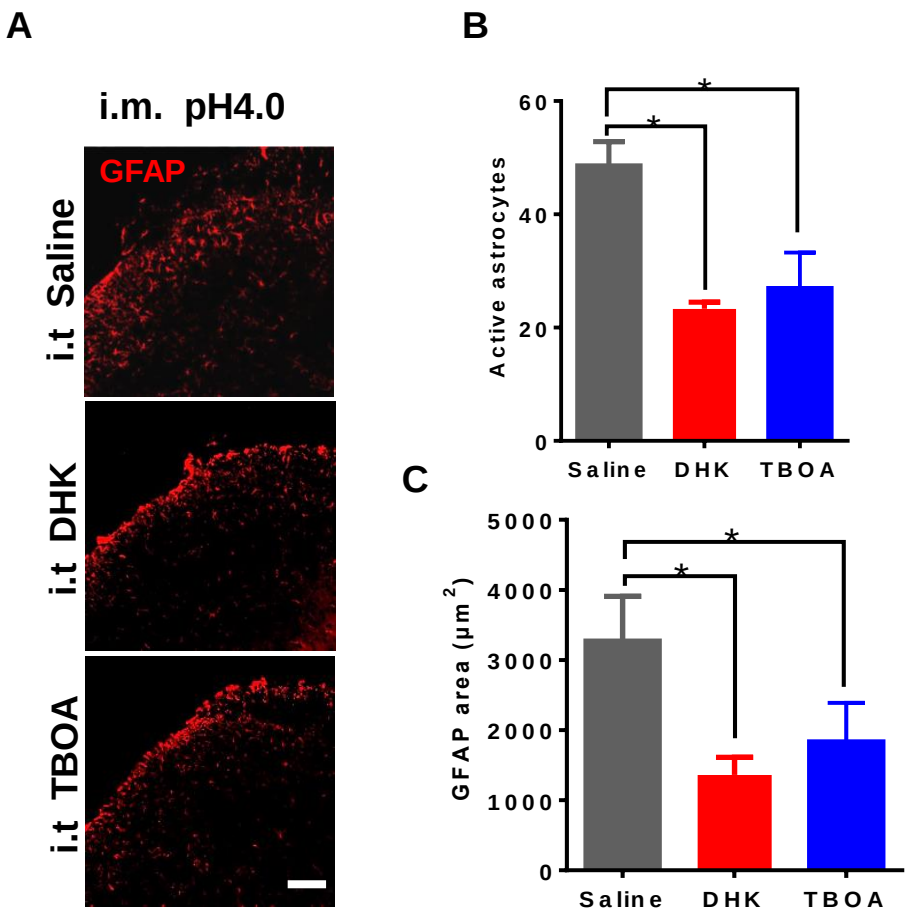
Supplementary figure 5



S5

Spinal neurons express hM3D/mCherry in the spinal dorsal horn of Vglu2-Cre mice.
(A) Representative confocal images show the expression of hM3D (red) and NeuN (green) neurons in the spinal dorsal horn 3 weeks after AAV5-hSyn-DIO-hM3D(Gq)-mCherry spinal injection in Vglut2 cre mice. (B) Quantification indicating the percentage of neurons expressed hM3D labeled with mCherry in the dorsal horn, data were presented as mean ± SEM (n = 3). (Scale bar, 200μm.).

Supplementary figure 6

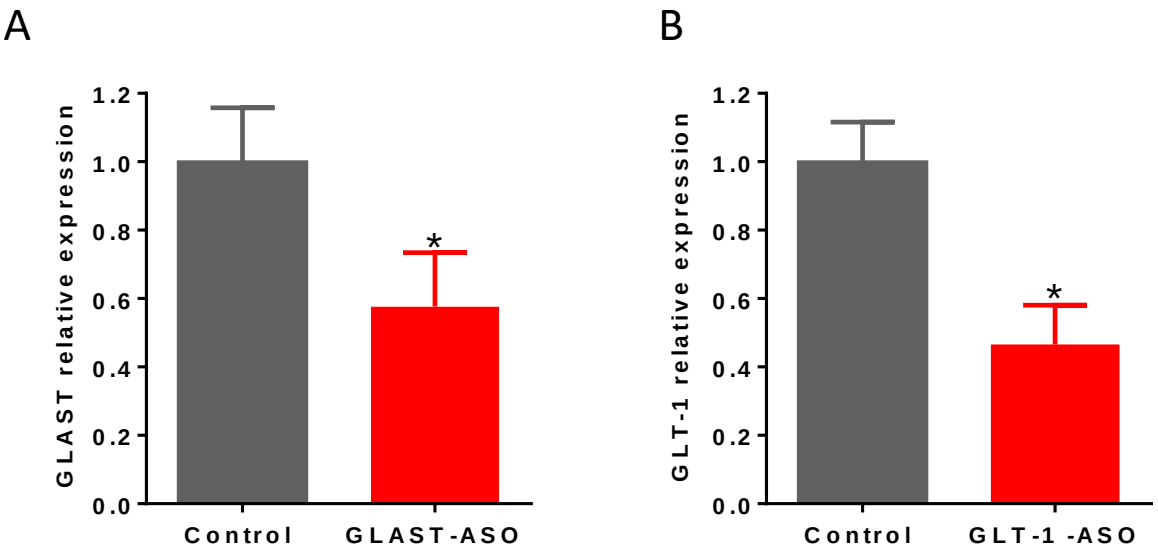


S6

Inhibition of spinal astrocytic glutamate transporters reduces the GFAP expression in the spinal cord after the first acid injection.

(A) Immunofluorescent staining of GFAP in lumbar spinal cord 4 hours after i.t. injection of DHK or TBOA followed by the acid injection, and (B) quantification of positive GFAP cells (activated astrocytes) and (C) the GFAP area in µm2 after the first acid injection in i.t. DHK and TBOA groups in comparison with i.t saline. n = 3 Data were presented as mean ± SEM vs. the control i.t. saline group ; (Scale bar, 200µm.).

Supplementary figure 7

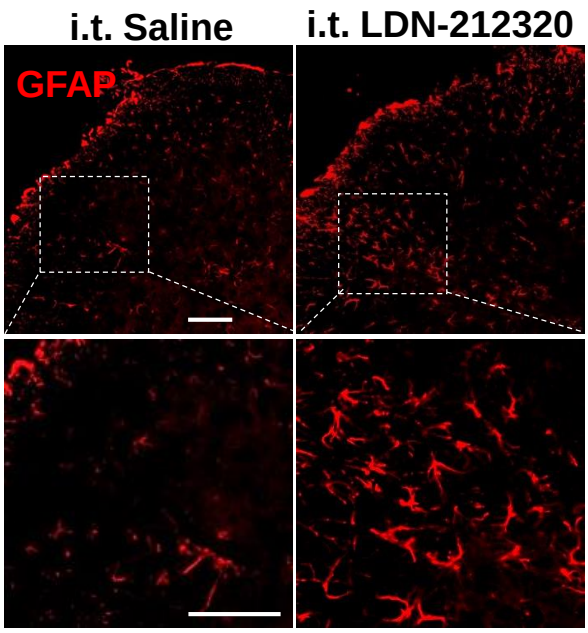


S7

The intrathecal infusion of (GLAST / GLT-1) ASOs reduces the mRNA expression of glutamate transporters

(A and B) Quantification show a decrease of GLT-1 and GLAST mRNA expression after intrathecal of ASO. Data were presented as mean ± SEM *, P<0.05 vs control; n=4/group.
The spinal cord RNA samples were extracted approximately one month after ASO injection. The qPCR primer sequences for EAAT1 were CAGTCTCGTCACAGGAATGGC and TTCCGGGGTGGATGATGATG, while those for EAAT2 were ATCAACAGAGGGTGCCAACA and GCTCCCAGGATGACACCAAA.

Supplementary figure 8



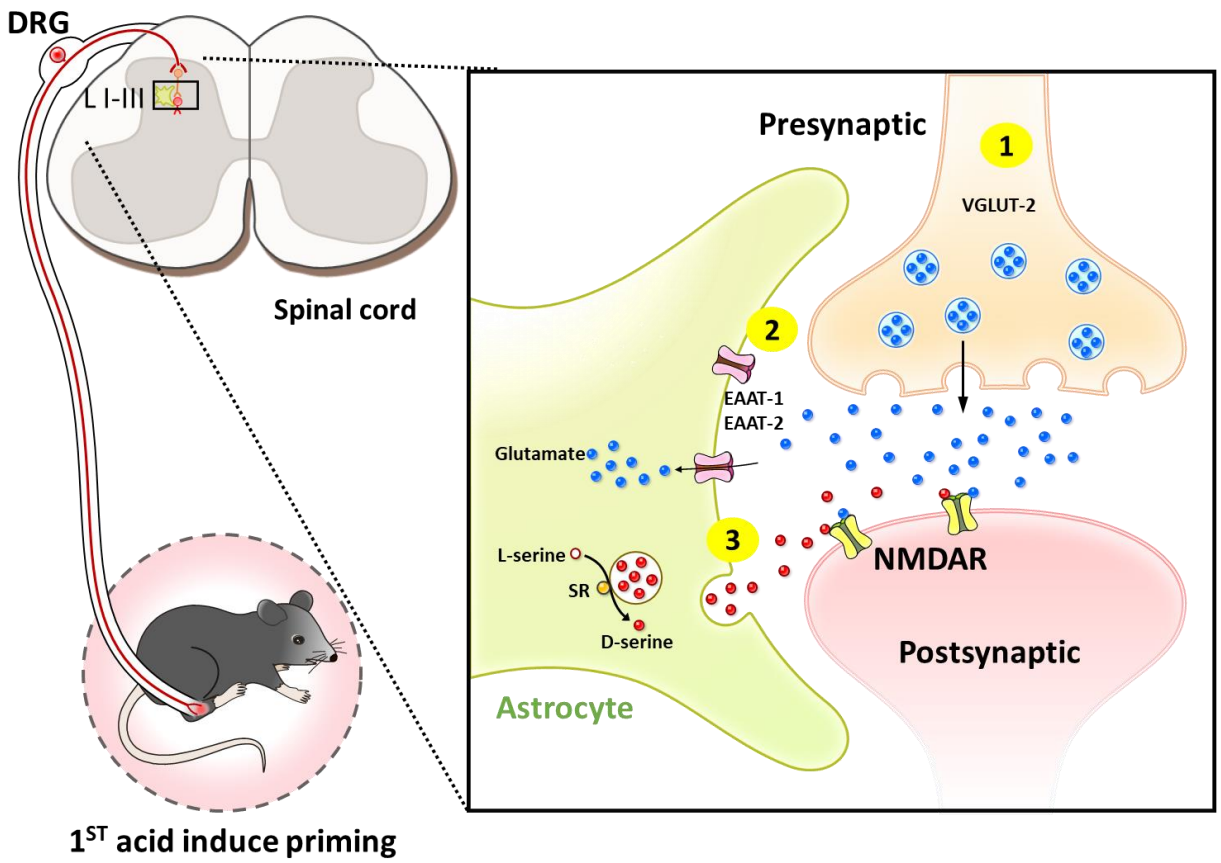
S8

Activation of spinal GLT-1 increases GFAP expression

Representative confocal images of GFAP immunofluorescent staining in lumbar spinal cord sections at 4 hours after i.t. GLT-1 activator LDN-212320 compared with i.t. saline. n=4. (Scale bar, 200μm.).

Supplementary figure 9

Graphical summary



S9

Graphical summary

1) The first intramuscular acid pH 4.0 injection activates spinal pERK expressing in Vglut2^{+} neurons.
2) The spinal astrocytes activation occurred 4 hours after the first acid injection. Dorsal horn Vglut2^{+} neurons are involved in astrocytes activation via GLT-1 or/and GLAST activity.
3) Spinal D-serine is required for hyperalgesic priming formation after astrocyte activation.