Therapeutic potential of β-lactam ceftriaxone for chronic pain in sickle cell disease

Sickle cell disease (SCD), one of the most common genetically inherited diseases, is the result of a point mutation in the β-globin gene that promotes hemoglobin polymerization and sickling of red blood cells.[1] No longer considered as a simple vasculopathy with anemia and hemolysis, SCD features the presence of complex pathophysiological changes.[2] Of the many clinical manifestations of SCD, chronic pain is among the most devastating complications lacking effective treatments.[3] In addition to intermittent acute pain crises, patients with SCD suffer from daily pain, which is reported as continuous, constant, and severe[4] and can present as spontaneous pain and hypersensitivity to thermal (cold and heat) and mechanical stimuli. We show here that ceftriaxone, a prototype β-lactam antibiotic, effectively alleviates both spontaneous ongoing pain and evoked pain in a humanized mouse model of SCD, through astrocytic inactivation. The pain reversal effect of ceftriaxone identified in this study is independent of the drug's antibacterial properties. Penicillin prophylaxis has been shown to be effective in preventing life-threatening pneumococcal infections in children with SCD between the ages of 2 months and 5 years,[5] highlighting a potential clinical strategy for treating or preventing chronic pain in SCD.

Most research on chronic pain in SCD has focused on neuronal mechanisms,[6,7] while the participation of glial cells,[8] specifically astrocytes, is less understood. Through dual connections to neurons and blood vessels in the central nervous system, astrocytes are positioned to play a vital role in maintaining glutamate homeostasis for neuronal signaling.[9] Glutamate transporter 1 (GLT1) is the major astrocytic glutamate transporter responsible for the uptake of over 90% of synaptically released glutamate to prevent excitotoxicity.[10] Dysfunction of astrocytic GLT1 has been associated with different neurological disorders including stroke and ischemia.[11] This study is the first to investigate the participation of GLT1 in the neuropathology of SCD. Given the emerging evidence suggesting the effectiveness of β-lactam antibiotics at restoring the expression and function of GLT1 *in vitro* and *in vivo*,[12] we aimed to examine the therapeutic potential of ceftriaxone for chronic pain in SCD.

We have previously carefully characterized chronic pain behaviors in a targeted knock-in mouse model of SCD (TOW mice) exclusively expressing human alleles encoding normal α- and sickle β-globin.[7] We therefore employed humanized TOW mice with SCD (8-10 weeks old) in this study after approval from the University of Illinois institutional animal care and use committee. As compared with age- and sex-matched non-sickle control mice ($h\beta^A/h\beta^A$), TOW mice ($h\beta^S/h\beta^S$) exhibited fully developed hypersensitivity to mechanical probing by von Frey filaments (Figure 1A) and to noxious thermal stimuli applied to the left hind paw (Figure 1B). After the baseline sensitivity testing on day 0, mice were treated with intraperitoneal (i.p.) ceftriaxone (200 mg/kg/day) for 7 consecutive days. Nociceptive responses to mechanical and heat stimuli were measured every other day. We found that ceftriaxone gradually reversed the mechanical allodynia and thermal hyperalgesia in TOW mice, without affecting mechanical and thermal sensitivity in control mice (Figure 1A, B). Significant suppression of mechanical and thermal hypersensitivity was observed after four injections of ceftriaxone (0.79±0.15 g in the ceftriaxone group *vs*. 0.10±0.03 g in the saline group, $P<0.001$ [Figure 1A]; 7.50 ± 0.44 s in the ceftriaxone group *vs*. 3.14 ± 0.35 s in the saline group, $P< 0.001$ [Figure 1B]). By day 6, ceftriaxone had completely restored the mechanical and thermal sensitivity in TOW mice ($h\beta^S/h\beta^S$) to levels which were indistinguishable from those in the non-sickle control mice ($h\beta^A/h\beta^A$). The anti-hyperalgesic/allodynic effect lasted for at least 24 days when the experiments stopped on day 28. A shorter period of treatment with ceftriaxone (200 mg/kg/day for 5 days, i.p.) produced a more transient effect that lasted for only 2-3 days (*Online Supplementary Figure S1*). These results demonstrated a potent and sustained effect of ceftriaxone in relieving evoked pain in mice with SCD.

Persistent ongoing pain, a main complaint of patients with SCD, is rarely studied in preclinical settings. We subjected TOW and control mice to a conditioned place preference (CPP) test to determine non-evoked ongoing pain. We have previously validated this negative reinforcement paradigm to unmask the presence of an aversive state as the result of non-evoked ongoing pain in mice.[7,13] After 7 days of treatment with ceftriaxone or saline in TOW mice, a single trial conditioning with saline and clonidine was performed on day 10. During the place preference test 20 h later, saline-pretreated TOW mice ($h\beta^S/h\beta^S$) spent significantly more time in the chamber that was paired with clonidine (551±58 s) than in the saline-paired chamber (259±43 s, $P<0.001$), indicative of clonidine-induced CPP (i.e., non-evoked spontaneous pain) in TOW mice with SCD (Figure 1C). In contrast, saline-pretreated non-sickle littermate mice ($h\beta^A/h\beta^A$) spent equal amounts of time in the saline- or clonidine-paired chambers (364±46 s *vs*.

353 ± 47 s, respectively), suggesting the absence of clonidine-CPP in the non-sickle mice (Figure 1C). Analysis of "difference scores" demonstrated a robust preference for chambers paired with clonidine in sickle mice ($h\beta^S/h\beta^S$), but not in non-sickle mice ($h\beta^A/h\beta^A$) (Figure 1D). Consistent with our previous findings in humans[4] and mice,[6,7] these results confirmed that spontaneous ongoing pain is a major feature in SCD. In TOW mice ($h\beta^S/h\beta^S$) that received ceftriaxone for 7 days, clonidine failed to generate CPP (341±21 s clonidine-paired chamber *vs*. 366 ± 32 s saline-paired chamber, $P>0.05$) (Figure 1E), similar to the absence of clonidine-CPP in the non-sickle, control mice ($h\beta^A/h\beta^A$). No groups of mice exhibited significant difference scores, indicating the absence of ongoing spontaneous pain in SCD mice after ceftriaxone treatment (Figure 1F). Therefore, ceftriaxone effectively blocked ongoing spontaneous pain, disrupting the clonidine-CPP behavior (i.e., ongoing pain) in TOW mice ($h\beta^S/h\beta^S$). As shown

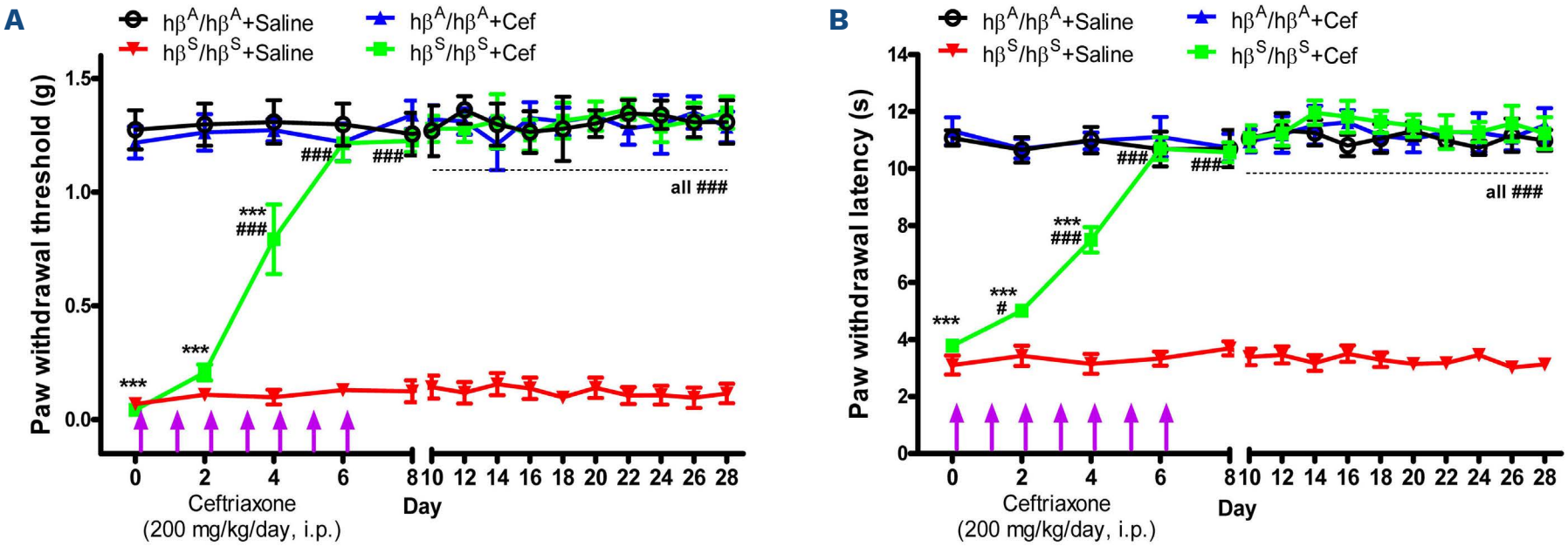

Figure 1. Ceftriaxone reversed chronic pain in TOW mice, an animal model of sickle cell disease. (A, B) Sensitivity to mechanical (A) and thermal (B) pain stimuli before and after intraperitoneal treatment with ceftriaxone (200 mg/kg/day.) was determined by calibrated von Frey filaments (Stoeling) using the "up-down" algorithm and a plantar tester (UGO Basile) with infrared light/heat stimuli, respectively. ***$P<0.001$ *vs*. "$h\beta^A/h\beta^A$ mice + saline" group; $^{\#}P<0.05$, $^{\#\#\#}P<0.001$ *vs*. "$h\beta^S/h\beta^S$ mice + saline" group; eight mice per group. (C) Intrathecal (i.t.) clonidine (1 µg)-induced conditioned place preference (CPP) in saline-treated TOW sickle cell disease mice. Mice were initially placed in the three-chamber CPP apparatus (San Diego Instruments) to record the "preconditioning" chamber preference. On the day of conditioning, the mice first received saline (5 µL, i.t.) paired with a randomly chosen end chamber and, 4 h later, clonidine (1 µg in 5 µL saline, i.t.) paired with the other end chamber. On the following day, 20 h after the afternoon pairing, mice were placed in the middle chamber of the CPP box with all doors open to enable free access to all chambers. Movement and duration of time each mouse spent in each chamber were recorded for 15 min for off-line analysis of chamber preference. $h\beta^S/h\beta^S$ mice spent significantly more time in the clonidine-paired chamber than in the saline-paired chamber, while $h\beta^A/h\beta^A$ mice spent similar amounts of time in either chamber. ***$P<0.001$, two-way analysis of variance followed by the *post hoc* Bonferroni test; eight mice per group. (D) Difference scores between test time and preconditioning (pre) time confirmed that $h\beta^S/h\beta^S$, but not $h\beta^A/h\beta^A$, mice developed clonidine-induced CPP. ***$P<0.001$ *vs*. $h\beta^A/h\beta^A$ mice; eight mice per group. (E) Clonidine (1 µg, i.t.) did not induce CPP in ceftriaxone-treated TOW $h\beta^S/h\beta^S$ mice or $h\beta^A/h\beta^A$ mice. $h\beta^S/h\beta^S$ and $h\beta^A/h\beta^A$ mice spent similar amounts of time in the saline- or clonidine-paired chambers. (F) Difference scores (test time – preconditioning time spent in the clonidine chamber) confirmed the absence of chamber preference. Cef: ceftriaxone; i.p.: intraperitoneal.

by the CPP test performed on day 30 (*Online Supplementary Figure S2*), the abolition of ongoing spontaneous pain by ceftriaxone was maintained for at least 3 weeks. The fact that ongoing pain and evoked pain hypersensitivity were no longer detected in TOW mice after 7 days of treatment with ceftriaxone indicates that ceftriaxone suppressed chronic pain in SCD.

To correlate pain-related behavioral changes with biochemical adaptations occurring in the central nervous system, the lumbar sections of the spinal cord were harvested for immunohistochemistry and western blotting analyses when the pain reversal effect plateaued on day 10. Compared with non-sickle control mice ($h\beta^A/h\beta^A$), TOW mice ($h\beta^S/h\beta^S$) exhibited substantially increased immunoreactivity of glial fibrillary acidic protein (GFAP),[14] demonstrating prominent astrocyte reactivity mainly in the superficial laminae of the dorsal spinal cord in mice with SCD (Figure 2). The enhanced astrocyte reactivity in TOW SCD mice was associated with reduced GLT1 immunofluorescent intensity in the spinal cord dorsal horn. Ceftriaxone significantly attenuated astrocyte reactivity, as demonstrated by a reduced number of GFAP-immunoreactive astrocytes (78 GFAP-immunoreactive cells in 15 regions of interest; 5 slides x 3 mice) in ceftriaxone-pretreated TOW mice ($h\beta^S/h\beta^S$) compared with that of saline-pretreated TOW mice ($h\beta^S/h\beta^S$) (175

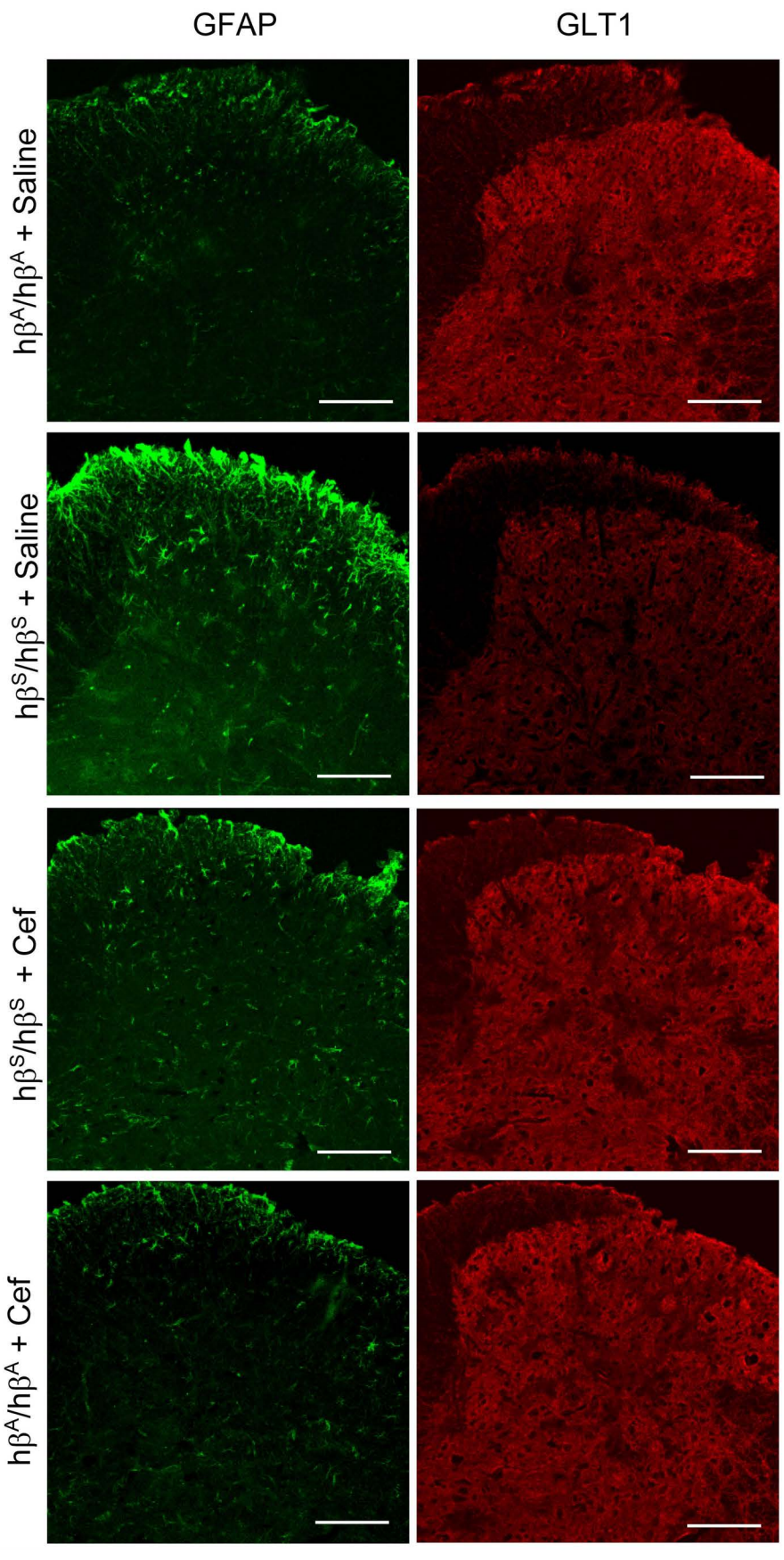

Figure 2. Immunoreactivity of GFAP and GLT1 in the superficial lamina region of the dorsal spinal cord in TOW mice. GFAP immunoreactivity was elevated, while GLT1 immunoreactivity was diminished in $h\beta^S/h\beta^S$ mice, in comparison with that in the control non-sickle $h\beta^A/h\beta^A$ mice. Ceftriaxone reduced GFAP immunoreactivity and increased GLT1 immunoreactivity in $h\beta^S/h\beta^S$ mice. Green: GFAP (1/500, Sigma-Aldrich); red: GLT1 (1/500, Sigma-Aldrich); blue: DAPI. Scale bar: 20 μm. Quantitation was performed by counting the number of positively stained cells using ImageJ software. Cef: ceftriaxone.

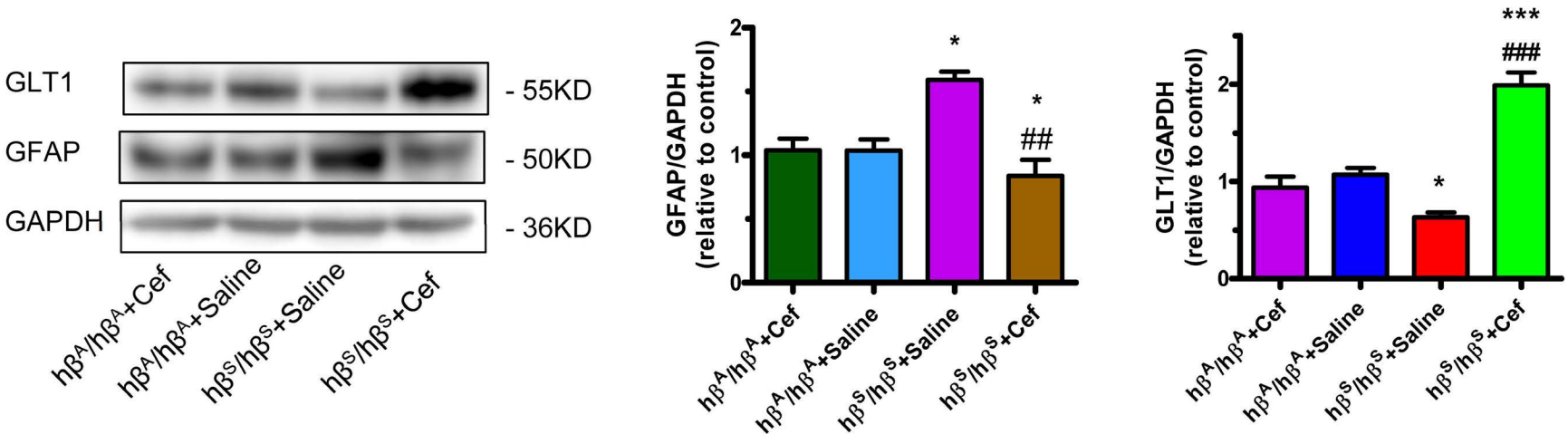

Figure 3. Expression of spinal GFAP and GLT1 in TOW hβ^S/hβ^S mice and control hβ^A/hβ^A mice, determined by the western blotting. Similarly to immunohistochemical analysis (Figure 2), the western blotting analysis also showed significantly increased expression of GFAP and decreased expression of GLT1 in the spinal cord of hβ^S/hβ^S mice. Ceftriaxone suppressed the upregulation of GFAP and promoted the expression of GLT1 in hβS/hβS mice. . *P<0.05, ***P<0001 *vs.* "hβ^A/hβ^A mice + saline" group. $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 *vs.* "hβ^S/hβ^S mice + saline" group; three mice per group. Cef: ceftriaxone.

GFAP-immunoreactive cells in 15 regions of interest; 5 slides x 3 mice). Meanwhile, reduced spinal GLT1 immunoreactivity in TOW mice (hβ^S/hβ^S) was restored by the treatment with ceftriaxone, which inversely correlated with the downregulation of GFAP immunoreactivity induced by ceftriaxone (Figure 2). Western blotting analysis demonstrated that the expression of spinal GFAP increased by 59%, while the expression of spinal GLT1 decreased by 41% in TOW mice (hβ^S/hβ^S) (P<0.5 *vs.* hβ^A/hβ^A -saline group) (Figure 3). Ceftriaxone completely blocked GFAP overexpression (P<0.01 *vs.* hβ^S/hβ^S-saline group) and abolished the repressive regulation of GLT1 expression in hβ^S/hβ^S mice (P<0.001 *vs.* hβ^S/hβ^S-saline group) (Figure 3). Collectively, these results demonstrated that astrocytes in the spinal dorsal horn became reactive in mice with SCD. Repeated intraperitoneal administration of ceftriaxone reduced astrocyte reactivity by increasing GLT1 expression. In addition to the spinal cord, similar biochemical changes were found, by western blotting analysis, in the dorsal root ganglia where ceftriaxone treatment (200 mg/kg, i.p. for 7 days) reversed GFAP upregulation and GLT1 downregulation in TOW mice (*Online Supplementary Figure S3*).

This is the first direct evidence that spinal astrocyte reactivity contributes to the development of chronic pain in SCD. Ceftriaxone (200 mg/kg, i.p. for 7 days) effectively blocked mechanical allodynia, heat hyperalgesia, and ongoing spontaneous pain associated with SCD. The effect of ceftriaxone is hypothesized to be mediated through the reversal of GLT1 dysfunction and the suppression of astrocyte reactivity. Ceftriaxone may induce the activation of transcription factor NF-κB, which then binds to and activates the GLT1 promoter.[15] The effect of ceftriaxone on chronic pain identified in this study was not related to the drug's antibacterial properties, because TOW mice did not have active bacterial infections during the experiment. Furthermore, it was shown that non-β-lactam antibiotics, such as doxycycline and kanamycin, had no effect on GLT1 expression and did not exhibit neuroprotective functions.[12] While there are concerns about the long-term usage of oral penicillin V in children with SCD, especially on the gut microbiota, our findings warrant further studies on the potential beneficial effect of ceftriaxone for chronic pain in patients with SCD. Moreover, GLT1 may serve as a new target for rational design of selective neuroprotective agents in SCD.

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Contributions

YH and XG performed the research; YH analyzed the data; and YH and ZJW wrote the manuscript and supervised the study.

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Data-sharing statement

The original data and protocols can be made available upon request.

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