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

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Sickle cell disease (SCD), one of the most common genetically inherited diseases, is the result of a point mutation in $\beta$-globin gene that promote hemoglobin polymerization and sickling of red blood cells\(^1\). No longer recognized as a simple vasculopathy with anemia and hemolysis, SCD features the presence of complex pathophysiologic changes\(^2\). Of the many clinical manifestations of SCD, chronic pain is among the most devastating complications lacking effective treatments\(^3\). On top of intermittent episodes of 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 hypersensitivities to the thermal (cold and heat) and mechanical stimuli. We show here that ceftriaxone, a prototype $\beta$-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 on its antibacterial property. Penicillin prophylaxis has been shown to be effective in preventing life-threatening pneumococcal infections in children with SCD between age two months and five years\(^5\), highlighting a potential clinical strategy of 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 (CNS), 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 to restore the expression and function of GLT1 in vitro and in vivo, we aim to examine the therapeutic potential of ceftriaxone for chronic pain in SCD.

Our previous work 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. Therefore, we employed the humanized TOW mice with SCD (8 - 10 weeks) in this study after approval by the University of Illinois IACUC. As compared with the age/sex-matched non-sickle control mice (hβA/hβA), TOW mice (hβS/hβS) exhibited fully developed hypersensitivity to mechanical probing by von Frey filaments (Figure 1A) and to the noxious thermal stimulus applied to the left hindpaw (Figure 1B). After the baseline sensitivity testing on Day 0, mice were treated with ceftriaxone (200 mg/kg/day, i.p.) consecutively for 7 days. Nociceptive responses to mechanical and heat stimuli were measured every other day. We found ceftriaxone gradually reversed the mechanical allodynia and thermal hyperalgesia in TOW mice, without affecting the mechanical and thermal sensitivities in control mice (Figure 1A-B). Significant suppression of mechanical and thermal hypersensitivities was observed after four injections of ceftriaxone (0.79 ± 0.15g in ceftriaxone group vs. 0.10 ± 0.03g in saline group, P < 0.001, Figure 1A; 7.50 ± 0.44s in ceftriaxone group vs. 3.14 ± 0.35s in saline group, P < 0.001, Figure 1B). On Day 6, ceftriaxone completely restored the mechanical and thermal sensitivities in TOW (hβS/hβS) mice to levels which were indistinguishable from those in the non-sickle control (hβA/hβA) mice. 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 (Figure...
S1). These results demonstrated a potent and sustained effect of ceftriaxone in relieving evoked pain in mice with SCD.

The persistent ongoing pain is a main complaint in patients with SCD, which is rarely studied in preclinical settings. Here, TOW and control mice were subjected to the 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. After 7 days’ 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 (hβS/hβS) mice spent significantly more time in the chamber that was paired with clonidine (551 ± 58s) than in saline-paired chamber (259 ± 43s, \( P < 0.001 \)), indicative of clonidine-induced CPP (i.e., non-evoked spontaneous pain) in TOW mice with SCD (Figure 1C). On the contrary, saline-pretreated non-sickle littermate (hβA/hβA) mice spent equal amount of time in the saline (364 ± 46s) or clonidine (353 ± 47s) paired chambers, 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 (hβS/hβS), but not non-sickle (hβA/hβA) mice (Figure 1D). Consistent with our previous findings in humans and mice, these results confirmed that spontaneous ongoing pain is a major pain feature in SCD. In TOW (hβS/hβS) mice that received ceftriaxone for 7 days, clonidine failed to generate CPP (341 ± 21s clonidine-paired chamber vs. 366 ± 32s saline-paired chamber, Figure 1E, \( P > 0.05 \)), similar to the absence of clonidine-CPP in the non-sickle (hβA/hβA) control mice. None of the mice groups exhibited significant difference scores, indicating the absence of ongoing spontaneous pain in SCD mice after ceftriaxone treatment.
Therefore, ceftriaxone effectively blocked ongoing spontaneous pain, disrupting the clonidine-CPP behavior (i.e. ongoing pain) in TOW (h\(\beta\)S/h\(\beta\)S) mice. As shown by the CPP test performed on Day 30 (Figure S2), the abolishment of ongoing spontaneous pain by ceftriaxone maintained for at least 3 weeks. Since ongoing pain and evoked pain hypersensitivities were no longer detected in TOW mice after 7-days’ treatment with ceftriaxone, these data indicated that ceftriaxone suppressed chronic pain in SCD.

To correlate mice pain behavioral changes with biochemical adaptations occurred in the CNS, the lumbar sections of the spinal cord were harvested for immunohistochemistry and western blotting analyses when pain reversal effect plateaued on Day 10. Compared with non-sickle control (h\(\beta\)A/h\(\beta\)A) mice, TOW (h\(\beta\)S/h\(\beta\)S) mice exhibited substantially increased immunoreactivity (IR) 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 displayed 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 reduced number of GFAP-IR astrocytes (78 GFAP-IR cells in 15 regions of interest or ROI, 5 slides x 3 mice) in ceftriaxone pretreated TOW (h\(\beta\)S/h\(\beta\)S) mice compared with that of saline pretreated TOW (h\(\beta\)S/h\(\beta\)S) mice (175 GFAP-IR cells in 15 ROIs; 5 slides x 3 mice). Meanwhile, reduced spinal GLT1-IR in TOW (h\(\beta\)S/h\(\beta\)S) mice was restored by the treatment with ceftriaxone, which inversely correlated with the downregulation of GFAP-IR induced by ceftriaxone (Figure 2). Western blotting analysis demonstrated the expression of spinal GFAP increased by 59%, while the expression of spinal GLT1 decreased by 41% in TOW (h\(\beta\)S/h\(\beta\)S) mice (\(P < 0.5\) vs. h\(\beta\)A/h\(\beta\)A - saline group; Figure 3). Ceftriaxone
completely blocked GFAP overexpression \( (P < 0.01 \text{ vs. } \text{hβ}^S/\text{hβ}^S\text{-saline group}) \) and abolished the repressive regulation of GLT1 expression in \( \text{hβ}^S/\text{hβ}^S \) mice \( (P < 0.001 \text{ vs. } \text{hβ}^S/\text{hβ}^S\text{-saline group}; \) 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 the western blotting analysis, in the dorsal root ganglia (DRG) where ceftriaxone treatment \( (200 \text{ mg/kg, } i.p. \text{ for 7 days}) \) reversed GFAP upregulation and GLT1 downregulation in TOW \( (\text{hβ}^S/\text{hβ}^S) \) mice (Figure S3).

This is the first direct evidence that spinal astrocyte reactivity contributed to the development of chronic pain in SCD. Ceftriaxone \( (200 \text{ mg/kg, } i.p. \times 7 \text{ days}) \) 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 GLT1 promoter\(^{15}\). The effect of ceftriaxone on chronic pain identified in this study was not related to its antibacterial properties, because TOW mice did not have active bacterial infections during the experiment. On the other hand, it has been shown that non-β-lactam antibiotics such as doxycycline and kanamycin had no effect on GLT1 expression nor exhibited neuroprotective function\(^{12}\). 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 potential beneficial effect of ceftriaxone for chronic pain in these patients. Moreover, GLT1 can serve as a new target for rational design of selective neuroprotective agents in SCD.
References


Figure legend

Figure 1. Ceftriaxone reversed chronic pain in TOW SCD mice. Mechanical (A) and thermal (B) sensitivities before and after the treatment with ceftriaxone (200 mg/kg/day, i.p.) were 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$\beta^A$ + saline” group; # $P < 0.05$, ### $P < 0.001$ vs. “h$\beta^S$/h$\beta^S$ + saline” group. n = 8/group. (C) Clonidine (1 µg, intrathecally or i.t.) induced CPP in saline-treated TOW SCD mice. Mice were initially exposed to the 3-chamber CPP apparatus (San Diego Instruments) to record the “preconditioning” chamber preference. On conditioning day, 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 have 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 the saline-paired chamber, while h$\beta^A$/h$\beta^A$ mice spent similar amount of time in either chamber. *** $P < 0.001$, two-way ANOVA followed by post hoc Bonferroni test; n = 8/group. (D) Different 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$\beta^A$; n = 8/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 amount of time in saline- or clonidine-paired chambers. (F) Different scores (test time – preconditioning time spent in the clonidine chamber) confirmed the absence of chamber preference.
Figure 2. Immunoreactivity (IR) of GFAP and GLT1 in the superficial lamina region of the dorsal spinal cord in TOW mice. GFAP-IR was elevated, while GLT1-IR was diminished in hβS/hβS mice, in comparison with those in the control non-sickle hβA/hβA mice. Ceftriaxone reduced the GFAP-IR and increased GLT1-IR in hβS/hβS mice. Green: GFAP (1/500, Sigma-Aldrich); Red: GLT1 (1/500, Sigma-Aldrich); Blue: DAPI. Scale bar: 20 µm. Quantitation analysis was performed by counting the number of positively stained cells using ImageJ.

Figure 3. Expression of spinal GFAP and of GLT1 in TOW hβS/hβS mice and control hβA/hβA mice, determined by the western blotting. Similar 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 hβS/hβS mice. Ceftriaxone suppressed the upregulation of GFAP and promoted the expression of GLT1. * P < 0.05, *** P < 0.001 vs. hβA/hβA mice+saline group. ## P < 0.01, ### P < 0.001 vs. hβS/hβS +saline group. n = 3/group.
Figure S1. Mechanical (A) and thermal (B) sensitivities before and after the treatment with ceftriaxone (200 mg/kg/day, i.p. × 5 days). *** P < 0.001 vs. “hβ^A/hβ^A” group; n = 8/group
Figure S2. When tested on Day 30, clonidine (1 µg, i.t.) did not induce CPP in ceftriaxone-treated TOW hβS/hβS mice or hβA/hβA mice. (A) hβS/hβS and hβA/hβA mice spent similar amount of time in saline- or clonidine-paired chambers. (B) Different scores (test time – preconditioning time spent in the clonidine chamber) confirmed the absence of chamber preference.
Figure S3

GLT1 - 55 KD
GFAP - 50 KD
GAPDH - 36 KD

Figure S3. Western blotting analysis of GFAP and GLT1 in the dorsal root ganglion of TOW hβ^S/hβ^S mice and control hβ^A/hβ^A mice. Ceftriaxone reversed the up-regulation of GFAP and the down-regulation of GLT1 in TOW hβ^S/hβ^S mice. * P < 0.05, vs. hβ^A/hβ^A mice+saline group. ## P < 0.01 vs. hβ^S/hβ^S +saline group, n = 3/group.