新北市政府 110 年度自行研究報告

研究報告名稱:
以 ENT1/ENT2 基因剔除小鼠模型及 ENT1/ENT2 抑制劑探討平衡型核苷轉運蛋白 ENT1/ENT2 於創傷性脊髓之角色

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新北市政府 110 年度自行研究成果摘要表

計畫名稱	以 ENT1/ENT2 基因剔除小鼠模型及 ENT1/ENT2 抑制劑探討平衡型核苷轉運蛋白 ENT1/ENT2 於創傷性脊髓之角色
期程	自 110 年 1 月 1 日至 110 年 12 月 31 日
經費	1,000,000 元
緣起與目的	探討 ENT1/2 基因剔除小鼠及給予抑制劑對創傷性脊髓損傷之影響，並觀察創傷性脊髓損傷之小鼠，在脊髓損傷後發炎反應是否下降，並評估是否對脊髓損傷復原有益。
方法與過程	將使用週齡 8-10 週之 C57BL/6 品系、ENT1 Knock out 或 ENT2 Knock out 之小鼠，進行脊髓損傷，使用 Spinal cord 損傷器、撞擊針 10 克重，從 37.5mm 高度自由落下損傷脊椎。依照不同組別給予 NBTI 藥物或是 J4，並預計脊髓損傷後 24 小時、72 小時以及 6 週予以犧牲，進行後續化學染色與免疫染色、mRNA 表現量測定以及 ELISA 檢測等，並將實驗數據統計分析，已完成結案。

研 究 發 現 及 建 議	Ent1 deletion/inhibition can improve functional recovery, ameliorate neuroinflammation and neuronal damage, and modulate astrocytic phenotype in mice with SCI. These findings indicate the important roles of Ent1 in the pathogenesis and treatment of SCI.
備 註	

(三) 研究內容：字體以標楷體為主，字體大小為14。標題依下列順序標明之：壹、一、(一)、1、(1)...等，並載列下列事項:

1.摘要(包含研究目的、研究方法、重要發現、主要建議及政策意涵)。

Background

Neuroinflammation plays a critical role in neurological recovery of spinal cord injury (SCI). Adenosine is an important modulator of neuroinflammation and neuronal function, whose cellular uptake is mediated by nucleoside transporters. This study was aimed to investigate the roles of equilibrative nucleoside transporter 1 (Ent1) in the inflammatory responses and functional recovery of SCI.

Methods

Spinal cord contusion at T10 dorsal portion was induced in mice to cause a complete paralysis of hindlimbs. Genetic deletion and pharmacological inhibition of Ent1 were used to evaluate the role of Ent1 in SCI. The outcomes were evaluated in terms of Basso Mouse scale (BMS), gait analysis, astrogliosis, microgliosis, and cytokine levels on day 14 post-injury (14 DPI). In addition, markers of astrocytic A1/A2 and microglial M1/M2 phenotypes were examined to investigate whether Ent1 inhibition may modulate the phenotypes of astrocytes and microglia/macrophage in SCI.

Results

The results showed that both genetic deletion and pharmacological blockage of Ent1 can improve the BMS score and stride length of mice, compared with the controls. Along with reduced size of lesion volume,

more preserved neurons were identified in the perilesional area of mice with Ent1 deletion/inhibition. Further examination showed that Ent1 deletion/inhibition significantly reduced the levels of inflammatory cytokines (IL-1β, TNF-α, or IL-6) and attenuated astrogliosis/microgliosis at the perilesional site of the spinal cord on 14 DPI. Moreover, Ent1 inhibition decreased the protein level of C3 (an A1 marker) but increased the levels of S100a10 (an A2 marker) and TGF-β, without changing the levels of iNOS (a M1 marker) and Arg1 (a M2 marker).

Conclusions

Ent1 deletion/inhibition can improve functional recovery, ameliorate neuroinflammation and neuronal damage, and modulate astrocytic phenotype in mice with SCI. These findings indicate the important roles of Ent1 in the pathogenesis and treatment of SCI.

2.主旨及背景說明(與現行業務關聯性)。

Spinal cord injury (SCI) is a devastating neurologic disorder that constitutes a considerable portion of the global injury burden and the number of individuals living with SCI is expected to increase in view of population growth [1]. SCI mostly occurs from a sudden and traumatic impact on the spine that dislocates or fractures the vertebrae. Following the initial mechanical impact that changes the vascular tissue and blood supply to the area of SCI, the secondary injury may include hemorrhage, ischemia, infiltration of immune cells, and the release of inflammatory cytokines [2]. SCI is generally considered as an irreversible neurological impairment and its treatment is still an unmet medical need. Neuroinflammation is a key component for the secondary injury mechanisms in SCI [3]. Interleukin-1 (IL-1), tumor necrosis factor alpha (TNF-α), and interleukin-6 (IL-6) are considered to be the critical mediators of the post-traumatic inflammatory reaction that may lead to neuronal death [4-6]. Also, the activation of microglia and astrocytes are notable responses of neuroinflammation. While activated microglia contribute secondary damage by releasing proinflammatory cytokines, microglia are also an essential component of the neuroprotective scar that forms after SCI [7]. The activated microglia can be polarized to a classically activated pro-inflammatory (M1) phenotype and an alternatively activated anti-inflammatory (M2) phenotype [8]. The M1 phenotype may initiate cascade of neurotoxic responses in secondary phase of SCI, whereas M2 phenotype is symbolized by the release of anti-inflammatory cytokines [9]. Likewise, while astrogliosis has been

regarded as a detrimental effect for SCI, emerging studies have suggested that reactive astrocytes also play a protective role in SCI [10, 11, 12]. Reactive astrocytes can be developed into two types, A1 and A2, respectively. A1 astrocytes have been reported to exert cytotoxic effects on local neurons and oligodendrocytes [13], whereas A2 astrocytes can promote neuron outgrowth and survival, and contribute to synapse formation and tissue repair [14].

This study was aimed to investigate the roles of equilibrative nucleoside transporter 1 (Ent1) in the inflammatory responses and functional recovery of SCI.

3.相關研究、文獻之檢討。

Adenosine, a purine nucleoside, is an important endogenous immunomodulator [15], whose functions also include neuroprotection [16]. Substantial level of adenosine was detected at the extracellular space of the spinal cord, following contusion SCI [17]. The implication of adenosine in SCI has been investigated in realted to its receptors. The activation of adenosine 2A receptor ($A_{2A}R$) may improve breathing in rats with cervical spinal injury [18] and reduce neuronal damage in mice with SCI [19]; adenosine 2B receptor ($A_{2B}R$) may be involved in the immunosuppressive effect of CD73 in SCI [20]. Nonetheless, it is noted that the modes of interaction between adenosine and the receptors depend on its extracellular concentrations [21]. The equilibrative nucleoside transporters (Ents) are facilitative transporters that play an important role in maintaining the homeostasis of adenosine by controlling the extracellular and intracellular adenosine levels [22]. Ents are divided into four subtypes, namely Ent1-4 (SLC29A1-4). Among these subtypes, only the expression of rat Ent1 has been reported at the spinal cord and the inhibition of rEnt1 at the spinal cord may modulate excitatory synaptic transmission by attenuating glutamate release [23]. While Ent1 has been investigated in related to disease progression in neurodegenerative diseases [24-26], its role in SCI is still unclear. The aim of the present study was to investigate the impacts of Ent1 deletion/inhibition on the inflammatory responses and functional recovery of SCI.

4. 研究方法(包含研究內容、範圍、對象、限制與過程)。

Animals

Male C57BL/6J mice were purchased from BioLASCO Taiwan (Taipei, Taiwan). Ent1$^{-/-}$ (B6.129X1-Slc29a1tm1Msg/J) mice were originally purchased from Jackson Laboratory (Bar Harbor, ME, USA). Ent1$^{-/-}$ mice were backcrossed with outbred C57BL/6 mice. The mouse colony was maintained through the breeding of heterozygous animals (Ent1$^{+/-}$) to obtain wild-type (Ent1$^{+/+}$) and knockout (Ent1$^{-/-}$) littermates. Both male and female transgenic mice (8-12 weeks old) were used in this study. The genotyping was conducted according to the procedure described previously [24]. All mice were maintained on a 12 h light/dark schedule and given food and water ad libitum in the Laboratory Animal Center (an AAALAC accredited experimental animal facility) at National Taiwan University. Procedures involving animals were conducted according to the ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines.

Induction of spinal cord contusion

Mice were anesthetized by intramuscular injection of Zoletil (50 mg/kg; tiletamine hydrochloride and zolazepam hydrochloride) and Rompun (12 mg/kg; xylazine hydrochloride) before the surgery. After laminectomy on the T9-T10 vertebral column, the spinal cord was exposed and contused by a 10 g rod with an impact head of 1.2 mm diameter at the height of 6.25 mm above the T10 spinal cord to allow it to drop using an Impactor model-II spinal cord contusion system. In the sham group, surgery procedures were identical except for the spinal contusion. After the injury,

the wound was quickly sutured using 4.0 silk thread (Unik Taiwan; New Taipei city, Taiwan). The bladders of mice were manually and gently massaged once a day to avoid retention of urine until the reflexive control of micturition was restored. Cefazolin (300mg/kg) was given for consecutive three days after surgery to prevent infection. Fourteen days after the surgery, mice were sacrificed for analyses.

Pharmacological treatment

For the administration of J4, an Ent1 inhibitor, male C57BL/6J mice (8-12 weeks old) were divided into three groups, the sham group, the SCI+J4 group and the SCI+vehicle group. In the J4-treated group, mice received intraperitoneal administration of 10 mg/kg J4 (an Ent1 inhibitor) twice a day for three days before the injury. Following the SCI, J4 (10 mg/kg) was administered to mice twice a day for consecutive 14 days. In vehicle-treated group, mice were treated with 5% DMSO, instead of J4, following the same protocol of the J4-treated group The sham group received the same treatment as the vehicle group.

Behavioral tests

The motor function after SCI was evaluated from day 3 to day 14 after the surgery by the Basso Mouse Scale (BMS) [27]. Scoring ranged from 0 for complete paraplegia to 9 for normal function. The footprint analysis was assessed on day 14 after the surgery. The hindlimbs of the mice were dipped in red and black dye for left and right foot, respectively. The mice were then placed on a piece of drawing paper and the stride length was defined as the distance between the middle point of two successive footprints.

RT-qPCR analysis

Mice were anesthetized by the inhalation of isoflurane. Spinal cords were frozen by flash freezing with liquid nitrogen and homogenized by sterile pellet pestles (Thermo Fisher Scientific, MA, USA) with 1 mL TRIzol™ Reagent (Thermo Fisher Scientific, MA, USA). Total RNA isolation, quality check, cDNA synthesis and SYBR green based quantitative real-time PCR assay were performed as described previously [28]. The forward/reverse primer sequences for *Slc29a1, Slc29a2, Slc29a3, Slc29a4, Cd39, Cd73, Ada, Adk, A_1R, $A_{2A}R$, $A_{2B}R$, A_3R, IL-1β, IL-6*, TNF-α, and Gapdh were listed in **Table 1**. The relative expression of target genes normalized to that of glyceraldehyde 3-phosphate dehydrogenase (Gapdh) was calculated by the comparative Ct (ΔCt) and was expressed as 2-ΔCt. The relative quantity was determined by the formula: 2-ΔΔCt, in which ΔΔCt values were obtained by subtracting the ΔCt values of mice with SCI from the sham controls (for J4 study) or the wild-type controls (for genetic knockout study).

Immunofluorescence staining

After being anesthetized by Zoletil (50 mg/kg) and Rompun (12 mg/kg), mice were intracardially perfused with ice-cold normal saline. The spinal cord (T9- T11) was removed and soaked in ice-cold 4% paraformaldehyde. The vertebrae were then placed in cassettes for paraffin embedding. The samples were cut into sagittal section of 4μm and placed in water bath at 42 ºC. The section was mounted onto slides and stored at room temperature until analysis. After deparaffinization, tissue retrieval was conducted in sodium citrate buffer (pH 6) at 90°C and

the spinal cord slices were then washed with phosphate-buffered saline (PBS). Sections were incubated in antibody dilution buffer (Roche, Basel, Swiss) at room temperature. The slides were immunostained by the following primary antibodies: chicken anti-NeuN (1:2000; Merk-millipore, Dramstadt, Germany), rabbit anti-ionized calcium-binding adapter molecule-1 (Iba-1) (1:1000; Abcam, Cambridge, MA, USA), or mouse antiglial fibrillary acidic protein (Gfap) (1:1000; cell signaling, Danvers, Massachusetts, USA) overnight at 4°C. Corresponding secondary antibodies conjugated with Alexa Fluor 488 or rhodamine (Jackson Immuno Research Laboratories, West Grove, PA, USA) were applied for the visualization of immunolabelling. After being washed with PBST (Phosphate Buffered Saline with Tween® 20) and PBS, the slices were mounted on a mounting gel (DAPI Fluoromount G; Southern Biotech Birmingham, Alabama, USA). Images were acquired by a Zeiss AXIO Imager M1 microscope (Carl Zeiss, Göttingen, Germany). In the perilesional area, the fluorescence intensity of Iba-1 and Gfap were quantified by the mean optical density (mean optical density = integrated optical density (IOD)/area) by ImageJ l.52 software (National Institutes of Health, Bethesda, Maryland, USA). The number of NeuN-positive cells in each sagittal section of the spinal cord was calculated at perilesional area at 20X magnification

Nissl staining

After the deparaffinization, the slices were placed in PBS and then in 1% cresyl violet solution. The slices were differentiated in 95% ethyl alcohol and dehydrated in 100% alcohol. After the dehydration, slices were

treated with xylene and mounted with dibutylphthalate polystyrene xylene. Regions of traumatic injury were identified by severe tissue destruction or staining loss. Six Nissl-stained sections by distance (100μm between the sections) were selected to estimate the proportional lesion size by ImageJ l.52 software (National Institutes of Health, Bethesda, Maryland, USA).

Western immunoblotting

The protein concentrations of the samples were determined by the Bio-Rad DC Protein Assay Kit (Bio rad, Hercules, CA, USA). Protein samples (10μg each) were diluted with loading buffer (200 mM Tris-HC1, 1.43% 2-mercaptoethanol, 0.4% bromophenol blue, and 40% glycerol) and heated at 98℃. The protein samples were then separated with 90 V for 10 minutes, followed by 130 V for 60 minutes on 12% SDS-polyacrylamide gel (for S100a10, c-caspase3, iNOS Arg-1 and Gapdh) or 8% SDS-polyacrylamide gel (for C3, NeuN) in running buffer (0.3% Tris base, 1.88% glycine, and 0.1% SDS). After electrophoresis, the gel was transferred onto a nitrocellulose membrane in transfer buffer (0.3% Tris base, 1.88% glycine, and 20% methanol; pH8.3) with 300 mA for 90 minutes. Nonspecific binding to membrane was blocked by BlockPROTM blocking buffer (Neihu, Taipei City, Taiwan) at room temperature on shaker at 25 rpm. For the detection of C3, NeuN, and Gapdh, the membrane was incubated overnight at 4℃ with antibodies for C3 (1: 200; Abcam, Cambridge, MA, USA), NeuN (1: 1000; Genetex, CA, USA), Arg-1 (1:500; Santa Cruz, Dallas, Texas, USA) or Gapdh (1:160000; Biodesign International, Saco, Maine, USA); for the detection

of S100a10 and c-caspases3, the membrane was incubated at 4℃ with S100a10 (1: 200; Abcam, Cambridge, MA, USA) or c-caspase3 (1:200; Merk-millipore, Dramstadt, Germany), all diluted in BlockPROTM blocking buffer; for the detection of iNOS the membrane was incubated for two nights at 4℃ with iNOS (1: 200; Abcam, Cambridge, MA, USA), diluted in BSA (Bovine serum albumin).The membrane was washed by TNT buffer (10mM Tris-HCl, 150mM NaCl, and 0.2% Tween 20; pH 7.4) and incubated with horseradish peroxidase (HRP)- conjugated anti-mouse IgG antibodies (1:5000; cell signaling, Danvers, Massachusetts, USA) or anti-rabbit IgG antibodies (1:2000; cell signaling, Danvers, Massachusetts, USA) in TNT buffer at room temperature. Bound antibodies were detected using Chemiluminescence reagent Plus (PerkinElmer Life Sciences, MA, USA) and Bio-rad ChemiDocTM XRS + Systems and Image LabTM Software to obtain images under appropriate exposure time.

Statistical analysis

All statistical analyses were conducted by GraphPad Prism v9.1 (GraphPad Software, San Diego, CA, USA). Data of alteration of adenosine-related genes in the SCI were analyzed by the unpaired t-test. For the effects of Ent1 inhibition, except that the data of lesion volume were evaluated by the unpaired t-test, all data (i.e., BMS, stride length, RT-qPCR, immunostaining, and Western blotting) were evaluated by the one-way ANOVA, followed by the least significant difference (LSD) test. For the effects of Ent1 knockout, all data (i.e., BMS, stride length, lesion volume, RTqPCR analysis, immunostaining, and Western blotting) were

evaluated by the unpaired t-test. The level of significance of 0.05 was considered statistically significant.

5.研究發現。

The expression of adenosine-related genes is altered in SCI

While it is known that extracellular adenosine level is highly increased during SCI [17], the regulation of adenosine-related genes in SCI remains unclear. Given that adenosine level is controlled by a number of proteins, including Ents, Cd39, Cd73, Ada, and Adk, gene expression of these proteins, along with that of adenosine receptors (A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R), were examined in B6 mice with and without SCI. As a result, following the SCI, the mRNA level of *Ent1* was significantly increased, but the expression of *Ent2* and *Ent4* was significantly decreased. The expression of *Ent3* was not altered (Figure 1A). In terms of the enzymes responsible for the formation of adenosine, the expression of *Cd39* and *Cd73* was significantly increased by SCI (Figure 1B). As for adenosine receptors, mRNA levels of $A_{2B}R$, and A_3R were significantly increased by SCI, whereas others remained unchanged (Figure 1C). These findings showed the aberrant expression of adenosine-related genes in SCI.

Genetic deletion of Ent1 improves functional recovery and reduced neuronal death in SCI

To investigate the role of Ent1 in the pathogenesis and treatment of SCI, $Ent1^{-/-}$ mice and the wild-type littermate controls were subjected to SCI. As shown in Figure 2A, the BMS score of the Ent1 knockout mice was significantly higher than that in the controls. The foot print analysis also showed that Ent1-/- mice had larger stride length than the controls (Figure 2B). Nissl staining demonstrated that the lesion area was reduced in $Ent1^{-/-}$ mice (Figure 2C). To evaluate the preserved neuron in the

perilesional area, immunostaining of NeuN was performed. As shown in Figure 2D, the number of NeuN-positive cells in the perilesional area was higher in Ent1$^{-/-}$ mice than in the controls. Similarly, protein level of NeuN was significantly higher and c-capaes3 was notably lower in Ent1-/- mice than in the control group (Figure 2EF). These findings showed that Ent1 deletion can improve functional recovery and reduce neuronal death in SCI.

Genetic deletion of Ent1 reduces the inflammatory responses in SCI

Neuroinflammation is important in the progression of SCI, in which IL-1β, IL-6 and TNF-α are considered to be critical for post-traumatic inflammatory reaction [4]. The effect of Ent1 deletion on the expression of these cytokines at the lesion and the perilesional site of spinal cord were examined. As a result, the mRNA levels of IL-1β, IL-6, and TNF-α were all significantly reduced in Ent1$^{-/-}$ mice (Figure 3A-C). In addition to the expression of inflammatory cytokine, astrogliosis and microgliosis are important benchmarks of neuroinflammation. As for astrogliosis, the Gfap-positive cells were less hypertrophic in Ent1$^{-/-}$ mice than in the wild-type controls (Figure 3D). Likewise, the density of the Iba-1-postivie cells were lower at around the lesion core of the Ent1$^{-/-}$ mice, compared with the controls (Figure 3E). These findings showed that Ent1 deletion exerted anti-inflammatory effect in SCI.

Pharmacological inhibition of Ent1 improves functional recovery after SCI

To confirm the effects observed in transgenic mice, further study was conducted to examine the effect of pharmacological inhibition of Ent1 on

SCI. J4, an Ent1 inhibitor, has been used to investigate the role of Ent1 in neurodegenerative diseases [24-26]. Therefore, it was given to B6 mice to investigate the effects of Ent1 inhibition on SCI (Figure 4A). As shown in Figure 4B, the BMS score of mice with SCI can be partially improved by the treatment of J4. Further examination on the coordination of hindlimbs movements showed that the treatment of J4 improved the stride length of the mice with SCI (Figure 4C). In terms of the lesion, the Nissl staining showed that the loss of spinal cord tissue was significantly reduced by the J4 treatment on 14 DPI (Figure 4D). To further evaluate the preserved neuron in the perilesional area, the immunostaining and Western blotting of NeuN were performed. As shown in Figure 4E, more NeuN-positive cells were identified in the perilesional area of the mice treated with J4. Likewise, protein level of NeuN was higher in the J4-treated group than in the vehicle group (Figure 4F). Since the cleaved caspase-3 (ccaspase3) is important for cell apoptosis [29], the protein level of c-caspase3 was examined. As shown in Figure 4G, the level of c-caspase3 was decreased by the treatment of J4 for continuous 14 days following the injury. These results showed that pharmacological inhibition of Ent1 can improve motor function and protect the neuronal cells from apoptosis in SCI.

Pharmacological inhibition of Ent1 reduces the inflammatory responses in SCI

Similar to the findings in mice with genetic deletion of Ent1, J4 treatment significantly reduced the expression of TNF-α, with a trend of reduction on the expression of IL-1β and IL-6, in mice with SCI (Figure 5A-C). For astrogliosis and microgliosis, as shown in Figure 5D, the Gfap-positive

astrocytes in the perilesional area were hypertrophied and their branches were thickened, showing morphological changes associated with the reactive status. Compared with that in the vehicle group, the average fluorescence intensity of Gfap in the J4 group was remarkably decreased on 14 DPI. For microgliosis, similar to astrocytes, the intensity of Iba-1-positive cells was lower in the J4-treated group, compared with the vehicle-treated group (Figure 5E). These findings showed that pharmacological inhibition of Ent1 can reduce the inflammatory responses, including the expression of inflammatory cytokines and the activation of astrocytes and microglia/macrophage at the perilesional site of the spinal cord.

Pharmacological inhibition of Ent1 modulates the phenotype of the activated astrocytes in SCI

To further examine the effect of Ent1 inhibition on the phenotype of activated astrocytes, the expression of C3 and S100a10, the makers of A1 and A2 astrocytes, respectively [30], was examined. As a result, the protein levels of C3 (Figure 6A) and S100a10 (Figure 6B) were significantly decreased and increased, respectively, by the treatment of J4. As astrocytes can produce transforming growth factor β (TGF-β) that has been implicated in inducing axon formation and neurogenesis [31, 32], the level of TGF-β was examined. As shown in Figure 6C, TGF-β protein was significantly increased by J4 treatment. On the other hand, similar to the differentiation of activated astrocytes, the activated macrophage/microglia can be polarized to the proinflammatory M1 type and anti-inflammatory M2 type, in which iNOS and Arg1 are considered

as the marker for M1 and M2 phenotype, respectively [8, 9, 33]. The results showed neither SCI nor J4 treatment changed protein level of iNOS (Figure 6D). This finding was in line with what has been reported [34]. On the other hand, the level of Arg-1 remained higher on 14 DPI and J4 treatment did not further increase the level of Arg-1 (Figure 6E). These findings showed that J4 treatment can elevate A2 astrocytes expression and lower the expression of A1 astrocytes without affecting the phenotype of macrophage/microglia.

6. 結論與建議(分立即可行之建議及長期性建議)。

Following the primary damage on the spinal cord, inflammation plays an important role in the development of secondary injury that may lead to neuronal death [3]. Targeting neuroinflammation may provide a way to improve neuronal function and the outcomes of SCI [35]. While methylprednisolone has been applied to reduce the inflammatory responses in SCI, its use is highly controversial and is not officially approved [36]. To date, the treatment of SCI is still considered to be an unmet medical need. Previous study has shown that extracellular level of adenosine, an important modulator of inflammatory responses, is highly increased after SCI [17].

Upon the increase of extracellular adenosine level, Ents plays an important role in mediating celluar uptake of adensoine. The present study showed that Ent1, Ent2, Ent3 and Ent4 are expressed at the spinal cord of mice, in which mRNA levels of Ents reacted differently upon SCI. Different from the down-regulated Ent2 and Ent4, Ent1 was significantly upregulated by SCI. Genetic deletion of Ent1 can reduce inflammatory responses and neuronal loss in SCI, improving the recovery of motor function. Pharmacological inhibition of Ent1 also showed beneficial effects in mice with SCI. Adenosine is an important endogenous immunomodulator that regulates cell functions through the interaction with the G-protein-coupled receptors. In related to SCI, previous studies mainly focused on the role of adenosine receptors, in particular the $A_{2A}R$, in which $A_{2A}R$ activation can provide neuroprotective effect on SCI [19]. Nonetheless, while the expression of

$A_{2A}R$ remained unchanged in mice with SCI, the expression of A_3R was highly increased (Figure 1C). The role of A_3R in SCI is worth an attention because A_3R has been proposed as a therapeutic target to reduce secondary events and improve neurocognitive functions following traumatic brain injury [37]. Furthermore, it has been demonstrated that the activation of A_3R can provide powerful analgesic effects in rodent models of experimental neuropathic pain [38].

It is noted that the modes of interaction of between adenosine and the receptors depend on its extracellular concentrations [21]. Extracellular level of adenosine is controlled by a number of proteins, including, Cd39, Cd73, Ada, Adk, and Ents. In terms of the regulation of ectonucleotidases in SCI, the expression of both Cd39 and Cd73 were both increased upon SCI (Figure 1). This is probably in response to the release of ATP by injured cells, enhancing the conversion of ATP to adenosine in the extracellular space. On the other hand, the expression of Adk and Ada, the enzymes responsible for lowering adenosine concentration, was not significantly changed by SCI. These findings suggest that the response of adenosine system to SCI favors the formation of extracellular adenosine. Corroboratively, evidence has suggested that modulating extracellular adenosine levels via overexpressing CD73 imparts neuroprotective effects in SCI by modulating the status of neuroinflammation [33].

Ents are facilitative transporters that mediate adenosine uptake by a concentration-dependent manner. Among Ents, our results showed that only Ent1 was up-regulated by SCI. The increase in Ent1 expression has been observed in Huntington's diseases [24, 39], alcohol use disorder

[40], and depression [41]. The increase of Ent1 can reduce extracellular adenosine concentration [39], whereas the inhibition of Ent1 results in the elevation of extracellular adenosine levels [24, 25]. In the spinal cord, selective inhibition of Ent1 by NBMPR can modulate glutamatergic synaptic transmission [23]. The present study further demonstrated that the deletion/inhibition of Ent1 exhibited anti-inflammation effect and improve functional recovery in SCI. In addition to the present finding in SCI, the beneficial effects of Ent1 inhibition has been demonstrated in animal models with neurodegenerative diseases [24-26].

Neuroinflammation involves microgliosis, the infiltration of macrophages, and
astrogliosis in SCI. Both the expression of Iba-1 (for microglia/macrophage), and Gfap (for astrocytes) were increased in SCI. The activation of microglia/macrophage and astrocytes can be reduced by either genetic deletion or pharmacological inhibition of Ent1 (Figure 3 and Figure 5). While the M1/M2 status of macrophage/microglia has been reported to play important roles in the pathogenesis of SCI [42], in the present study, pharmacological inhibition of Ent1 did not change the status of M1/M2 (Fig. 6D and E). Nonetheless, it was previously reported that mRNA level of iNOS (a marker of M1) peaked at 6 hours after SCI and it declined until 72 hours after SCI [43], suggesting that iNOS is expressed at the acute phase of the injury, but not on later day (e.g., 14 DPI of the present study).

In terms of the activated astrocytes, similar finding in astrocytes has been reported, in which Gfap expression is reduced in Ent1 null mice [44]. In

addition, the present results showed that Ent1 inhibition significantly reduced protein levels of C3 (a A1 marker) (Figure 6A) but significantly increased protein level of S100a10 (a A2 marker) (Figure 6B). A1 astrocytes have shown neurotoxic effect to oligodendrocytes and neurons in vitro [13], whereas A2 astrocytes promote neuronal survival and tissue repair [14]. It was previously reported that the functional recovery in mice with SCI can be improved by reducing astrocytes with A1 phenotype [45, 46]; the increase of A2 astrocytes may provide beneficial effect on reducing the pro-inflammatory cytokines production [47]. Given that decreasing A1/increasing A2 astrocytes may be beneficial to the injury, the modulation of A1/A2 phenotype by J4 treatment support the beneficial effect of Ent1 inhibition in the treatment of SCI. In addition to the change in astrocytic phenotype, Ent1 inhibition also increase the level of TGF-β, which has been implicated in regulating adult neurogenesis and mediating changes in neurogenesis in response to injury [48]. Except for the A1/A2 phenotype, the reactive astrocytes surrounding the SCI lesion may further develop to form the glial scar, providing a physical barrier to restrict the expansion of the lesion area. The formation of glia scar is considered as a part of endogenous mechanisms to limit the initial tissue damage to the spinal cord and prevent extension of injury into adjacent sites. However, in the present study, the expression of N-cadherin (a marker of scarforming astrocytes) [49] was not increased by SCI. J4 treatment did not change the expression of N-cadherin in SCI mice.

Conclusions

Either pharmacological inhibition or genetic ablation of Ent1 can attenuate the inflammation, reduce the lesion, and improve functional recovery in mice with thoracic spinal cord contusion. These results suggest that Ent1 is a potential target for the pathogenesis and treatment of SCI.

7. 參考文獻。

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8. 表

Supplementary Table 1. Primary sequences for RT-qPCR analysis

Genes		Primer sequence
mouse *Slc29a1*	Forward	5-‘CAAGTATTTCACAAACCGCCTGGAC-3’
	Reverse	5-‘GAAACGAGTTGAGGCAGGTGAAGAC-3’
mouse *Slc29a2*	Forward	5-‘CATGGAAACTGAGGGGAATCGA -3’
	Reverse	5-‘GTTCCAAAGGCCTCACAGAG -3’
mouse *Slc29a3*	Forward	5-‘TTGGGCTCTGTATGGGACTC-3’
	Reverse	5-‘TTCTTCAGGATGGGTCCAAG-3’
mouse *Slc29a4*	Forward	5-‘GCGGCTGTGCTCCTAAAC-3’
	Reverse	5-‘CGTAAGCCTGGTCGTGAG-3
mouse *Cd39*	Forward	5-‘GTACCTGAGTGAGTACTGCTTCTC -3’
	Reverse	5-‘GCTGGGATCATGTTGGTCAAGTTC -3’
mouse *Cd73*	Forward	5-‘CAAATCCCACACAACCACTG -3’
	Reverse	5-‘TGCTCACTTGGTCACAGGAC -3’
mouse *Ada*	Forward	5-‘GAAGGCAAAGGAGGGCGTGGTCTA -3’
	Reverse	5-‘GATGTCCACAGCCTCACGCACAA -3’
mouse *Adk*	Forward	5-‘GTGCTATTTGGAATGGGGAAT -3’
	Reverse	5-‘CAACCACTGAGCCACTTTCAT -3’
mouse *Adora1*	Forward	5-‘GCTTAGTCCCTCAGAATCACG -3’
	Reverse	5-‘CCCTTGTCCTTAGAGGTTCCA -3’
mouse *Adora2a*	Forward	5-‘CTGCTTTGTCCTGGTCCTCAC -3’

9.圖

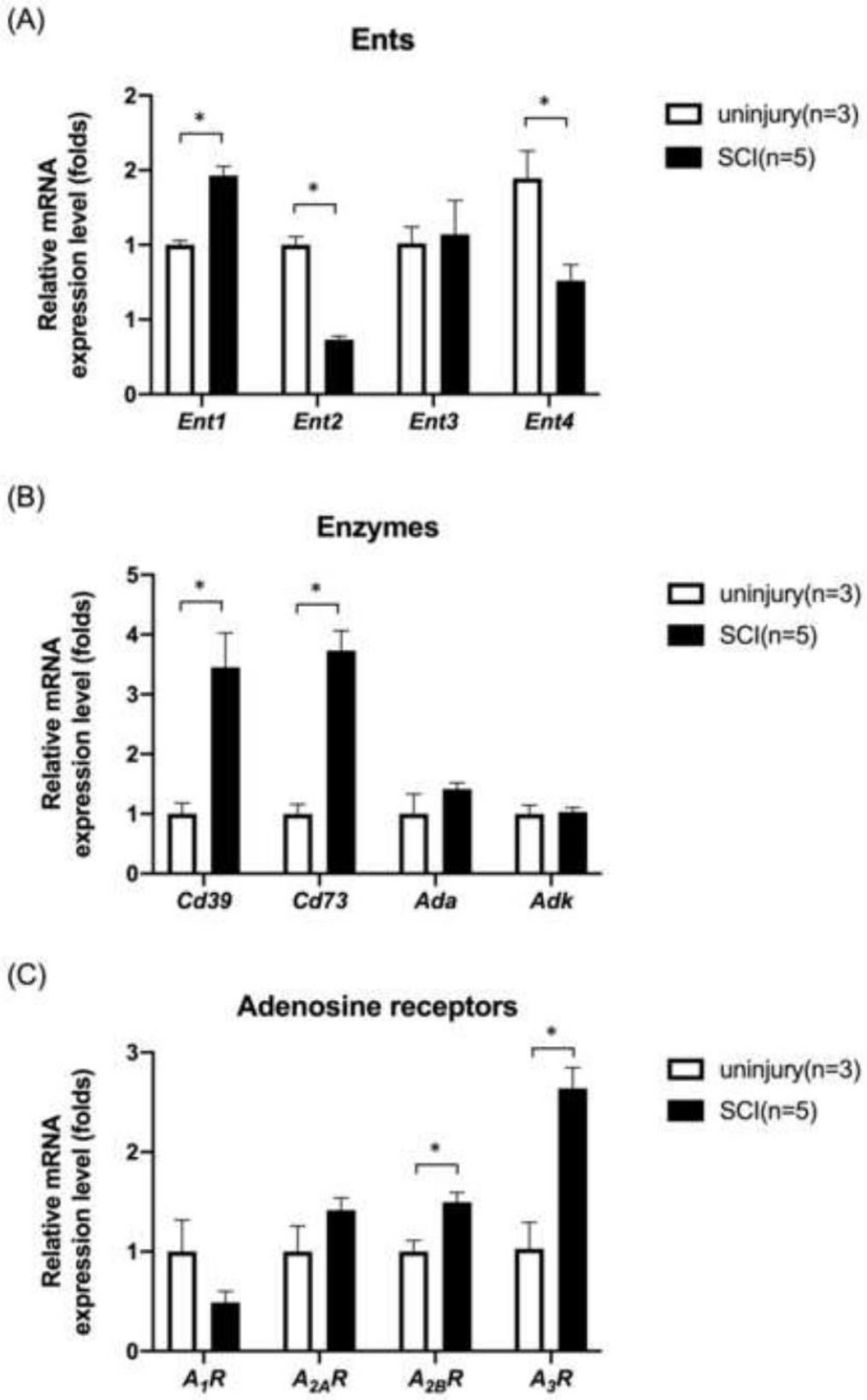

Figure 1. Changes in the mRNA levels of equilibrative nucleoside

transporters, *Ent1-4*. (A) Enzymes responsible for the formation/conversion of adenosine (B), and adenosine receptors (C) in mice with spinal cord contusion. Data are expressed as means ± SEM of 3-5 animals. *$p < 0.05$.

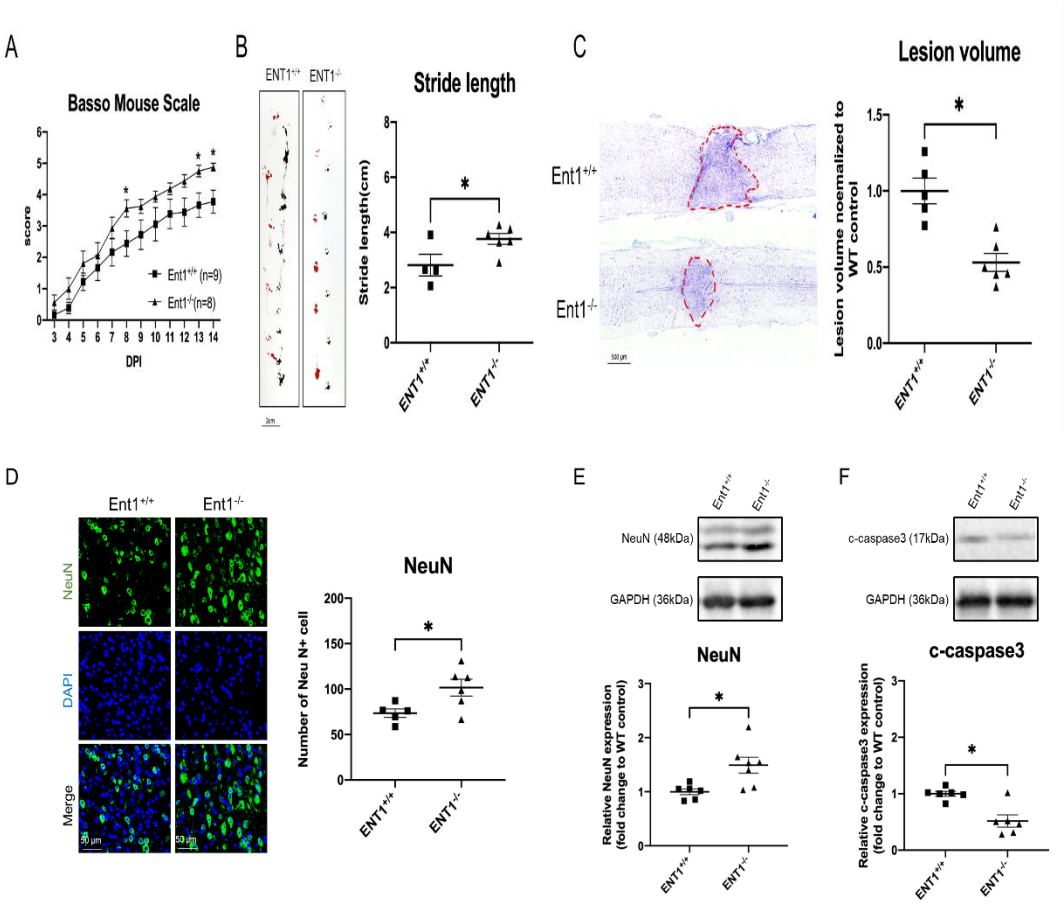

Figure 2. Improved motor function and reduced neuronal death in Ent1$^{-/-}$ mice with spinal cord contusion. (A) Basso mouse scale (BMS) at different days following spinal cord contusion in Ent1$^{+/+}$ (n=9) and Ent1$^{-/-}$ (n=8) mice (B) Representative images of footprint on day 14 post-injury (14 DPI) and quantitative results of the footprint analysis. Scale bar, 2cm. (C) Nissl-staining of sagittal section of spinal cord at 14 DPI and the quantitative analysis of lesion volume. Scale bar, 500μm. (D) Representative images of NeuN (green) in the perilesional area and the quantitative results. Scale bar, 50μm. (E and F) Protein levels of NeuN (E) and c-caspase 3 (F) at 14 DPI and the quantitative densitometric analysis of these proteins. Data are presented as mean ± SEM of 4-6 animals. *$p < 0.05$.

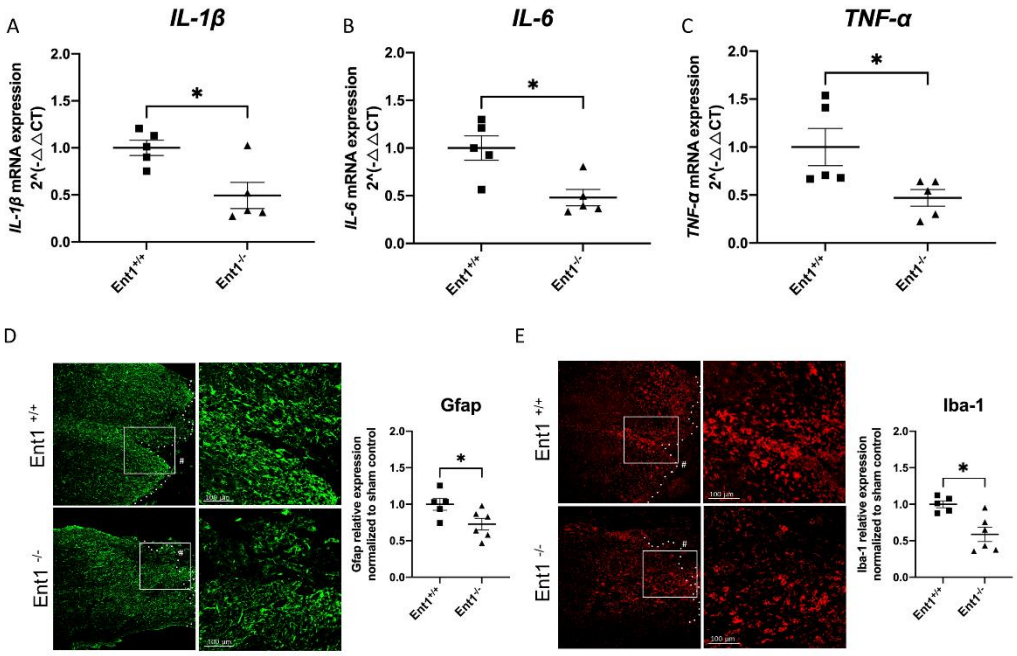

Figure 3. Reduced inflammatory responses in Ent1-/- mice with spinal cord contusion. (A-C) mRNA levels of IL-1β (A), IL-6 (B), and TNF-α (C) at T9-T11 of Ent1$^{-/-}$ mice and the wild-type controls. (D and E) Representative images of Gfap (D; green) and Iba-1 (E; red) in the perilesional area of Ent1$^{-/-}$ mice and the wild-type littermate controls (scale bar, 100μm). The quantitative results were shown on the right-hand side of the images. The data are presented as mean ± SEM of 5-6 animals. *$p < 0.05$. # Stands for the lesion core.

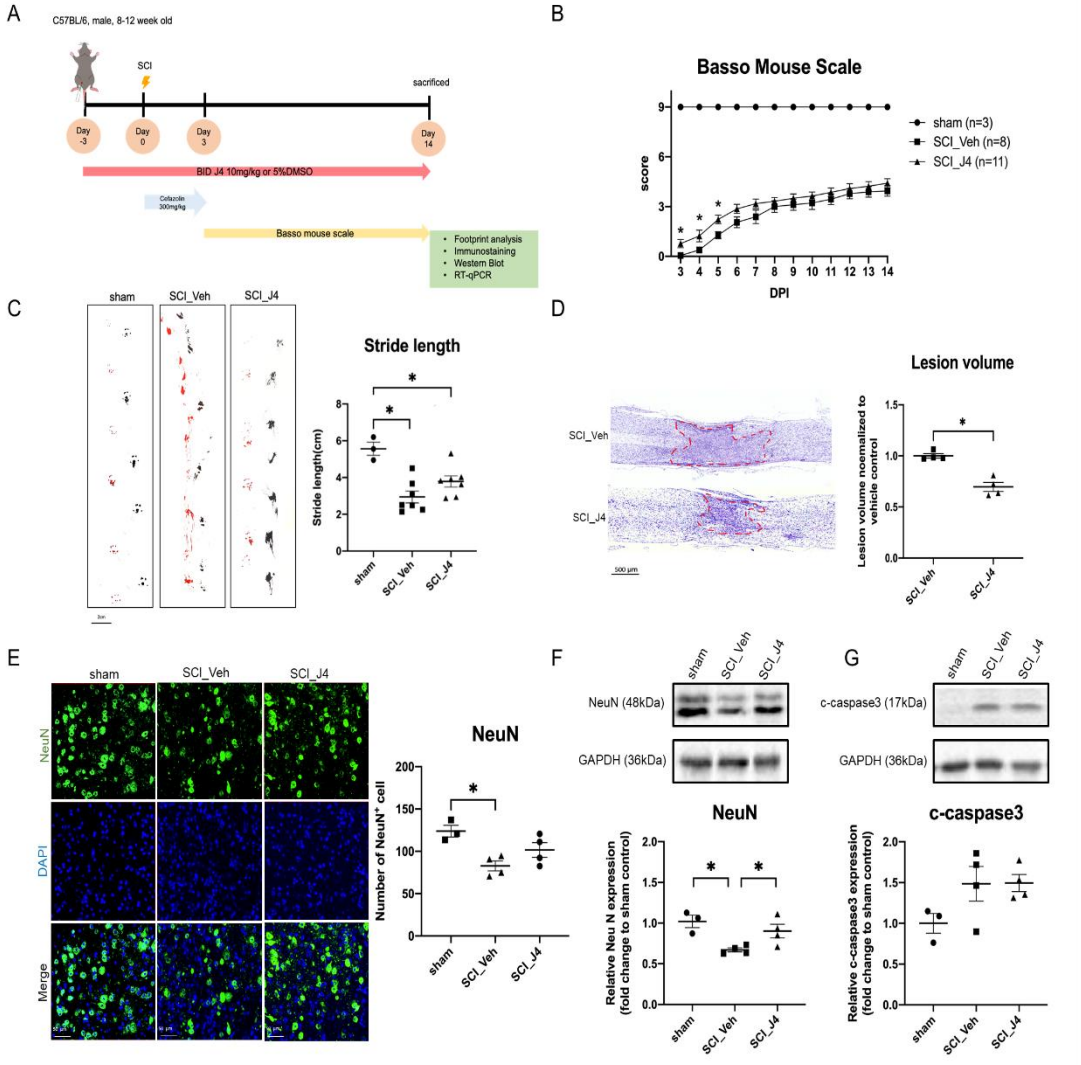

Figure 4. Improved motor function and reduced neuronal death in J4-treated mice with spinal cord contusion. (A) J4, an Ent1 inhibitor, was intraperitoneally injected three days before injury and then continuously injected daily for 14 days following the spinal cord injury. (B) Basso mouse scale (BMS) at different days following the spinal cord contusion. (C) Representative image of footprint analysis on day 14 post injury (14 DPI) and the quantitative results. (D) Nissl-stained of sagittal section of spinal cord on 14 DPI and the quantitative results of the lesion volume. Scale bar, 500 μm. (E) Representative images of NeuN (green)

in the perilesional area and the quantitative results. Scale bar, 50μm. (F and G) Protein level of NeuN (F) and ccaspas3 (G) on 14 DPI at T9-T11 and the quantitative densitometric analysis of these proteins. Data are given as the mean ± SEM of 3-7 animals. *$p < 0.05$.

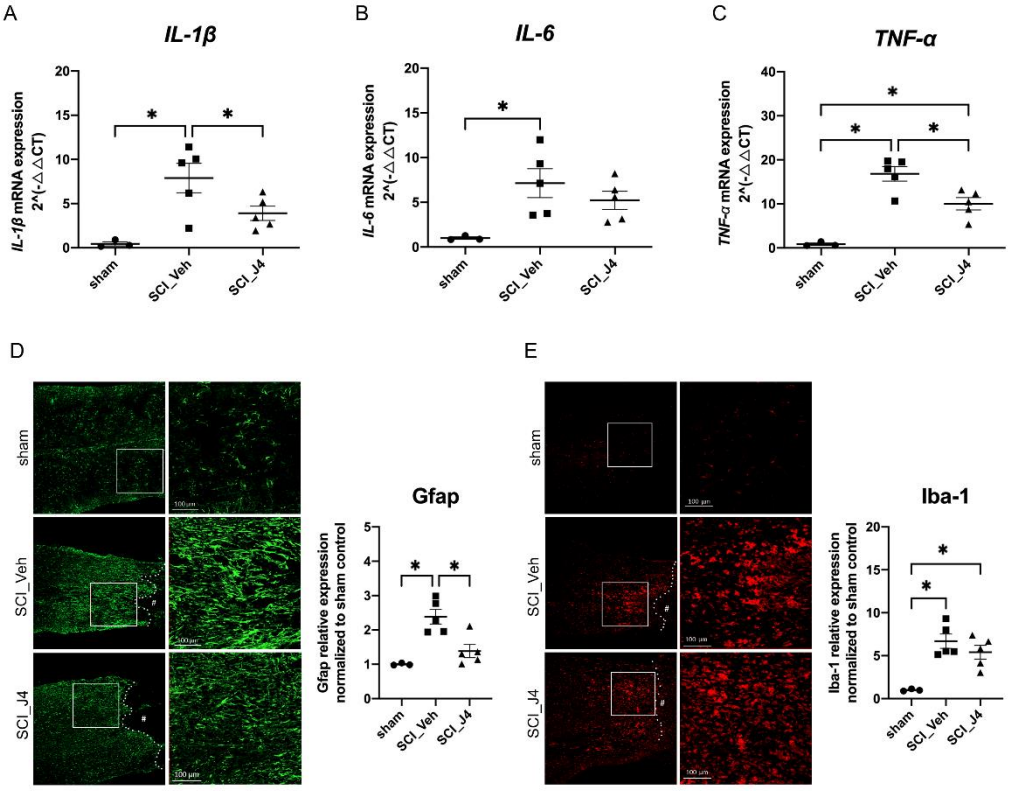

Figure 5. Reduced inflammatory responses in J4-treated mice with spinal cord contusion. (A-C) The effects of J4, an Ent1 inhibitor, on the mRNA levels of IL-1β (A), IL-6 (B), and TNF-α (C) at perilesional area of sham mice and mice with SCI on day 14 postinjury. (D and E) Representative images of Gfap (D; green) and Iba-1 (E; red) at the perilesional area and the quantitative results. Scale bar, 100μm. Data are presented as mean ± SEM of 3-5 animals. *$p < 0.05$. # Stands for the lesion core.

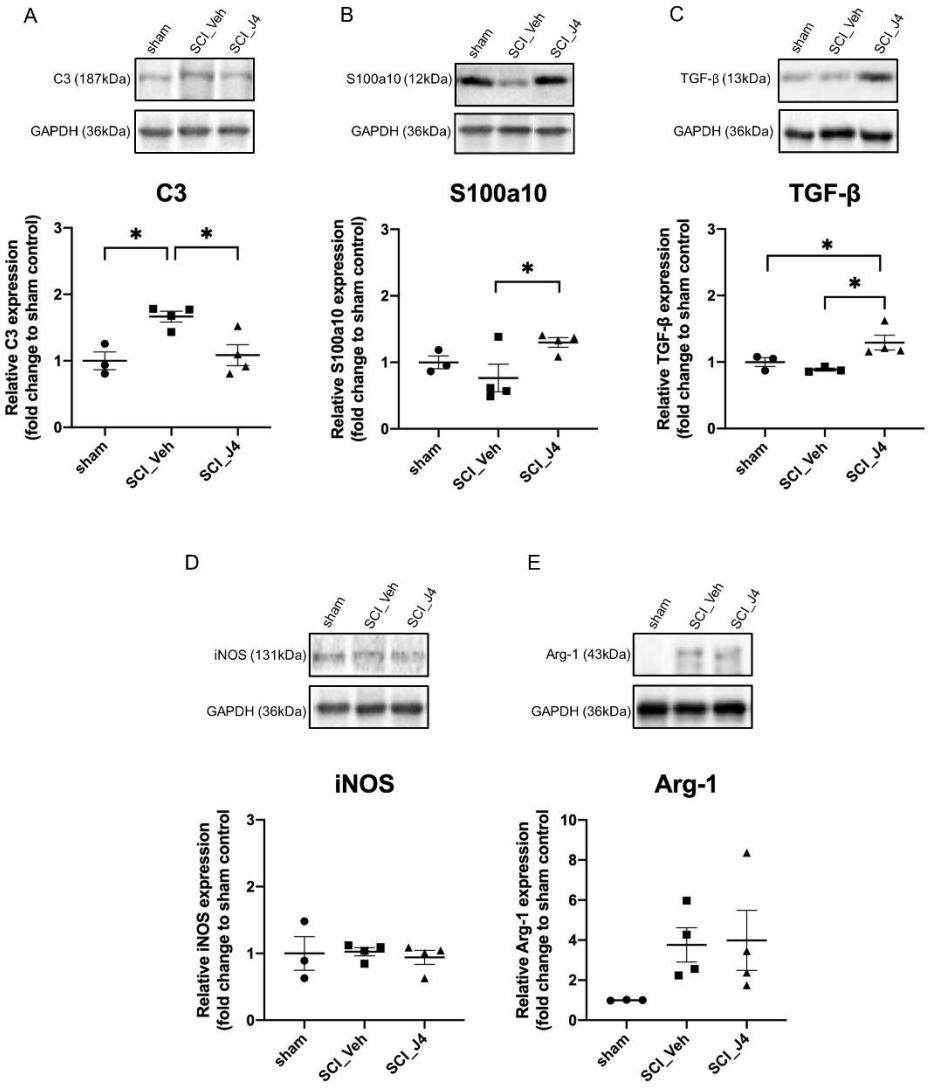

Figure 6. Changes in astrocytic A1/A2 phenotypes in J4-treated mice with spinal cord contusion. (A and B) The effects of J4, an Ent1 inhibitor, on the astrocytic A1 marker C3 (A), A2 marker S100a10 (B), and TGF-β (C). (D and E) Protein levels of M1 marker iNOS (D)and M2 marker Arg-1 (E) in the sham and SCI mice on day 14 postinjury and the quantifications of densitometry of these proteins. Data are presented as mean ± SEM of 3-4 animals. *$p < 0.05$.