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Cell cycle inhibition limits development and maintenance of neuropathic pain following spinal cord injury

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Abstract

Chronic pain after spinal cord injury (SCI) may present as hyperalgesia, allodynia, and/or spontaneous pain and is often resistant to conventional pain medications. Identifying more effective interventions to manage SCI-Pain requires improved understanding of the pathophysiological mechanisms involved. Cell cycle activation (CCA) has been implicated as a key pathophysiological event following SCI. We have shown that early central or systemic administration of a cell cycle inhibitor reduces CCA, prevents glial changes, and limits SCI-induced hyperesthesia. Here we compared the effects of early versus late treatment with the pan-cyclin dependent kinase inhibitor flavopiridol on allodynia as well as spontaneous pain. Adult C57BL/6 male mice subjected to moderate SCI were treated with intraperitoneal injections of flavopiridol (1 mg/kg), daily for 7 days beginning either 3 h or 5 weeks after injury. Mechanical/thermal allodynia was evaluated, as well as spontaneous pain using the mouse grimace scale (MGS). We show that sensitivity to mechanical and thermal stimulation, and locomotor dysfunction were significantly reduced by early flavopiridol treatment compared to vehicle treated controls. SCI caused robust and extended increases of MGS up to 3 weeks after trauma. Early administration of flavopiridol significantly shortened duration of MGS changes. Late flavopiridol intervention significantly limited hyperesthesia at 7 days after treatment, associated with reduced glial changes, but without effect on locomotion. Thus, our data suggest that cell cycle modulation may provide an effective therapeutic strategy to reduce hyperesthesia after SCI, with a prolonged therapeutic window.

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Authors' contributions JW contributed to the conception and design of the studies, analysis and interpretation of data, carried out SCI surgery and behavioral test, writing and revising the manuscript; ZZ contributed to behavioral tests, analyzed and interpreted data; XZ participated in behavioral tests, data analysis, and tissue processing; CLR carried out behavioral experiments; SGD conceived research and revised manuscript; AIF conceived research, participated in interpretation of data and revised the manuscript. All authors read and approved the final manuscript.

Keywords

Spinal cord injury; Neuropathic pain; Spontaneous pain; Cell cycle inhibition; inflammation

1. Introduction

Spinal cord injury (SCI) causes not only motor and sensory deficits, but also chronic, neuropathic pain. In most patients, pain begins weeks to months after injury and is persistent [47]. This unremitting pain can occur above, below or at the SCI lesion level [17; 47; 49] and includes increased sensitivity to noxious stimulation (hyperalgesia), pain in response to previously innocuous stimuli (allodynia), and/or spontaneous pain [29; 46–48]. The latter is often characteristic of central pain. In humans, facial expression of pain can be used to quantify spontaneous pain in individuals who are unable to express themselves verbally such as infants, young children, those with verbal or cognitive impairments [9]. However, characterizing facial expression of pain following SCI has not been examined. The mechanisms underlying the development and persistence of SCI-induced neuropathic pain remain unclear.

Glial cell activation (termed gliopathy [27]) after SCI has been implicated in the induction and maintenance of SCI pain. After SCI, activation of microglia and astrocytes occurs in regions near, as well as, remote from the spinal lesion including caudal dorsal horn and thalamus [14; 21; 23; 33; 60; 61; 70]. Inhibition of glia activation improves chronic and persistent pain syndromes in remote segments below the level of lesion after SCI and in other central neuropathies [27]. Thus, identification of molecular mechanisms underlying this “gliopathy” may help to clarify the pathobiology of SCI pain and lead to the elucidation of novel therapeutic targets.

Cell cycle activation (CCA) contributes to both astroglial and microglial activation after SCI and cell cycle inhibition facilitates motor functional recovery [7; 51–53; 60; 64; 65]. Little is known about the role of CCA in neuropathic pain. We have recently examined the role of CCA in the pathophysiology and modulation of SCI-Pain. We found that critical cell cycle components- such as E2F1, PCNA, CDKs 1,4, and cyclin D1 - are up-regulated after SCI, not only at the thoracic spinal lesion site but also in the lumbar spinal dorsal horn, the posterior nucleus (PO) of the thalamus and the hippocampus [60–63]. Early central or systemic administration of a selective cyclin-dependent kinase (CDK) inhibitor reduced CCA, gliopathy and neuronal activity in the dorsal horn or PO, as well as limiting chronic SCI-induced hyperesthesia. Thus, early cell cycle modulation may provide an effective therapeutic strategy to improve reduce both hyperesthesia and motor dysfunction after SCI.

Early intervention after injury may eliminate the development of central sensitization, key to reducing long term SCI-Pain. However, given the persistent activation of cell cycle pathways after SCI and its association with chronic neuroinflammation [59], it is plausible that even relatively late intervention may reduce SCI-Pain. Moreover, it is not always possible to intervene at early time points. In present study, we compared the effects of early versus late treatment with the CDK inhibitor flavopiridol on both hyperesthesia and motor dysfunction.

In addition, we reported for the first time that SCI induces spontaneous emitted pain evaluated by a mouse grimace scale that is suppressed by flavopiridol.

2. Materials and methods

2.1. Animals

All subjects were male C57BL/6 mice, aged 8–10 weeks (20–25 g), purchased from Taconic (Rensselaer, NY). The animals were housed in groups of 2–5, under a 12/12 hour light/dark cycle with food and water freely available. All procedures were approved by the University of Maryland School of Medicine Animal Care and Use Committee.

2.2. Spinal cord contusion and drug treatment

Mice were deeply anesthetized with isoflurane before laminectomy at T10, followed by a spinal impact using an Infinite Horizon Spinal Cord Impactor (Precision Systems and Instrumentation) with a force of 60 kdyn, a moderate injury [61; 63]. Sham-treated animals received laminectomy and identical procedures except for spinal impact. Manual bladder evacuations were performed twice daily until neurogenic bladder voiding returned. After SCI, mice were assigned to a treatment group according to a randomized block experimental design. The number of mice at various time points in each study is indicated in the figure legends. All SCI animals were injured by the same surgeon and at the same period of time.

Flavopiridol (Santa Cruz Biotechnology, Inc.) was dissolved completely in DMSO, further diluted in sterile saline to a final concentration of 2.28% DMSO, and administered intraperitoneally once daily beginning 3 h post-injury and continuing for 7 days. Groups of mice received 1 mg/kg flavopiridol or the equivalent volume of vehicle. This dose was based upon prior investigations in SCI model [7; 65]. A separate cohort of mice received 1 mg/kg flavopiridol or the equivalent volume of vehicle intraperitoneally once daily beginning 36 days post-injury and continuing for 7 days. The timeline of the experimental design is shown in Fig. 1. The number of mice in each group of each study is indicated in Table 1.

2.3. Nocifensive tests

The von Frey filament method is used to detect hind-paws withdrawal from a mechanical stimulus according to a method published previously [61]. Briefly, each mouse was placed on a wire mesh platform in individual Plexiglass cubicles and allowed to acclimate for one hour. The von Frey filaments (MyNeuroLab, St. Louis, MO), with incremental stiffness ranging from 0.04 g to 2.0 g, were applied serially to the plantar surface of each hind paw in ascending order of stiffness. Each trial was consisted of five perpendicular applications (one application every 5 seconds) of a von Frey filament to the hind paw for 2–3 seconds. A positive response is brisk paw withdrawal (at least 3 times out of 5 applications) in response to the filament. No paw withdrawal was considered a negative response and the next filament in ascending order was applied to the paw. A valid response is defined as a brisk, complete withdrawal of the hind paw from the platform. In the event of an ambiguous paw response, the application of the filament was repeated. Threshold was defined as the filament with the lowest bending force that elicited at least 3 positive responses of 5 trials [41].

Hot and cold plate tests were used to assess the thermal hyperesthesia [8]. An incremental hot/cold plate (PE34; IITC Life Sciences, Woodland Hills, CA) with a starting temperature of 30°C and the hot and cold ramps, set at the maximum rate of 10 °C/min, was used to induce the nocifensive behaviors of licking a hind paw and jumping to identify the thresholds for noxious heat and cold, respectively. Briefly, each mouse was allowed to acclimate for 30–60 seconds in a Plexiglas cylinder on the 30°C metal plate prior to the onset of the stimulus trial. The temperature of the plate at the time when the licking (hot) or the jumping (cold) occurred was recorded as the outcome measure. Automatic cut-off temperatures of 0°C (cold stimulus) and 50°C (hot stimulus) were used to avoid tissue injury.

2.4. The mouse grimace scale (MGS)

To minimize stress and fatigue, a separate cohort of mice was used for this study. The MGS was performed as described previously with some modifications [31; 32; 50]. Briefly, each mouse was placed on wire mesh platform in a Plexiglas observation cubicle and allowed to acclimate for five minutes. A high-resolution digital video camera showing clear mouse facial expression was positioned in front of each cubicle. Mice were video-taped for 30 minutes before injury, day 1 post injury and weekly thereafter for up to 5 weeks. The clear face images were selected every 3 minutes. Identifications were removed to ensure that subsequent coding was performed blind by a single, experienced experimenter. Randomized and unlabeled photos were presented on a large, high-resolution computer monitor, one at a time. For each photo, the orbital tightening, nose bulge, and ear position were scored based on the values 0 (no pain present), 1 (moderately visible), and 2 (severe). Orbital tightening is narrowing of the orbital area, with a tightly closed eyelid or an eye squeeze (denoted by wrinkle around eye). Nose bulge is a rounded extension of skin visible on the bridge of the nose. Ear position refers to ears pulled apart and back from their baseline position or featuring vertical ridges that form owing to tips of ears being drawn back. The individual and composite score were calculated from the mean of the values from 10 images for each mouse per each time point.

2.5. Locomotion test

Mice were tested for hind limb function in open field locomotion on day 1 post injury and weekly thereafter for up to 6–7 weeks using the Basso Mouse Scale (BMS) for locomotion [3].

2.6. Tissue processing and histopathology

Mice were perfused with 4% buffered paraformaldehyde at indicated time. The dissected spinal cords were post-fixed for overnight and cryoprotected through a sucrose gradient. After embedding in OCT, 20-µm-thick coronal sections were cut and placed serially on set of 10 slides. A representative slide from each set was stained with eriochrome cyanine (ECRC) to determine the location of the injury epicenter with the least amount of spared white matter (WM) [65]. Images were taken at 4× magnification and analyzed using NIH ImageJ software. WM area is expressed as a percentage of ECRC-positive area in total area of each section which was averaged from 2-3 adjacent sections at the lesion epicenter.

2.7. Immunohistochemistry and quantitative image analysis

Lumbar enlargement 4-5 was sectioned for immunofluorescence staining followed procedures described previously [61]. The following primary antibodies were used: mouse anti-cyclin D1 (1:500; Thermal); rabbit anti-CDK4 (1:500; Santa Cruz Biotechnology); rat anti-ED1 (1:500; AbD Serotec); rabbit anti-Ionized calcium binding adaptor molecule 1 (Iba-1, 1:1000, Wako Chemicals); rabbit anti-glial fibrillary acid protein (GFAP, 1:500, Millipore); and mouse anti-GFAP (1:500, Sigma). Images were acquired using a Leica TCS SP5 II Tunable Spectral Confocal microscope system (Leica Microsystems Inc) and processed using Adobe Photoshop 7.0 software (Adobe Systems). All immunohistological staining experiments were performed with appropriate positive control tissue as well as primary/secondary-only negative controls. For quantitative image analysis, digital images at 63× magnification were captured from both sides of spinal dorsal horns. The number of cyclin D1, CDK4, ED1/Iba-1, and GFAP labeled cells was quantified for each animal by a total # of both the left and right sides of dorsal horns (four images from 4 sections per mouse). All images were captured from n=5–6 mice per group.

2.8. Western blot analysis

Mouse spinal cord tissue (three-millimeter segments) centered at the injury site was obtained at indicated time for Western blot analysis performed as described previously [61]. Primary antibodies included: mouse anti-cyclin D1 (1:500; Thermal); rabbit anti-CDK4 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, UAS); rabbit anti-Iba-1 (1:1000; Wako Chemicals), and mouse anti-Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:2000; Millipore). Immune complexes were detected with the appropriate HRP conjugated secondary antibodies (KPL, Inc., Gaithersburg, MD) and visualized using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, Rockford, IL). Chemiluminescence was captured on a Kodak Image Station 4000R station (Carestream Health Inc., Rochester, NY) and protein bands were quantified by densitometric analysis using Carestream Molecular Imaging Software (Carestream Health Inc., Rochester, NY). The data presented reflects the intensity of target protein band compared to control and normalized based on the intensity of the endogenous control for each sample (expressed in fold of sham).

2.9. Statistical analysis

Quantitative data are plotted as mean $\pm$ standard error of the mean. For all behavioral tests repeated measures 2-way analyses of variance (ANOVAs) were conducted, followed by Bonferroni post-hoc test to compare the differences between each group. Statistical significance was evaluated between 2 individual samples using Student unpaired t tests. For multiple comparisons, one-way analysis of variance (ANOVA) followed by Student Newman-Keuls-post-hoc test. For non-parametric data the Kruskal–Wallis one-way ANOVA was used followed by multiple pairwise comparisons using Dunn's post hoc test. Statistical analysis was performed using SigmaPlot Program, Version 12 (Systat Software) or GraphPad Prism software, version 4.00 for windows (GraphPad Software, Inc). A p value of <0.05 was considered statistically significant.

3. Results

3.1. Early or late systemic treatment with flavopiridol limits SCI-induced hyperesthesia

To test whether SCI-induced CCA is involved in the development of post-injury allodynia evoked by mechanical and thermal stimuli, flavopiridol (1 mg/kg) was administrated systemically by ip beginning at 3 h post-injury and daily for 7 days (Fig. 2A). There was no difference in mechanical/thermal threshold between the groups before the SCI. By 4 weeks of SCI, mice regained adequate locomotor function to be able to withdraw a hind paw from a stimulus and undergo nocifensive behavioral testing. On day 28 after SCI, flavopiridol treated-mice (n=10) had a significantly higher mechanical threshold than the vehicle mice (n=9, **p<0.01), indicating less mechanical hyperesthesia, and these differences persisted to day 56 (*p<0.05). The threshold for hot plate temperature was decreased in vehicle group, whereas flavopiridol reversed the hot threshold to the basal level (*p<0.05). The threshold for cold plate in the SCI/vehicle group was increased at 4 weeks post-injury and declined at 8 weeks. In contrast, flavopiridol treated-mice had similar cold threshold of the baseline level up to 8 weeks post-injury.

To test whether delayed cell cycle inhibition reverses the disrupted mechanical/thermal thresholds after SCI, flavopiridol was administrated ip at 36 days post-injury and daily for 7 days. Nocifensive behaviors were tested at 37, 39, and 42 days post-injury (Fig. 2B). There was no difference in mechanical/thermal threshold between the groups at 35 days post-injury. After 4-7 days following flavopiridol treatment (n=9), the hind paws withdrawal thresholds responses to mechanical and heat stimuli were significant improved compared to SCI-vehicle group (n=8, *p<0.05, **p<0.01). The threshold for cold plate in both groups was declined after 35 days post-injury. These findings demonstrate that CCA is participated, at least in part, in maintaining ongoing pain after SCI.

3.2. SCI induces facial expressions of pain which is mitigated by CCA inhibition

To address spontaneous pain, and to reduce the potential confounding effects of reflex and motor deficits after SCI, we used the MGS to examine the time course of post-injury pain and evaluated the efficacy of CCA inhibitor against spontaneous pain. Preliminary attempts to use five facial features including orbital tightening, nose bulge, cheek bulge, ear position, and whisker change [31; 32; 50], but we noticed a poor visibility in cheek and whisker change in C57BL/6 black mice that is a common mouse strain used in SCI study. Thus, we analyzed the three features in our study which are orbital tightening, nose bulge, and ear position. Figure 3 is representative mouse face images corresponding to the three features with a three-point scale. 0 (not present) represent a mouse at baseline without facial grimace. 1 illustrates a mouse with moderate facial grimacing. 2 displays a mouse with obvious facial grimacing. The extent and time course of the MGS in SCI mice was quantified as the individual and composite score (Fig. 4). Baseline MGS scores were the same in different groups before injury. At day 1 post-injury, MGS scores in both sham-injured groups (n=5) slightly increased and then returned to baseline levels. Remarkably, MGS scores in SCI/Veh group increased from baseline levels by 2-4-fold, remained elevated for a few weeks, and returned to baseline levels after 4 weeks of SCI. Repeated measures ANOVA followed by posthoc testing revealed highly significant effect in SCI/Veh (n=9) vs. Sham/Veh group

(n=5, *p<0.05, **p<0.01, ***p<0.001). In contrast, flavopiridol treated-mice (n=10) appeared significantly reduced MGS scores at 1-3 weeks post-injury (*p<0.05, ***p<0.001). MGS scores were detected at baseline levels by 4 weeks in all SCI mice. The MGS individual scores among the three action units showed the similar pattern as composite score, attesting to their individual utility. Notably, all mice in SCI/Veh group had higher MGS scores than that in Sham/Veh group from d1 through week 3 post-injury, indicating spontaneous pain sign (Fig. 5). The MGS scores in all injured mice with flavopiridol treatment were also higher than that in Sham/Veh group during first week. By 2-3 weeks post-injury, half of mice from SCI/Fla group did not show spontaneous pain.

3.3. SCI-induced CCA at both lesion area and lumbar SDH were attenuated by flavopiridol

To confirm the effect of SCI on CCA, Western blot was performed with the 3 mm spinal cord tissue centered on the injury site at 7 weeks after SCI. In agreement with previous studies [7; 60; 61; 64], SCI upregulates expression levels of key cell cycle proteins including cyclin D1 and CDK4 at the lesion site (Fig. 6A). Quantitative analysis of Western blots showed that cyclin D1 expression was significantly increased for a 3-4-fold in injured spinal cord extracts (n=5) compared with sham tissue (n=4, p<0.001, Fig. 6B). The expression level of CDK4 indicated a 2-3-fold increase (p<0.01, Fig. 6C). The up-regulation of these cell cycle proteins was significantly attenuated in early flavopiridol-treated mice (n=5) in comparison with vehicle-treated tissue (n=5, p<0.05). However, SCI-induced the up-regulation of these cell cycle-related proteins was not attenuated in mice with delayed flavopiridol treatment (Fig. 6D-F).

Further, the expression of cell cycle-related proteins was examined in the lumbar spinal dorsal horn by immunohistochemistry. In the intact spinal cord, only few cyclin D1⁺ and CDK4⁺ cells were detected (Fig. 7-8). At 7-8 weeks after SCI, cyclin D1- and CDK4-positive cells were significantly increased in the vehicle-treated mice (p<0.01, p<0.001 vs. Sham/vehicle), consistent with our prior studies [60; 61]. Importantly, the up-regulation of these cell cycle-related proteins was clearly attenuated in the lumbar spinal dorsal horn in mice with both **early** (Fig. 7) and delayed flavopiridol treatment (n=6, p<0.05, p<0.001, Fig. 8). Thus, our data suggest that increased CCA contributes to, at least in part, maintenance of hyperesthesia following SCI.

3.4. Flavopiridol treatment results in reduced glial reactivation after SCI

Western blotting was performed at the lesion site for the activated microglia marker Iba-1 after SCI. Very low levels of Iba-1 expression were found in uninjured controls from both groups of sham/vehicle and sham/flavopiridol (Fig. 9A), but 7 weeks after SCI, Iba-1 protein expression showed a 8-9-fold increase in injured spinal cord extracts from the vehicle-treated mice (n=5) compared with sham tissue (n=4, p<0.01, Fig. 9B). Spinal cords from the early flavopiridol-treated mice (n=5) had significantly less up-regulation of Iba-1 protein expression compared with vehicle/SCI mice (p<0.05). However, SCI-induced the up-regulation of Iba-1 protein expression was not attenuated in mice with delayed flavopiridol treatment (Fig. 9C-D).

To determine if the observed attenuation of below-level hyperesthesia in flavopiridol-treated mice may be related to the reduced activation of gliopathy, spinal cord lumbar 4/5 sections from injured mice perfused at 7-8 weeks after SCI were stained for ED1/Iba-1 as well as GFAP, and activated microglia and astrocytes within the SDH were quantified. ED1 is a cellular marker specific for activated rat microglia, monocytes, and macrophages. We found that ED1 positive reactive microglia were rarely seen in uninjured tissue (Fig. 10–11), but 7-8weeks after SCI, ED1⁺/Iba1⁺ cells were significantly increased in the vehicle/SCI tissue (n=5) compared with sham tissue (n=5, $p<0.01$, Fig. 10–11), consistent with other reports [21; 61]. Immunohistochemistry also showed an increase in immune-labeling of GFAP in contrast to sham tissue ($p<0.01$, $p<0.001$, Fig. 10–11). Notably, quantitative image analysis showed that there were significant reduction in positively-stained cells in both early and delayed flavopiridol-treated animals (n=5–6, $p<0.05$, $p<0.01$, Fig. 10–11). Thus, decreased numbers of reactive microglia/astrocytes are found in the lumbar spinal dorsal horn in flavopiridol-treated mice associated with reduced below-level mechanical/thermal allodynia in these animals.

3.5. Early but not delayed cell cycle inhibition after SCI improves recovery and reduces tissue damage

To examine the effect of inhibiting the cell cycle on locomotor functional recovery in mice contusion model, the C57BL/6 mice subjected to a moderate contusion SCI or sham received an ip injection of flavopiridol (1 mg/kg) or vehicle beginning at 3 h post-injury and once daily for 7 days. At day 1 after SCI, all mice had a BMS locomotor score of 0 or 1, indicating nearly complete loss of motor function (Fig. 12A). By 3 weeks after injury, flavopiridol-treated mice (n=10) had significantly improved BMS scores compared with vehicle-treated animals (n=9, $p<0.05$) which remained through 6 weeks after injury. We also compared the extent of WM sparing in SCI mice that received either flavopiridol treatment or vehicle injections. Early flavopiridol treatment (n=7) significantly increased WM area compared with vehicle-treated animals (n=7, $p<0.05$, Fig. 12B). Figure 12C depicts representative eriochrome stained sections and illustrates the differences in spared WM area between vehicle- and flavopiridol-treated animals.

To test whether delayed administration of flavopiridol improves locomotor function, a separate cohort of mice received 1 mg/kg flavopiridol (n=9) or the equivalent volume of vehicle (n=8) intraperitoneally once daily beginning 36 day post-injury and continuing for 7 days. BMS was assessed on day 1 post injury and weekly thereafter for up to 7 weeks. All SCI mice were then assigned to a treatment group according to a randomized block experimental design. At day 35 post-injury, no significant difference of the BMS scores between two groups were observed (Fig. 12D). During or after flavopiridol treatment, BMS was recorded at days 38, 42, and 49 after injury. No significant difference in BMS scores was observed at all these time-points between two groups (Fig. 12D). WM sparing was also compared between groups at 49 days post-injury. Delayed administration of flavopiridol did not alter spared WM area at the lesion center (Fig. 12E).

4. Discussion

Systemic administration of the pan-CDK inhibitor flavopiridol attenuated both the development (early treatment) and the maintenance (delayed treatment) of mechanical/thermal allodynia, and gliopathy in the lumbar dorsal horn following thoracic spinal cord contusion in mice. Moreover, we quantified for the first time the time course of spontaneous pain via facial expression following SCI. Early flavopiridol treatment significantly decreased the MGS as well as CCA in the lesion site after SCI. Locomotor recovery as well as spared white matter area was improved by early cell cycle inhibition, but not with delayed treatment.

SCI triggers secondary injury processes that include post-mitotic cell death (neurons and mature oligodendrocytes) and mitotic cell activation/proliferation (microglia and astrocytes). Growing evidence [6; 7; 15; 16; 24; 35–37; 55] indicates that abnormal cell cycle re-entry or re-expression of cell cycle proteins in neurons can cause cell death which has been associated with neurodegenerative conditions including Alzheimer's disease as well as CNS injury such as stroke, brain injury and SCI. Up-regulation of cell cycle proteins in microglia or astrocytes leads to cell proliferation and activation that contribute to inflammation and glial scar formation. We and others [7; 40; 51; 52; 64; 65] have shown CCA in neurons and glia in injured spinal cord and that administration of structurally different cell cycle inhibitors reduces neuronal death and microglial/astrocyte reactivity, and improves motor function recovery after SCI.

A prior study reported that administration of the pan-CDK inhibitor flavopiridol reduced tactile allodynia after spinal nerve injury through inhibition of astrocyte proliferation [54]. CDK5 inhibitors such as roscovitine can reduce heat hyperalgesia in rat models of complete Freund's adjuvant-induced inflammation or formalin-induced nociceptive responses [56; 66; 68]. More recently, we demonstrated [58; 60; 61] that contusion SCI in both rat and mouse causes dysregulated CCA in neurons and glia of the spinal dorsal horn (SDH), which is critical for central sensitization [29]. Moreover, elevated expression of cell cycle proteins is observed chronically in the L4/5 spinal dorsal horn after thoracic SCI, consistent with our previous studies [60; 61]. Cell cycle inhibition by intrathecal or systemic administration of the selective CDK inhibitor CR8 reduces hind paws mechanical allodynia after contusion SCI [60; 61]. Our present data shows that intraperitoneal administration of flavopiridol attenuates hind-paw hyperesthesia in response to mechanical or thermal stimuli. These results suggest that increased CCA in the lumbar SDH contributes to below-level neuropathic pain induced by thoracic SCI. Given the persistent CCA in the lumbar SDH and reduced ongoing mechanical/heat allodynia by delayed cell cycle inhibition, CCA may contribute to persistent pain induced by SCI.

Following SCI, dysfunctional microglia and astrocytes in the SDH are key contributors in the underlying cellular mechanisms contributing to both the development and the maintenance of above-, at-, and below-level neuropathic pain [20; 22; 23; 43]. In agreement with these findings, the present data showed that SCI increased activation of microglia and astrocytes in both the injury site and lumbar SDH. However, microglia and astrocytes may play distinct roles in neuropathic pain induction and maintenance [39; 45]. Microglia play a

critical role in the development of neuropathic pain, but its role in reducing the established late-phase neuropathic pain is limited [39]. Dissociation between microglial marker expression and pain behaviors has been reported in peripheral nerve injury models [5; 10; 71]. Accumulating evidence suggests that proliferation and re-activation of spinal cord astrocytes are important both for the induction and maintenance of inflammatory and for neuropathic pain [28]. The astrocytic marker GFAP is better correlated with pain behaviors after inflammation [26; 45; 69]. Inhibiting astrocyte proliferation in the spinal cord by flavopiridol was shown to reduce neuropathic pain after spinal nerve injury [54]. It is well documented that CCA after SCI contributes strongly to glial activation [22]. Numbers of ED1⁺/Iab-1⁺ activated microglia and GFAP⁺ astrocytes were significantly reduced with both early and delayed flavopiridol treatment and changes were associated with decreased CCA. Thus, inhibiting activation of both microglia and astrocytes may be a mechanism by which flavopiridol reduces neuropathic pain following SCI. However, intraperitoneal administration of CDK inhibitors may also alter changes in the sensitivity of peripheral nociceptors, dorsal root ganglia or spinal cord circuits. Our previous studies [60; 61] show that systemic and intrathecal administration of CDK inhibitors produce similar effects on the attenuation of neuropathic pain, suggesting that inhibition of neuropathic pain by CDK inhibitors may via CNS mechanisms.

Rodent pain behavioral tests after SCI have been criticized because they typically rely on stimulus-response behaviors to mechanical and thermal stimuli [1; 34; 44]. This can be problematic because SCI has the potential confounding effects of causing reflex and motor deficits [62; 63]. In particular, neuropathic pain behavioral assessments become difficult when animals subjected to moderate/severe injury are not able to withdraw their hind paws from a stimulus during first few weeks after SCI. Clinical studies demonstrate that SCI patients display spontaneous pain. Facial expressions of common emotional states have been well characterized in humans and also evidenced in nonhuman mammals including rats [19]. Recently, Mogil's lab developed grimace scale in both mouse [31; 32] and rat [50] for evaluating the efficacy of postoperative analgesics. The grimace scales have subsequently been developed for the rabbit [30], horse [12], and cat [25]. Facial pain expression offers an assessment of early-phase spontaneous pain following SCI. The MGS is a standardized behavioral coding system with high accuracy and reliability [31; 32]. In the present study, the MGS with some modification has been adopted to evaluate effects of SCI, as well as cell cycle modulation, on SCI-Pain. After SCI, the MGS remained elevated over three weeks. Early administration of flavopiridol robustly shortened the duration of elevated MGS in SCI mice, suggesting a reduction of spontaneous pain.

Mechanical and thermal hypersensitivity in the hind-paws after SCI continues for months, far longer than do spontaneous pain as shown by the MGS. One possibility is that prey animals presumably are highly motivated not to display facial grimacing and may eventually learn to control their facial musculature even as pain persists, as do patients with chronic pain [11]. Thus, the disappearance of the facial grimacing may not necessarily represent the disappearance of spontaneous pain. Compared to other spontaneous pain measurements used in SCI studies, such as conditioned place preference [13; 67], facial expression coding has the considerable advantage that no subject training and special equipment (except for a video camera) are required. It may also have the advantage of more complete blinding of the

experimenter [42], as during scoring the presence or absence of an inflamed or guarded hind-paw is obscured.

Intrathecal or systemic administration of flavopiridol at early phase has been shown to promote locomotor functional recovery after SCI in rats [7; 40; 65]. In agreement with these findings, the present data showed that early systemic administration of flavopiridol after SCI in mice produced greater locomotor recovery than vehicle group which associated with reduced inflammation and increased spared whiter matter area. It is well known that tissue sparing is correlated with locomotor function in contusion SCI models [2; 3]. Delayed treatment with flavopiridol in the present study did not alter spared white matter area that is correlated with the BMS scores. SCI-induced inflammation and astrogliosis play a significant role in delayed secondary tissue damage that occurs over days, weeks and even months after the initial injury [4; 18]. We reported previously [64] that SCI is accompanied by a prolonged, sustained up-regulation of cell cycle-related protein expression that may contribute to the development of glial scar formation, chronic inflammation and progressive tissue loss at the lesion site. Such changes in the present study were not reduced at the lesion area with delayed flavopiridol treatment. However, delayed CCA inhibition reduced CCA and gliopathy at the lumbar SDH which are sufficient for attenuation of below-level neuropathic pain but not for locomotor recovery. One possible explanation is that the extent of pathological changes in the two regions differs markedly following SCI, and that the dose paradigm used in the current study may not be sufficient to modulate chronic cell cycle and inflammatory changes at the more severely damaged lesion site. The absence of such effects is consistent with the finding of unchanged spared whiter matter in the injury region or the lack of effect on locomotion function. Differences between the early and delayed treatment for locomotion improvement in part may reflect the fact that dose response studies were not performed to optimize the systemic dose required. In addition, the permeability of the blood-brain barrier is likely less in week 5 than it in the first week after SCI [38; 57].

In summary, thoracic SCI produces facial expression of pain as well as below-level hyperesthesia in association with cell cycle mediated microglial/astrocytes activation. Cell cycle inhibition by flavopiridol limited SCI-induced spontaneous pain as well as the development and maintenance of hyperesthesia. These results suggest that cell cycle modulation may be a potential target to treat chronic SCI pain.

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Fig. 1.

Timeline of the *in vivo* experimental design. (A–B) Early treatment. The C57BL/6 mice subjected to a moderate contusion SCI or sham received an intraperitoneal (ip) injection of flavopiridol (1 mg/kg) or vehicle beginning at 3 h post-injury and once daily for 7 days. (C) Delayed treatment. Flavopiridol (1 mg/kg) or vehicle was administered ip at 36 days post-injury and daily for 7 days.

Fig. 2.

Early and delayed treatment with flavopiridol attenuated mechanical/thermal allodynia following SCI. (A) pan-CDK inhibitor flavopiridol (1 mg/kg) was administrated systemically by intraperitoneal (ip) beginning at 3 h post-injury and daily for 7 days. There was no difference in mechanical/thermal threshold between the groups before the SCI. Flavopiridol-treated mice (n=10) had significantly less post-SCI mechanical and thermal hyperesthesia than vehicle-treated mice (n=9, * $P < 0.05$, ** $P < 0.01$). (B) Flavopiridol was administrated ip at 36 days post-injury and daily for 7 days. Nocifensive behaviors were tested before injury, 35 days post-injury, and at 37, 39, and 42 days post-injury. There was no difference in mechanical/thermal threshold between the groups at 35 days post-injury. After 4-7 days following flavopiridol treatment (n=9), the hind paws withdrawal thresholds responses to mechanical and heat stimuli were significant improved compared to SCI-vehicle group (n=8, * $p < 0.05$, ** $p < 0.01$).

Fig. 3.

SCI induces spontaneous emitted pain evaluated by Mouse Grimace score (MGS).

Representative mouse face images corresponding to the three facial features (orbital tightening, nose bulge, and ear position) with a three-point scale. 0 (not present) represents a mouse at baseline without facial grimace, 1 shows a mouse with moderate facial grimacing, and 2 illustrates a mouse with obvious facial grimacing.

Fig. 4.

SCI induces facial expressions of pain which is mitigated by CCA inhibition. Individual score of the three facial features (A–C) was recorded and analyzed before injury, at day 1, 3, and weekly up to 5 weeks after SCI. The MGS was calculated by the average score of three features. Baseline MGS scores were the same in different groups before injury. At day 1 post-injury, MGS scores in both sham-injured groups ($n=5$) slightly increased and then returned to baseline levels. MGS scores in SCI/Veh group ($n=9$) increased from baseline levels by 2–4-fold, remained elevated for 3 weeks, and returned to baseline levels after 4 weeks of SCI. Q-Q plots demonstrated that the MGS score data approached normality and showed equal variance; therefore these data were subsequently analyzed using parametric statistics. Repeated-measure two-way ANOVA was used followed by Student's Newman-Keuls post-hoc test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs SCI/Veh. In contrast, flavopiridol treated-mice ($n=10$) appeared significantly reduced MGS scores at 1–3 weeks post-injury ($*P < 0.05$, $***P < 0.001$). MGS scores were detected at baseline levels by 4 weeks in all SCI mice.

Fig. 5.

The MGS composite score of individual mouse is indicated as scatter plot. All mice in SCI/Veh group had higher MGS scores than that in Sham/Veh group from d1 through week 3 post-injury. The MGS scores in all injured mice with flavopiridol treatment were also higher than that in Sham/Veh group during first week. By 2-3 weeks post-injury, half of mice from SCI/Fla group did not show spontaneous pain. n=5 (Sham/Veh), 5 (Sham/Fla), 8 (SCI/Veh), and 9 (SCI/Fla).

Fig. 6.

Upregulation of cell-cycle-related proteins at the lesion site after 7 weeks of SCI is attenuated by early cell cycle activation inhibition, but not by delayed treatment. Three-millimeter segments of spinal cord tissue representing the injury epicenter were collected for Western blot analysis. (A) Early treatment. Representative immunoblots for cyclin D1, CDK4, and GAPDH are indicated. (B–C) Western blot analysis showed that expression levels of cyclin D1 and CDK4 were markedly increased in the vehicle/SCI mice but significantly lower in early flavopiridol-treated mice. $n=4-5$ mice/group. $**P < 0.01$, $***P < 0.001$ vs. Sham/Veh; $\#P < 0.05$ vs. SCI/Veh. (D) Delayed treatment. Representative images are indicated for cyclin D1, CDK4, and GAPDH. (E–F) Western blot analysis showed that SCI-induced the up-regulation of cyclin D1 and CDK4 was not attenuated in mice with delayed flavopiridol treatment. $n=4$ mice/group. $*P < 0.05$, $***P < 0.001$ vs. Sham/Veh.

Fig. 7.

Early administration of flavopiridol significantly reduces SCI-induced cell cycle activation at lumbar 4/5 spinal dorsal horn (SDH). (A) Representative coronal sections of lumbar spinal cord stained for cyclin D1 and CDK4. Scale bar = 100 μ m. (B–C) Quantification analysis showed that few cyclin D1⁺ and CDK4⁺ cells were found in the SDH of sham-injury mice. These positive cells were significantly increased in vehicle-treated mice at 8 weeks after SCI compared with sham-injury mice, which were attenuated in flavopiridol-treated mice. $n=5$ mice/group. $**P<0.01$, $***P<0.001$ vs. Sham/Veh; $##P<0.01$ vs. SCI/Veh.

Fig. 8.

SCI-induced cell cycle activation at lumbar 4/5 spinal dorsal horn (SDH) was attenuated by delayed administration of flavopiridol. (A) Representative images for cyclin D1 and CDK4. Scale bar = 100 μ m. (B–C) Quantification analysis showed that cyclin D1⁺ and CDK4⁺ cells were significantly increased in vehicle-treated mice at 7 weeks after SCI compared with sham-injury mice, which were attenuated in flavopiridol-treated mice. A few cyclin D1⁺ and CDK4⁺ cells were found in the SDH of sham-injury mice. $n=4-6$ mice/group. ** $P < 0.01$, *** $P < 0.001$ vs. Sham/Veh; # $P < 0.05$, ### $P < 0.001$ vs. SCI/Veh.

Fig. 9.

Early cell cycle activation inhibition by flavopiridol, but not delayed treatment, attenuates microglial activation at the lesion site. Three-millimeter segments of spinal cord tissue representing the injury epicenter were collected for Western blot analysis. (A) Early treatment. Representative immunoblots for Iba-1 and GAPDH are indicated. (B) Western blot analysis showed that expression levels of Iba-1 were markedly increased in the vehicle/SCI mice but significantly lower in flavopiridol-treated mice. $n=4-5$ mice/group. $**P < 0.01$ vs. Sham/Veh; $\#P < 0.05$ vs. SCI/Veh. (C–D) Delayed treatment. SCI-induced the up-regulation of Iba-1 protein expression was not attenuated in mice with delayed flavopiridol treatment. $n=4$ mice/group. $*P < 0.05$ vs. Sham/Veh.

Fig. 10.

Early administration of flavopiridol significantly decreases gliopathy at the lumbar 4/5 spinal dorsal horn (SDH) after SCI. (A) Representative coronal sections of lumbar spinal cord stained for ED1/Iba-1 and GFAP. Scale bar = 100 μ m. (B–C) Quantification of ED1(green)/Iba-1(red) activated microglial cells showed that after SCI, there were significantly more activated microglia in the lumbar SDH of vehicle-treated mice compared with sham-injured mice. The number of GFAP-positive cells (green) was significantly increased at 8 weeks after injury in SCI/Veh mice. Early treatment with flavopiridol significantly reduced the number of reactive microglia/astrocytes. $n=5$ mice/group. $**P < 0.01$, vs. Sham/Veh; $\#P < 0.05$, $##P < 0.01$ vs. SCI/Veh.

Fig. 11.

SCI results in decreased gliopathy at the lumbar 4/5 spinal dorsal horn (SDH) after SCI which was attenuated by delayed administration of flavopiridol. (A) Representative coronal sections of lumbar spinal cord stained for ED1/Iba-1 and GFAP. Scale bar = 100 μm. (B–C) Quantification of ED1(green)/Iba-1(red) activated microglial cells showed that after SCI, there were significantly more activated microglia in the lumbar SDH of vehicle-treated mice compared with sham-injured mice. The number of GFAP-positive cells (green) was significantly increased at 7 weeks after injury in SCI/Veh mice. Delayed treatment with flavopiridol significantly reduced the number of reactive microglia/astrocytes. n=4-6 mice/group. ** $P < 0.01$, *** $P < 0.001$ vs. Sham/Veh; # $P < 0.05$, ## $P < 0.01$ vs. SCI/Veh.

Fig. 12.

Early administration of flavopiridol, but not delayed treatment, improves locomotor functional recovery and spared white matter (WM) after SCI. (A) BMS scores in early flavopiridol-treated mice (n=10) were significantly higher than in vehicle-treated mice (n=9) starting at day 21 after SCI (* $P < 0.05$). (B) Quantification of the spared WM area at the lesion epicenter showed a significant increase in early flavopiridol-treated mice (n=7) compared with vehicle mice (n=7, * $P < 0.05$). (C) Representative sections show the lesion epicenter stained with eriochrome cyanine (ECRC) for myelinated areas of spared WM. (D) Delayed treatment. Mice received 1 mg/kg flavopiridol (n=9) or the equivalent volume of vehicle (n=8) intraperitoneally once daily beginning 36 day post-injury and continuing for 7 days. BMS was assessed on day 1 post injury and weekly thereafter for up to 49 days. No significant difference of the BMS scores was observed between two groups before or after treatment at all time-points. (E) Delayed administration of flavopiridol did not alter spared WM area at the lesion center at 49 days post-injury. n=5 mice per group.

Table 1

Definition of the groups

Groups/mice #	Functional assessment	Outcome measures in subgroups (subgroups, randomly selected)
Early treatment		
Sham/Veh	5	IHC
Sham/Fla	5	IHC
SCI/Veh	9	IHC, SWM
SCI/Fla	10	IHC, SWM
Early treatment for MGS		
Sham/Veh	5	WB
Sham/Fla	5	WB
SCI/Veh	9	WB
SCI/Fla	10	WB
Delayed treatment		
Sham/Veh	8	IHC, WB
Sham/Fla	8	IHC, WB
SCI/Veh	8	IHC, SWM, WB
SCI/Fla	9	IHC, SWM, WB

SCI: spinal cord injury; Veh: vehicle; Fla: flavopiridol; MGS: mouse grimace scale; IHC: immunohistochemistry; SWM: spared white matter; WB: western blot.