Intrathecal Acetyl-L-Carnitine Protects Tissue and Improves Function after a Mild Contusive Spinal Cord Injury in Rats

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Abstract

Primary and secondary ischemia after spinal cord injury (SCI) contributes to tissue and axon degeneration, which may result from decreased energy substrate availability for cellular and axonal mitochondrial adenosine triphosphate (ATP) production. Therefore, providing spinal tissue with an alternative energy substrate during ischemia may be neuroprotective after SCI. To assess this, rats received a mild contusive SCI (120 kdyn, Infinite Horizons impactor) at thoracic level 9 (T9), which causes loss of ~80% of the ascending sensory dorsal column axonal projections to the gracile nucleus. Immediately afterwards, the energy substrate acetyl-L-carnitine (ALC; 1 mg/day) or phosphate-buffered saline (PBS) was infused intrathecally (sub-arachnoid) for 6 days via an L5/6 catheter attached to a subcutaneous Alzet pump. ALC treatment improved overground locomotor function (Basso-Beattie-Bresnahan [BBB] score 18 vs. 13) at 6 days, total spared epicenter (71% vs. 57%) and penumbra white matter (90% vs. 85%), ventral penumbra microvessels (108% vs. 79%), and penumbra motor neurons (42% vs. 15%) at 15 days post-SCI, compared with PBS treatment. However, the ascending sensory projections (anterogradely traced with cholera toxin B from the sciatic nerves) and dorsal column white matter and perfused blood vessels were not protected. Furthermore, grid walking, a task we have shown to be dependent on dorsal column function, was not improved. Thus, mitochondrial substrate replacement may only be efficacious in areas of lesser or temporary ischemia, such as the ventral spinal cord and injury penumbra in this study. The current data also support our previous evidence that microvessel loss is central to secondary tissue degeneration.

Key words: ALC; axon; contusion; dorsal column; ischemia; mitochondria; spinal cord injury

Introduction

TRAUMATIC SPINAL CORD INJURY (SCI) leads to the immediate and secondary loss of many cells, including long projecting axons.¹ SCI rarely is complete, with some axons typically being spared at the epicenter.² Preservation of even a small portion of axons is associated with neurologic function in humans,^{3,4} making axon protection an important therapeutic target for treatments. SCI causes immediate microvascular damage leading to hemorrhage⁵ and vasospasm,⁶ and is followed by secondary microvascular loss over at least 2 days.⁷ This microvascular loss has serious consequences, since we have shown that pure ischemic injury to the spinal cord leads to ascending dorsal column sensory axon degeneration.⁸ Moreover, treatment with a protein tyrosine phosphatase inhibitor, which protects dorsal column blood vessels after contusive SCI, corresponds with tissue and ascending sensory axon sparing.⁹

The downstream mechanism(s) of ischemia-induced axon loss after SCI remains unclear but may involve mitochondrial dysfunction. Mitochondria have the important role of producing most

of the adenosine triphosphate (ATP) required for normal cellular activity.¹⁰ The central nervous system (CNS) does not have glucose stores and ischemia caused by a SCI likely deprives axonal mitochondria of energy substrates needed for ATP production. Insufficient ATP, in turn, would lead to Na⁺/K⁺ ATPase dysfunction causing axonal swelling and subsequent reverse operation of the Na⁺/Ca²⁺ exchange, leading to detrimental axonal Ca²⁺ influx,¹¹ which also can be toxic to mitochondria.¹² This cascade may drive axon degeneration, especially because CNS axons lack intrinsic energy reserves.¹³

One therapeutic strategy to maintain ATP production would be to provide an alternative energy source for mitochondria. Acetyl-L-carnitine (ALC) donates acetyl groups to coenzyme A (CoA) to form acetyl-CoA, which is used by mitochondria for ATP production.¹⁰ Systemic ALC treatment improves mitochondrial respiration and bioenergetics and protects tissue following severe contusion to the thoracic (T10, 200 kdyn)¹⁴ or lumbar (L1/2, 250 kdyn)¹⁵ spinal cord. However, locomotor function (Basso-Beattie-Bresnahan [BBB] score) improved only in ALC-treated rats following lumbar injury (a predominantly gray matter injury) but not thoracic injury (a predominantly white matter injury), which

the authors suggested may be due to inadequate protection of white matter.¹⁵ Such severe thoracic injuries may preclude any functional white matter sparing. Alternatively, therapeutic levels of ALC may not have been achieved quickly or long enough due to the intermittent systemic administration and/or due to the temporary vasospasm at the injury site. Therefore, we chose to use a mild contusion (120 kdyn) combined with continuous intrathecal infusion of ALC. This more mild injury also permits assessment of ascending sensory axons in the dorsal column and their function separately from the overground locomotor function, which is served by more ventral pathways.¹⁶ The intrathecal administration ensures that the ALC concentrations are much higher within the spinal cord tissue and surrounding dorsal root ganglions than in the rest of the body. Thus, any beneficial effects observed are most likely mediated by mechanisms within the nervous system.

Methods

Animals

Nineteen female Sprague-Dawley rats were used (~200 g; Harlan). Animal procedures and care was in accordance with the guidelines of the National Institutes of Health and were approved by the University of Louisville Institutional Animal Care and Use Committee. Animals habituated to their cages for a minimum of 7 days and were given free access to food and water except during behavioral testing.

General surgical procedures and post-operative care

Animal surgeries were performed according to a standardized protocol as described elsewhere.⁸ In short, after intramuscular (i.m.) injection of an anesthetic mixture containing ketamine, acepromazine and xylazine, ophthalmic ointment was placed on the eyes and 5 mL saline was injected subcutaneously before and after surgery. Surgery and recovery was performed on a 37°C heating pad. After surgery, the wound was rinsed with sterile saline and closed in layers, and bacitracin applied topically and gentamicin injected i.m. immediately and 2 and 4 days later. Buprenorphine was injected i.m. for pain relief once each animal awoke and four times every 12 hours. Bladders were manually expressed twice a day until spontaneous voiding occurred.

Spinal cord injury and intrathecal drug delivery

Contusive SCI was performed as described elsewhere.⁸ Briefly, after a skin incision, the T9 vertebra was stabilized, a laminectomy performed and a 120 kdyn contusion delivered using the Infinite Horizons impactor (Precision Systems and Instrumentation, LLC, Lexington, KY). Sham animals were treated similarly, but not contused.

Intrathecal polyethylene catheters with 100 μ m diameter tips attached to Alzet osmotic pumps (model 2001, 7d, 1 μ L/h; Durect, Cupertino, CA) were prepared and implanted at vertebral level L5/6 as described elsewhere.⁹ The only differences were that the pumps were incubated overnight in sterile saline before being filled immediately before surgery, under sterile conditions, with sterile 0.1 M phosphate-buffered saline (PBS) or with PBS containing freshly dissolved ALC (1 mg/day; 41.67 mg/mL, Cat#A6706, Sigma-Aldrich, St. Louis, MO). After SCI, the thin part of the catheter was inserted into the subarachnoid cerebrospinal fluid space through a small hole in the dura, the thick part of the catheter sutured to the muscles, and the pump placed subcutaneously. Sham operated rats received a catheter attached to a PBS-filled pump. Drug treatment was randomized beforehand, but the sequence altered as needed to ensure that the impactor displacement (a measure of injury severity) between groups was not significantly different. On post-operative Day 6, after the first behavioral assessments, the subcutaneous pump was removed by cutting and cauterizing the catheter.

This intrathecal route and dose (1 mg/day) is similar to that used by others (0.9 mg/day) to show neuroprotection of motor neurons after L5 hemisection in rats.¹⁷ Others have found protective effects of intraperitoneal injection of 300 mg/kg ALC (~60–75 mg per rat) after a severe spinal cord injury on mitochondrial function during the acute injury phase, as well as white matter sparing and improved locomotor function.¹⁵ This seems comparable to our 1 mg/day dose because drugs can be similarly active after intrathecal administration at 100-fold lower doses than after intraperitoneal injection, probably due, at least in part, to the much smaller enclosed spinal cord tissue volume, compared with the whole body. An example is the daily dosing of our intrathecal protein tyrosine phosphatase inhibitor (0.485–1.455 μ g/day),⁹ compared with that of a closely related inhibitor administered intraperitoneally (40–100 μ g/day).¹⁸

Locomotor assessment

Rats were acclimated to handling and the testing equipment three times prior to baseline assessments. To assess overground locomotor function, rats were placed in an 85 cm diameter circular tub and allowed to walk for 4 min while two certified examiners scored locomotor performance according to the BBB scale.¹⁹ Ninety minutes later, grid walking was assessed on a sensitive elevated plastic chain link fence grid as described elsewhere,⁸ a task we have shown is dependent on the dorsal column.^{8,9} Rats walk voluntarily for a total of 90 sec and footfalls are counted. Observers were blinded to treatment condition.

Ascending sensory axon tracing

After the second behavioral assessment (Day 12; 3 days before analyses), ascending dorsal column sensory axons from the sciatic nerves projecting to the gracile nucleus that pass through the injury site were traced bilaterally with cholera toxin B (CTB) as described previously.²⁰ Briefly, CTB was injected into both sciatic nerves at mid-thigh level and the nerve crushed to increase tracer uptake.²¹ A ligature around the nerve prevents CTB leakage.

Intravital perfused blood vessel labeling and preparation for histology

Twenty minutes before processing for histology, perfused blood microvessels were labeled by injecting 200 μ g of Texas Red conjugated lycopersicon esculentum agglutinin (LEA; Vector Laboratories, Burlingame, CA) into the left jugular vein as described elsewhere.⁸ Injected LEA only binds to endothelial cells in perfused microvessels.^{22,23} After 20 min, the rats were perfused with PBS followed by 4% paraformaldehyde. Tissue was post-fixed overnight and then cryoprotected in 30% sucrose.

Histological analyses

Eleven millimeter spinal cord segments centered on the injury epicenter from all subjects were mounted together in a block to obtain 20 μ m transverse sections using a cryostat and perform the different histological staining procedures as described in detail elsewhere.⁸ Unless otherwise noted, each histological assessment used slides with sections spaced 200 μ m. Spared white matter was identified by eriochrome cyanine (EC) staining and was used to determine the maximum injury epicenter for each rat and alignment of sections from other histological markers. To identify cell bodies of lower motor neurons in the ventral horn at the injury site, cresyl violet staining was performed as previously described.⁷ To identify blood vessels, double immunofluorescence was performed as described⁸ using goat anti-Texas Red immunoglobulin G (IgG) for perfused and mouse anti-rat RECA-1 IgG primary antibodies for total (RECA-1+) blood vessels, combined with appropriate secondary antibodies. The goat anti-Texas Red antibody was used to

amplify the Texas Red-conjugated LEA signal, which helps with counting of the blood vessels. CTB-traced dorsal column sensory axon terminals were identified in 30 μm sagittal sections through the medulla (gracile nucleus). Lumbar spinal cord sections (L4/5 afferents) were used to confirm successful injection. CTB immunohistochemistry on every third section was performed as described⁸ using a well-defined protocol and highly specific primary antibody.

Quantitative measurements

Mosaic images of the entire section were taken at 10 $\times$ (bright field for CTB and motor neurons) or 20 $\times$ (fluorescent) objective and white matter, microvessels and CTB+ terminals quantified as described.⁸ Briefly, the percentage of EC-stained pixels for each spinal cord section was calculated as a percentage of sham, with the three sections with the lowest percentage defined as the epicenter. Averages of three sections 1 mm rostral and caudal were defined as the penumbra. Dorsal column white matter sparing was measured separately. To quantify motor neuron survival, cresyl violet-stained cell bodies of the ventral horn were counted in the epicenter and penumbra and expressed as a percentage of sham. The percentage of spared ascending dorsal column sensory terminals in the gracile nuclei was determined by the combined number of pixels of all sections on both sides. Only rats with normal CTB staining of the uninjured lumbar enlargement were included. One ALC-treated and three PBS-treated rats were excluded for this reason. To quantify blood vessels, LEA+ or RECA-1+ microvessels were counted in high resolution images of the spinal cord injury site as described previously.⁸ Briefly, an 800 $\times$ 800 μm grid with a 100 μm cell size was centered directly above the central canal (dorsal assessment) or placed directly below with the left side of the grid vertically aligned with the central canal (ventral assessment).^{7,24} Thin tube-like structures (blood vessels) that intersect the horizontal or vertical grid lines were counted and the average number in three sections at the epicenter and penumbra expressed as a percentage of sham.

Statistical analysis

All analyses were done blinded to the treatment. Data are group averages $\pm$ standard error of the mean, and in most instances are expressed as a percentage of sham. All images were selected from rats and sections that represented their approximation to the group average. Student's *t*-test was used to assess statistical significance, except for BBB and footfall data which was analyzed using two-way repeated measures analysis of variance followed by Tukey *post hoc* tests. Statistical analyses were performed using Prism 6 (GraphPad, La Jolla, CA). A *p* value <0.05 was considered statistically significant.

Results

T9 contusion injury severity and animal exclusion(s)

Rats were subjected to a mild T9 120 kdyn contusion injury. Two rats with low displacements (550 μm and 560 μm) were not analyzed because we find this level of injury to be too mild to produce sufficient tissue damage and locomotor dysfunction. Our (unpublished) and other studies^{25–27} show that spinal cord (impactor) displacement is an excellent predictor of white matter sparing and locomotor function, and we have used that for many years to make sure that injury severity is similar between groups. Group sizes were based on our lengthy experience with this well-established model, detecting significant changes with CTB tracing^{9,21} and different vascular and white matter lesions.⁸ Spinal cord displacements ranged from 600 μm to 880 μm and did not differ between treatment groups (ALC 680 $\pm$ 36 μm , PBS 700 $\pm$ 37 μm ; *p* > 0.05), therefore

any differences observed are not attributable to different injury severities. In addition, one ALC-treated rat exhibited signs of stress (poor grooming, touch sensitivity, fever) and was excluded from the experiment. The cause of this was unclear, but no other subjects in either treatment group exhibited these problems post-operatively.

Intrathecal ALC protects white matter and lower motor neurons after T9 contusive SCI

To test the neuroprotective effects of ALC, rats received a continuous intrathecal infusion of ALC (*n* = 6) or PBS (*n* = 7) for 6 days starting immediately after the T9 contusion at a similar dose and route to what others found can increase L4–6 tibial motor neuron survival after L5 hemisection in rats.¹⁷ At 15 days, when all the rats were processed for histological analyses, myelin EC staining revealed increased spared white matter following ALC (Fig. 1B), compared with PBS treatment (Fig. 1C). Most sparing by ALC was observed in the ventral and lateral portions of the spinal cord (Fig. 1C vs. Fig. 1B). ALC treatment resulted in an $\sim$ 14% and $\sim$ 5% increase in epicenter and penumbra spared white matter, respectively, compared with PBS (*p* < 0.01 epicenter; *p* < 0.05 penumbra; Fig. 1G). ALC also rescued lower motor neurons in the ventral horn of the penumbra as shown in cresyl violet-stained sections (Fig. 1F vs. Fig. 1E). While only $\sim$ 15 $\pm$ 8% of penumbral motor neurons were present in PBS rats, remarkably this was increased to $\sim$ 42 $\pm$ 9% in the ALC treated group (*p* < 0.05; Fig. 1H). ALC-treated rats did not have significantly more motor neurons at the epicenter (Fig. 1H). In many rats, no motor neurons were present at the injury epicenter. It is possible that epicenter motor neurons were lost due to the primary injury, whereas penumbra motor neurons were lost due to the secondary injury and thus rescued by ALC treatment.

Intrathecal ALC transiently improves overground locomotion after T9 contusive SCI

To test whether ALC treatment improved locomotor function, overground walking was assessed 6 and 12 days after SCI. Consistent with its neuroprotective effects of white matter and motor neurons, 6 day ALC treatment was associated with significant improvements in walking after T9 SCI, compared with those receiving intrathecal PBS infusions (*p* < 0.001; Fig. 1I). The $\sim$ 5 point increase in BBB scores in the ALC treated group corresponds to these animals having more consistent weight supported steps and superior forelimb-hindlimb coordination than PBS-treated rats. By 12 days, no significant overground walking differences were observed between these two groups because the mild severity of the injury led to significant locomotor recovery in the PBS-treated rats from 6 to 12 days (*p* < 0.001; Fig. 1I); ALC-treated rats were not different between 6 and 12 days (Fig. 1I).

Dorsal column sensory axons and their function are not protected by intrathecal ALC after SCI

We have extensively used a well-characterized dorsal column model to study long-projecting ascending sensory axons of passage through the injury site after contusive SCI.^{8,9,20,21} Transganglionic anterograde tracing with CTB from the sciatic nerves to the gracile nucleus in the medulla is used to quantify axonal sparing. As expected, the mild contusion caused a substantial but not complete loss of sensory axons. However, no obvious differences were observed in CTB-traced projections between PBS- and ALC-treated rats (Fig. 2B vs. Fig. 2C). PBS- and ALC-treated rats had $\sim$ 15 $\pm$ 7%

FIG. 1. Acetyl-L-carnitine (ALC) rescues white matter, protects motor neurons, and transiently improves overground walking after T9 contusive spinal cord injury (SCI). Compared with sham rats (**A**, **D**), rats treated with intrathecal PBS for 6 days and analyzed at 15 days after SCI showed a loss of eriochrome cyanine (EC)-stained epicenter white matter (**B**) and penumbra (1 mm from epicenter) cresyl violet (CV)-stained ventral horn motor neurons (**E**). Compared with PBS-treated rats, those with intrathecal ALC infusions had greater epicenter white matter sparing of the ventral and lateral spinal cord (**C**) and had increased numbers of surviving ventral motor neurons in the injury penumbra (**F**). Scale bar 400 μ m (**A**–**C**) and 200 μ m (**D**–**F**). (**G**) SCI induces significant white matter loss at the injury epicenter and penumbra 15 days after T9 SCI. Intrathecal ALC infusion ($n=6$) resulted in increased epicenter and penumbra white matter sparing, compared with PBS-treated rats ($n=7$). (**H**) SCI induces significant ventral motor neuron loss at the injury epicenter and penumbra. ALC-treated rats ($n=6$) had an increase in the total number ventral horn motor neurons in the penumbra, compared with PBS-treated rats ($n=7$) 15 days after T9 SCI. (**I**) Open field overground locomotion assessed using the Basso-Beattie-Bresnahan (BBB) scale was significantly improved at 6 days in ALC-treated rats ($n=6$) versus PBS-treated rats ($n=7$) after T9 SCI. Improved function in the PBS-treated group occurred between Days 6 and 12 post-SCI, which contributed to the lack of a significant difference observed between ALC- and PBS-treated rats on Day 12 post-SCI. In (**G**, **H**) the rostral and caudal penumbra data were averaged and expressed as a single value. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with PBS treatment using Student's t -test (**G**, **H**) or two-way repeated measures analysis of variance (ANOVA) with Tukey's multiple comparison test (**I**). #### $p < 0.001$, 6 day compared with 12 day within PBS-treated rats using two-way repeated measures ANOVA with Tukey's multiple comparison test.

and $\sim 30 \pm 21\%$ sensory axon sparing, respectively, which was not significantly different (Fig. 2D). Note that one ALC-treated rat had normal ($\sim 115\%$ of sham) CTB accumulation, but because we could not find a methodological reason for this high value, its data were included in the data set; excluding its value did not change the lack of statistical significance.

We have shown that a grid walking task is dependent on ascending sensory axons⁸ and that their protection after SCI is associated with grid walking improvements.⁹ Here, the same injury decreased grid walking performance; however, no differences were observed between ALC-treated rats (6 day, 30 ± 11 footfall errors per 90 sec walking; 12 day, 22 ± 7 footfall errors per 90 sec walking) and PBS-treated rats (6 day, 37 ± 5 footfall errors per 90 sec walking; 12 day, 21 ± 5 footfall errors per 90 sec walking; Fig. 2E). These data are consistent with the lack of sensory axon sparing

observed (Fig. 2D), and further support our previous findings suggesting that grid walk is dependent on ascending dorsal column sensory axons.

Dorsal column white matter is not spared by intrathecal ALC after contusive SCI

Observation of the EC stained sections (Fig. 1) suggested that most tissue sparing was of the ventral and lateral spinal cord. Given that dorsal column sensory axon sparing and gridwalk were not improved by ALC, we decided to specifically look at dorsal column white matter sparing as we did before.⁹ Quantitative analysis revealed that dorsal column spared white matter at the epicenter and penumbral was not significantly improved by ALC treatment (Fig. 2F). As was the case with sensory axons, one ALC-treated rat (the same one) had

FIG. 2. Acetyl-L-carnitine (ALC) does not protect dorsal column sensory axons after T9 contusive spinal cord injury (SCI). Cholera toxin B (CTB) was injected bilaterally into the sciatic nerves on Day 12 after the final behavioral assessment was performed, and histology of CTB labeled axonal projections to the medulla assessed on Day 15 post-injury. Compared with sham-operated rats (A), far fewer CTB+ projections are seen in the gracile nuclei in rats treated for 6 days with either phosphate-buffered saline (PBS; B) or ALC (C). Scale bar, 200 μ m. (D) SCI induces significant ascending dorsal column sensory axon loss as measured 15 days after T9 SCI. Intrathecal ALC ($n=5$) delivered via a lumbar catheter for 6 days did not protect sensory axons, compared with PBS-treated rats ($n=4$). Note that one ALC-treated and three PBS-treated rats data were excluded due to insufficient lumbar CTB staining of uninjured sensory afferents and motor neurons from the sciatic nerves, which is a positive control ensuring successful tracer uptake and transport. (E) SCI induces significant deficits on a gridwalking task (footfalls) 6 and 12 days post-injury, deficits which are not improved following ALC treatment ($n=6$), compared with PBS ($n=7$). (F) SCI induces significant loss of dorsal column white matter at the epicenter and penumbra, and 6 day ALC treatment ($n=6$) did not improve dorsal column white matter sparing, compared with PBS ($n=7$). In (F), the rostral and caudal 1 mm penumbra, data were averaged and expressed as a single penumbra value.

an unusually high (65%) amount of dorsal column white matter sparing at the injury epicenter, and was included in the analysis; exclusion of this rat did not alter the lack of statistical significance.

Ventral blood vessels in the penumbra are protected by intrathecal ALC after T9 contusion

Lastly, we wanted to determine whether microvessels were protected by ALC treatment since we have shown in the same model that a neuroprotective treatment that protects blood vessels is associated with tissue sparing and functional recovery.⁹ Compared with sham-operated rats (Fig. 3A), those with a contusion and PBS or ALC treatment had extensive damage to microvessels as indi-

cated by the loss of LEA+ (perfused) microvessels (Fig. 3B, 3C). The most severe loss was observed in the dorsal column at the epicenter, and far less in the ventral and lateral white matter, or in the penumbra (not shown). A similar pattern and extent of loss of RECA-1+ (total) blood vessels was observed (data not shown). Quantification showed that the ALC treatment did not significantly affect epicenter-perfused (LEA+) blood vessels in either the dorsal (ALC $21 \pm 6\%$, PBS $23 \pm 4\%$) or ventral spinal cord (ALC $46 \pm 16\%$, PBS $39 \pm 8\%$, Fig. 3D). ALC also did not significantly affect the number of penumbral-perfused (LEA+) blood vessels in the dorsal spinal cord (ALC $83 \pm 10\%$, PBS $71 \pm 13\%$), but did protect penumbral ventral spinal cord-perfused (LEA+) blood vessels (ALC $108 \pm 11\%$, PBS $79 \pm 4\%$; $p < 0.05$; Fig. 3D), which is

FIG. 3. Acetyl-L-carnitine (ALC) does not protect epicenter blood vessels after T9 contusive spinal cord injury (SCI). Rats received intravenous injections of lycopersicon esculentum agglutinin (LEA) 20 min prior to euthanasia in order to identify perfused (LEA+) blood vessels. Compared with sham (A), SCI resulted in extensive loss of perfused blood vessel as seen at 15 days after T9 contusion. Severe damage to blood vessels is observed at the injury epicenter, particularly to the dorsal columns, and this appeared similar between rats receiving 6 day intrathecal phosphate-buffered saline (PBS; B) or ALC (C). Similar effects were observed for total (RECA+) blood vessels (not shown). Scale bar, 200 μm. (D) SCI-induced loss of perfused (LEA+) blood vessels was most extensive at the injury epicenter, where it was more severe in the dorsal than in the ventral spinal cord. No significant differences were observed between intrathecal ALC ($n=6$) versus PBS ($n=7$) treatment in the epicenter; however, significantly more total (RECA-1+) and perfused (LEA+) blood vessels were present in the ventral penumbra of ALC-treated rats. In the dorsal column, the total number (RECA-1+) blood vessels was greater than the perfused (LEA+) vessels, consistent with an angiogenic response, which ALC did not affect. Note: for the rostral and caudal penumbra (1 mm from epicenter) data were averaged and expressed as a single value. * $p < 0.05$. ** $p < 0.01$, compared with PBS treatment.

consistent with the ventral motor neuron sparing observed in ALC-treated rats. Similar effects were seen in the total (RECA-1+) blood vessels (Fig. 3D). Collectively, the microvascular data suggest that protection of penumbral blood vessels contributes to the improved locomotor and tissue sparing effects, particularly the observed ventral motor neuron sparing in the penumbra.

Discussion

Our previous studies suggest that degeneration or rescue of dorsal column microvessels in rats leads to dorsal column white matter and sensory axon loss⁸ or preservation,⁹ respectively. Ischemia causes loss of oxygen and glucose, resulting in mitochondrial dysfunction, which leads to tissue loss after SCI.¹⁰ Systemic treatment with ALC can improve mitochondrial respiration and bioenergetics after severe contusions in rats.^{14,15} Here, intrathecal infusion of ALC after a T9 contusive SCI promoted survival of white matter, motor neurons, and blood vessels in the ventral spinal cord and injury penumbra, and improved overground locomotion, which is served by ventral pathways.^{16,28} However, ALC did not rescue dorsal column microvessels, sensory axons, or white matter, nor did it improve the dorsal column-dependent grid walking function.

White matter rescue by ALC may be dependent on injury severity

This study expands previous observations that ALC is neuroprotective after SCI. Daily bolus intraperitoneal injections of ALC protect tissue¹⁴ and improve overground locomotor (BBB) function¹⁵ after a severe L1/2 contusion. The same investigators found that ALC did not protect white matter or locomotor function after a severe T10 contusion.^{14,15} In contrast, we find in our milder T9 contusion model that continuous intrathecal ALC treatment results in ~14% white matter sparing at the epicenter and hindlimb locomotor function. Collectively, these data suggest that ALC is neuroprotective after predominantly gray matter injuries, whereas it can only be efficacious after predominantly white matter injuries when they are mild. Our finding that ALC did not protect the dorsal columns, where injury is severe, compared with the ventral spinal cord, is consistent with this idea. It is also possible that our drug delivery route (intrathecal vs. systemic) or delivery method (continuous vs. bolus) contributed to better protection reported here. However, this seems less likely because previously white matter sparing was observed with intermittent systemic ALC treatment after L1/2 SCI.¹⁵

It was previously shown that continuous intrathecal infusions of ALC protects local motor neurons after L5 hemisection.¹⁷ Here, a similar treatment rescued ventral horn motor neurons in the penumbra of the thoracic cord, which is further support for the notion that ALC can be used to protect gray matter neurons after SCI. Both of these studies suggest that ALC treatments, including systemic ones are neuroprotective after SCI due to their effects within the spinal cord.

ALC cannot rescue long-projecting axons in severely injured spinal cord tissue

To our knowledge, the effects of ALC on survival of long projecting axons of passage through the spinal cord injury site has not been tested directly. We used our well-developed mild T9 contusion injury model for quantification of anterogradely traced ascending primary sensory axons.²⁰ The mild contusion, unlike more severe injuries, spares a portion of these sensory axons,²¹ many of

which are initially intact but are lost during the secondary injury phase. Thus, they can be rescued when intrathecal treatment with a broad spectrum tyrosine phosphatase inhibitor is started 4 h post-contusion at T9.⁹ Despite sparing of ventral white matter, motor neurons, and blood vessels, along with improved overground walking with ALC treatment, we did not find any differences in the survival of dorsal column sensory axons using the anterograde tracing method.²¹ We did not use epicenter histological measures of axon survival (e.g., neurofilament or amyloid precursor protein staining)²⁹ because contusions result in axonal swelling making it difficult to determine actual severing of individual axons. Further, the fact that we did not observe functional improvements on the grid walking task suggests that these axons were not spared. We^{8,9} and others³⁰ have shown that this task is dependent on dorsal column sensory axons. The current results, which showed positive effects of ALC on the BBB test, further confirm the use of grid walking as a specific behavioral tool to assess dorsal column sensory axon function. It is possible that sensory axons were lost after treatment was stopped on Day 6, but we do not think this is the case because of the lack of behavioral improvement on grid walking on Day 6 and the protection observed with other histological measures.

In our previous study showing full protection of dorsal column sensory axons,⁹ a different impactor was used which combines higher velocity with lower spinal cord displacement (0.3 mm, compared with 0.6–0.8 mm here). Following contusive SCI somatosensory evoked potentials (SSEPs) correlate strongly with spinal cord displacements.²⁷ SSEPs are detected in two thirds of rats with spinal displacements of 0.6 mm, albeit at lower amplitudes than control, and displacements below (0.2 and 0.4 mm) or above (0.8, 1.0, and 1.2 mm) 0.6 mm result in either normal or absent SSEPs, respectively.²⁷ This agrees with our current data that many, but not all, of the dorsal column sensory axons are lost with the displacement severity used in the current study; here, we found that ~15% of dorsal column axons were spared in PBS-treated rats, which is similar to our previous work (~16%)⁸ using the same injury severity and CTB tracing technique in control rats. Thus, it appears that consistent effects across experiments can be achieved when controlling for spinal displacement. Several studies have found a strong correlation between displacement and white matter loss.^{26,27,31} Therefore, we suggest that ALC could not rescue the dorsal sensory axons because the injury severity, from the dorsal column's perspective, was too severe.

The overground locomotor BBB score is largely dependent on axons coursing through the ventral and lateral funiculi.^{16,28} Therefore, the current BBB results do suggest that ALC treatment can protect long-projecting axons that course through the rescued ventral spinal cord tissue, which was less severely injured. Due to the mild severity of the injury, PBS-treated rats had significant locomotor improvements from Day 6 to 12, leading to no differences in BBB scores between PBS- and ALC-treated rats on Day 12. Therefore, we suggest that the similar BBB's observed on Day 12 between groups is not indicative of loss of fibers after the ALC treatment was stopped.

Neuroprotection by ALC requires perfused microvessels

Endothelial cell loss and ischemia is one of the earliest and key causes of tissue degeneration after SCI.^{8,24,32} The ALC treatment did not significantly affect the loss of perfused (LEA-labeled) microvessels in either the dorsal or ventral spinal cord at the injury epicenter, consistent with the limited tissue-preserving effects.

However, ALC treatment provided significant protection of ventral blood vessels in the injury penumbra, which corresponds with the observed protection of ventral motor neurons, as well as the ventral spinal cord-dependent BBB function. Most of the protection was observed in the ventral spinal cord, where overall less microvascular loss was observed. Notably, the dorsal column microvessels were not protected, consistent with their proposed role in preserving dorsal column white matter and sensory axons. The differences in severity of microvessel loss between the dorsal and ventral cord reflect the fact that this is a dorsal impact injury. Moreover, dorsal column blood vessels derive from the dorso-lateral side of the spinal cord, which are damaged to a greater extent than the ventral blood vessels that serve the ventral spinal cord and all gray matter.^{33,34} Collectively, these data suggest that ALC can rescue endothelial cells and microvessel perfusion in mildly but not severely injured tissues. In support of this, in an unpublished pilot study, we found that continuous intrathecal ALC did not protect sensory axons from an experimental model of pure ischemia of the dorsal column, which we found caused near complete dorsal column microvascular loss.⁸ This also is consistent with tissue protection primarily in the penumbra after severe SCI.¹⁵

It is possible that ALC promoted the angiogenic response which peaks at 7 days in rats after SCI.²⁴ In the dorsal column, the total number blood vessels was greater than the perfused vessels, consistent with the finding that many angiogenic vessels are not perfused.²² However, the ALC treatment did not affect this. Moreover, the effect of ALC was seen only in the ventral penumbra, whereas angiogenesis occurs throughout the spinal cord.²⁴ This supports the idea that early protection of microvessels by ALC contributed to the neuroprotective effects.

Mechanisms of protection by ALC

Mitochondrial dysfunction after SCI is a key contributor to secondary degeneration.¹⁰ ALC is known as a substitute substrate for mitochondria by donating acetyl groups to CoA to form acetyl-CoA which is used by mitochondria for ATP production and for its positive effects on mitochondrial bioenergetics.¹⁰ Thus, although we did not test for this, ALC most likely exerted its neuroprotective effects in this study by preserving mitochondrial function, including bioenergetics as has been found after SCI,^{14,15} as well as reducing ultrastructural abnormalities, loss of mitochondrial membrane potential, and alterations in fusion-fission proteins.³⁵ However, ALC also might act through other mechanisms (e.g., by its antioxidant properties).³⁶ Others have suggested ALC to act against excitotoxicity³⁷ or even to indirectly inhibit mitochondrial permeability transition.³⁸ All of these other potential additional mechanisms of ALC either directly or indirectly target mitochondrial function.

Conclusion

In conclusion, the neuroprotective actions of ALC are evident from several studies and seem to be most pronounced in mildly injured tissue around the core of the injury. We propose that without resolving ischemia, ALC cannot provide permanent protection by being a bioenergy replacement. Our data also suggests that ALC might exert its effects at least in part by protecting endothelial cells of microvessels. Therefore, ALC may be more efficacious when combined with treatments which target and protect blood vessels. Since ALC is safe for human consumption, it would be easily translatable to the clinic and is therefore a drug candidate worthy of continued evaluation.

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Author Disclosure Statement

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